CHAPTER: 04

VARIABLES IN URINE METABOLOMICS

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Urine is highly non-invasive in nature as compared to other bio-fluids and is very popular for metabonomic analysis due to its ease of availablity. It has been a powerful tool for probing the metabolic responses under different stresses like chemical induced toxicity(Holmes, Bonner et al. 1992; Holmes, Sweatman et al. 1995; Holmes, Loo et al. 2007), cancer (Carrola, Rocha et al. 2010; Srivastava, Roy et al. 2010), inborn errors of metabolism (Holmes, Foxall et al. 1997) and nutritional interventions(Lenz, Bright et al. 2004; Edmands, Beckonert et al. 2011). Many studies have documented the effect of sample handling has upon the metabolic make-up of urine. Different methods of sample preparation and sample storage (room temperature (22°C), refrigerator (4 °C), or the deep-freeze -80°C have significant effect upon the changes of urine metabolites over time, as detected by ^{1}H-NMR. The inter-personal variations in pH of urine have strong effect on the chemical shift of sensitive molecules like citrate, trimethylamine-oxide, dimethylamine and N-methylhistidine leading to false interpretation of data during multivariate analysis and fallacious grouping. The addition of optimal quantity of buffer solution to the urine samples increases the chemical shift consistency of a sample. By outlining proper sample handling, including filtration, buffer solution addition for effective pH control, preservation etc. and storage techniques for urine samples in metabolomic studies may ensure that sample reflects the original metabolic state of subject only and not any analytical error.

EFFECT OF TEMPERATURE

The effect of temperature on urine storage was observed at room temperature for 24 hr and variations were observed in concentration of creatinine, hippurate and acetaminophen (paracetamol) glucuronide and sulphate conjugated by Barton et al. on comparision of raw urine at t= 0 hr and t = 24 hrs at 4°C prior to freezing at -80°C or -196°C. The authors have reported that urine samples remain stable at 4°C for 24 hrs and should be stored in deep freezer within this duration as small variations were observed by 36 hrs (Barton, Waterman et al. 2010; Rasmussen, Savorani et al. 2011). The effect of temperature and its duration had also been reported in another study as well while co-consideration of gender differences among the healthy volunteers. In female urine acetate, benzoate, creatine, glycine, lactate, malonate, succinate, trimethylamine, and formate increased, while creatinine, urea, guanidinoacetate, hippurate and citrate decreased whereas in male urine, only three metabolites creatinine, creatine, and phenylacetylglycine showed significant variations over the four weeks after a 4-week period of storage at room temperature (22°C), which indicated that male urine was more stable at room temperature. The same study had also reported the effect of urine sample pre-preparation on metabolite concentration of female urine by analyzing the raw urine

sample, centrifuged urine, filtered urine and addition of preservative sodium azide (0.1, 1 and 10mM). The same study reported the effect of various temperatures (room temperature, 4°C and -80°C), various freeze-thaw cycles and different storage conditions during the period of 4 weeks on urine stability. Storage at 4°C produced slight variations in urine metabolic profile but its storage at -80°C reflected the best original composition with minimal metabolic perturbation (Saude and Sykes 2007). The alterations in urine metabolome over time were considered to be a consequence of oxidation reactions, bacterial degradation and/or enzymatic conversion of certain metabolites. A reduction in increase of acetate and succinate concentration under inert environment at room temperature was observed as compared to the corresponding urine samples kept under normal atmosphere. Decrease in glutamate/glutamine and lactate concentration were observed due to bacterial degradation as these alterations were completely removed when samples were pre-centrifuged with addition of sodium azide in it and stored at -80^{o}C.

The centrifugation of urine at 10,000 rpm for 10 min was reported to reduce the degree of sample decomposition over four weeks (Saude and Sykes 2007) which might be attributed to the presence of bacteria, erythrocytes, fungi and other non-cellular debris causing the sample degradation. The above observation suggested that the speed of centrifugation of sample may also effect the sample composition, for example, centrifugal speed of 1600 RCF avoids the breaking of cellular components present in urine (Koukoulaki, O'Donovan et al. 2008) while at the speed of 14,000 RCF, cellular components may break and get released in the sample. Therefore, the optimization of centrifugal speed for removal of cellular and non-cellular components from urine at different pre-centrifugal speeds became an important task to be answered. A study conducted by Bernini et al. had focused upon the optimal pre-centrifugation speed for urine samples so as to avoid cell breakage and improved spectral quality. The spectra obtained from raw urine samples (without any pre-centrifugation) were compared with the samples centrifuged at 450, 1000, 3000 and 11,000 RCF. The speed > 11,000 RCF showed closer correlation with the raw samples. The authors have suggested that initial centrifugation between 1,000 to 3,000 RCF would spun down the cellular components and following centrifugation of samples at 14,000 RCF would eliminate the suspended inorganic particles from the supernatant. The larger pH variations in chemical shifts of histidine and methyl-histidine and lack of new metabolites were reported in samples with higher cellular components. These two observations led to two conclusions: firstly that the concentration of cellular metabolites released during centrifugation were either below the detection limit of the instrument or high molecular weight metabolites and secondly, the presence of these metabolites cause the resultant pH variations in a sample

