CHAPTER: 02

VARIABLES IN SERUM METABOLOMICS

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Ch.Id:-ASU/GRF/EB/IASINAM/2022/Ch-02

DOI: https://doi.org/10.52458/9789391842741.2022.eb.grf.asu.ch-02

The metabolomics is an inter-disciplinary field of technology, biology and data mining. There are numerous variables which can significantly impact the data generated and may lead to erroneous results. In this chapter, we will talk about effect of temperature on metabolomic data.

2.1 EFFECT OF TEMPERATURE AND ITS DURATION

Room temperature: The time and temperature of separation of serum/ plasma from blood may introduce several metabolic perturbations. Therefore, a study was performed to analyse the quality of serum and plasma samples as a function of elapsed time (between 0 to 4 hrs) between blood collection and processing and temporal changes on serum/plasma from sample processing to freezing (up to 24hrs). The incubation of blood at 25°C caused significant changes in NMR profile of the serum/plasma which includes an increase in lactate, decrease in glucose concentration and variations in pyruvate concentration was observed linearly from 0-4 hours of sample collection and processing at room temperature. Decrease in glucose was found to be more significant in serum than plasma as EDTA has an inhibitory effect on metallozymes and metal dependent enzymes of glycolysis. The increase in lactate concentration was also quite obvious as it is an end product of anaerobic glycolysis. The trend in concentration of pyruvate increased in plasma at 25°C but remained constant in serum. Also, decrease in concentration of triglycerides, proline, choline and histidine and an increase in citrate concentration was observed in serum with increase in time duration for centrifugation. Similar trend was also observed in glucose and its derivatives when citrate, EDTA and oxalate/fluoride plasma was observed. Therefore, for a reliable evaluation of glucose, lactate and pyruvate whole blood should be processed within 2 hr from the time of collection, keeping it at 4°C, although the latter may still not be the best procedure for pyruvate. A decrease in intensities of fatty acids and VLDL/LDL was also observed due to the oxidation reactions at room temperature. There was no significant effect on proline resonance in plasma- EDTA /citrate samples while it was observed in plasma oxalate and fluoride samples and variations in only choline and proline resonances was observed in plasma. Thus, it was proposed that plasma is more stable at room temperature than serum. The authors had also considered the effect of light on storage conditions and found that light does not play a significant role in sample storage (Bernini, Bertini et al. 2011).

The serum and plasma samples after their separation from blood are analysed by 1H NMR spectroscopy and therefore, need to be kept in autosampler at room temperature for few hours. Therefore, the optimization of duration of time for non-significant changes in serum/plasma is crucial for minimal pre-analytical errors.

Considering this aspect of sample storage, effect of room temperature at biological samples was studied by Deprez et al. while performing optimization of protocols of serum and urine. The effect of room temperature on serum samples of rats which were kept in auto-sampler of the NMR spectrometer for 15hr and 24 hr was evaluated for corresponding metabolic perturbations. An increase in glycerol, tyrosine and phenylalanine resonances was observed at both the time points. The increase was attributed to consequence of degradation of macromolecules and subsequent release of bound aromatic amino-acids with time. The increase in glycerol signal occurred due to the lipase activity and therefore, study had suggested for addition of a lipase inhibitor to prevent lipolysis. (Lenz, Bright et al. 2004).

Plasma stored at room temperature for over twenty-four hours shows increase in intensity of lactate doublet at δ1.33ppm and has line broadening effect on the measurement of plasma lipoprotein methylene resonance line width (Lim, Price et al. 1991). The temperature dependence of plasma was investigated at temperature 292K and 310 K in a recent study, which has also reported that ^1H NMR signals from the fatty acyl side chains of the lipoproteins increased substantially with temperature (hence also molecular mobility), with a disproportionate increase from lipids in low-density lipoprotein. "The increase in intensity of lipid resonances with significant sharpening in line widths was observed relative to the resonances from the small molecules. This implies, therefore, that the lipoprotein line widths are dominated by T_2 relaxation rather than a dispersion of chemical shifts, and from the inverse relationship between T_2 and the rotational correlation time, as the particles become more motionally mobile at the higher temperatures, as expected, the T_2 values are increased. Selective changes are also visible, e.g., the cholesteryl C-18 axial CH_3 group of HDL progressively sharpens and increases in intensity (intermediate 298 and 304 K steps not shown), as do the signals from some of the ring current shifted CH_3 groups of amino acids in albumin. These HDL and albumin signals are not normally resolved in NMR spectra measured at lower frequencies. Other lipid resonances also increase in intensity with temperature, particularly those from the CH_2 protons at δ 1.2ppm, the $CH_2CH{=}CH$ signal at δ 2.0ppm, the $HC{=}CHCH_2CH{=}CH$ signal at δ 2.7ppm (which completely obscures the high frequency citrate signals at 310 **K),** the choline $N^+(CH_3)_3$ signal at δ 3.25, and the $CH{=}CH$ signal at δ 5.1. Two partially resolved signals from glycerol also sharpen with increasing temperature and the spin-spin coupling pattern for the γ-CH_2 of glutamine also changes, probably indicating a slight change in conformation of the molecule or of the no-equivalence of the methylene ^1H chemical shifts" (Carrola, Rocha et al. 2010).

4°C, -20°C and -80°C: The effect of temperature on serum separation from blood also plays an important role in introducing pre-analytical variations in metabonomics data. A

study has examined the effect of pre-centrifugation time and temperature on serum metabolites and the conditions were as follows: blood was allowed to stand for 4 hrs and 24 hrs at both the room temperature as well as at 4°C. The serum separated under these conditions was used for ^{1}H NMR spectroscopic analysis and observed an increase in lactate and decrease in glucose concentration is serum samples (Cheng, Chang et al. 1998). A validation study for high throughput ^{1}H NMR-based metabolic analysis of human serum and urine for large-scale epidemiological studies was performed by R.H. Barton et al. The first part of study was implemented as a blinded split-specimen exercise between the specimens frozen at t=0 hr (40 urine; 40 serum) and their matched aliquots held for t=24 hr at 4ºC prior to freezing. This exercise was difficult for serum due to the potential for variations in wetting, homogeneity and minor freeze