(Bernini, Bertini et al. 2011). The presence of γ-glutamyltransferase in the urine of healthy adults was responsible for disappearance of glutamate/glutamine. Similarly, the increase in succinate level and decrease of urea in urine was attributed to the presence of urease and isocitrate lyase (enzymes of bacterial origin). The addition of EDTA in urine samples resulted in inhibition of these enzymes and consequent metabolic perturbations. Thus, authors have suggested the management of urine samples as follows: (i) removal of cells and particulate matters through the combined use of a mild pre-centrifugation 1,000–3,000 RCF (5 min at 4°C) and filtration; (ii) long-term storage of samples in liquid nitrogen (or liquid nitrogen vapour) to avoid breaking of residual cells; (iii) fast processing (within 2 h for collection); (iv) storage at 4°C between collection and processing. Recommendations at points (iii) and (iv) aim at reducing the effects of any possible enzymatic/cellular activities (Bernini, Bertini et al. 2011).

The increase in acetate concentration and decrease in intensities of citrate, hippurate, benzoic acid, glycine and histidine were observed in the long-term storage of urine sample at 4°C. While at -25°C and -80°C, no significant variations were observed for 26 weeks (Lauridsen, Hansen et al. 2007). Another study on biofluids analysis by liquid chromatography and mass spectrometry has also reported that urine samples stored at -20°C do not show any significant variations in metabolic configuration for up to 6 months. Similarly, the number of freeze–thaw cycles (up to 9) did not seem to affect sample integrity. For short-term storage the use of 0–4°C in a fridge or a cooled auto-sampler for up to 48 h still seems to provide useable samples and may provide meaningful results (Gika, Theodoridis et al. 2008).

Effect of freeze drying and freeze thaw cycle: The raw urine samples that underwent repeated cycles of freeze/thaw over the 4-weeks had an intermediate degree of changes in concentrations of acetate, benzoate, citrate, creatine, creatinine, formate, glycine, hippurate, lactate, malonate, succinate, trimethylamine and urea, when these metabolites were compared with raw urine stored at room temperature and in the deepfreeze. There was no observed change in metabolites levels at -80°C for six months and non-significant minor changes were observed only after nine-months of storage at the same temperature (Saude and Sykes 2007). The effect of freeze drying on urine samples includes the loss of volatile metabolites of urine and its re-dissolution in D2O results in decrease of intensity of creatinine and hippurate resonances due to incomplete re-dissolution of these abundant metabolites. The creatinine CH2 signal at δ 4.06 disappeared almost completely in freeze-dried samples reconstituted with the D2O-based buffer, evidently due to deuteration at this position. Since deuteration of creatinine is subject to general-acid as well as general-base catalysis, it is expected to be fast in a complex solution such as urine, especially at high urine and buffer

concentrations. The changes in the 1H NMR profiles are due to freeze-drying and reconstitution themselves and not to prolonged storage of the freeze-dried samples. Therefore, although freeze-dried urine samples are expected to have excellent long-term stability, and redissolving in D2O makes water peak suppression easier, the process introduces alteration to metabolite profiles, in particular due to deuteration of enolizable metabolites via buffer catalyzed exchange. Therefore, freeze-drying and reconstitution of urine is not recommended (Lauridsen, Hansen et al. 2007).

Effect of buffer solution, filteration and preservatives: The chemical shift variations arising either due to pH change or due to ionic strength variation result in spurious interpretations. Such variations can be minimized with various methods like addition of metal chelators like EDTA, buffer solutions and preservatives. Therefore, systematic study of effect of these agents at different concentrations and determination of optimal concentration and quantity becomes an important issue for minimisation of pre-analytical bias in a study. The optimum pH value determination for a sample is not only necessary for chemical shift consistency but also for avoiding the degradation caused by strong acids. Similarly, due to low solubility of $Na_2HPO_4.12H_2O$ in water limits the use of high concentration buffer solution for metabonomic studies. Keeping these aspects in mind, the effect of pH, dilution and ionic strength on chemical shift variability was studied by Xiao et al. on nine urinary metabolites including acetate, hippurate, citrate (carboxylic acids), DMA, TMA (amines), creatinine, glycine, histidine (amino acids) and urea. The presence of ionisable groups in these metabolites made it liable to chemical shift variations occurring due to pH change. The chemical shifts of citrate, creatinine, DMA and TMA were highly sensitive for ionic strength and that of glycine and histidine showed only a mild salt sensitivity and at a given salt concentration, chemical shift variation was found to be inversely proportional to pH, as observed in resonances of citrate, creatinine, hippurate and glycine. These alterations were attributed to the ionization processes of functional groups present in above metabolites. For carboxyl and amino groups, the increase of pH leads to the increase of $RCOO^-$ or the decrease of $RNH3^+$ concentration which enhances field shielding effects on the protons in the molecules, and thus moving the proton peaks to upper field (or smaller chemical shift values). Moreover, different proton resonances of the same metabolite also showed the different changes in chemical shift values. For example, the ionization of the carboxyl group in hippurate resulted in much greater chemical shift changes for CH2 (0.22 ppm) than for aromatic protons (0.01 ppm); the ionization of –CONH in creatinine led to larger chemical shift changes for CH2 (0.23 ppm) than for CH3 (0.08 ppm). These shift occurred due to proximity effects of the ionizable groups on the observed protons, which is in reasonable agreement with previous findings from the pH effects on 13C

chemical shifts for short chain fatty acids. By taking chemical shift consistency, the signal-to-noise ratio, sample dilution effects and low temperature buffer storage into consideration, authors have suggested that K_2HPO_4/NaH_2PO_4 buffer (pH 7.4, 1.5 M) with the buffer–urine volume ratio of 1 : 10 was optimal for the NMR-based urinary metabonomic studies. With such preparation, the sample pH values were adequately controlled to pH 7.1–7.7 for normal human urine, and chemical shifts of most metabolites with ionizable groups were controlled within 0.002 ppm (1.2 Hz on 600 MHz data) the only exception of that for citrate and histidine. High resolution bucketing and full resolution data analysis can be carried out with some corrections for the chemical shifts of citrate resonances and two singlets of histidine in the aromatic region using already reported peak alignment methods. (Xiao, Hao et al. 2009).

The bivalent ions like Ca^{2+} and Mg^{2+} in urine are reported to bind with EDTA in urine sample. A study has explored the effect of this metal chelator on the frequency shifts observed in urine NMR spectra and proposed that EDTA improves both peak alignment and peak widths by efficiently reducing the metal-metabolite interaction (Asiago, Nagana Gowda et al. 2008). But Bernini et al had recommended that the addition of additives (like enzyme inhibitors) should be avoided because the required concentrations will introduce signals in the NMR spectra covering the resonance of metabolites and may also induce changes in pH, ionic strength, etc. thus further affecting the original NMR profiles (Bernini, Bertini et al. 2011). Therefore, optimal buffer addition and lesser time duration between sample collection and its acquisition will provide optimal results for metabonomic data.

Apart from raw urine analysis, urine is oftenly diluted or lyophilized for the identification and quantification of metabolites present in low concentration. While the raw urine defines the lower limit of concentration, the concentrated and lyophilised urine defines its upper limit and therefore, the varying degree of concentration require different buffer concentration. Therefore, a study was conducted for effect of buffer solutions on raw urine and ten times concentrated urine. Both states of urine were analysed with different concentrations of buffer solution and it was found that minimal pH variation and the consequent chemical shift variability was observes when 0.33M buffer solution was added to raw urine and 1.0M phosphate buffer solution to concentrated urine. The effect of sodium azide NaN_3 and sodium fluoride NaF was also assessed at 4^oC, -25^oC and -80^oC for 7 days and 26 weeks. The stability of urine samples could be extended with preservatives for short period of time but long storage of non-frozen samples is not recommended. Also, a downfield shift of 0.008ppm in citrate resonances was observed in urine samples with sodium fluoride. Shift of the citrate resonances was expected due to the binding of fluoride ion to metal ions, such as Ca2+

and Mg2+, present in urine. These metal ions form complexes with a variety of compounds, notably citrate, resulting in variations of chemical shifts. These apparent complexation effects suggest the possibility of stabilization of citrate chemical shift by addition of NaF or another complexing agent, especially when the concentration of metal ions is expected to be substantial and varying between samples. Whereas, the preservation of urine with 0.1% NaN3 gave 1H NMR patterns identical to those observed for non-preserved urines (stored at -25 and -80 °C). The significance of this finding was twofold. First, human urine samples stored frozen at -25 °C or below for up to 26 weeks do not appear to require preservation. Second, the presence of NaN3 did not cause any observable changes in the metabonome composition, despite the reactive nature of azide (a powerful nucleophile and potential participant in redox and complexation reactions). Use of this preserving agent is especially recommended when working with animal urine, where the risk of bacterial contamination is substantially higher than with human urine. (Lauridsen, Hansen et al. 2007). The increase in benzoate, creatinine, formic acid and trimethylamine concentration with time at room temperature and corresponding decrease in creatine, hippurate and citrate occurs due to bacterial activity in urine sample. This bacterial degradation can be minimized by addition of sodium azide in urine samples while the collection of urine samples. Addition of increasing concentration of sodium azide (0.1, 1 and 10mM) had shown inversely proportional relationship with sample stability. Also, the filtration of urine samples through a 0.22μm syringe followed by their storage at room temperature greatly reduced the degree of these metabolic change (Saude and Sykes 2007). The filtration with 0.22 μm syringe ensured the removal of cell debris from the urine sample (Bernini, Bertini et al. 2009; Bernini, Bertini et al. 2011).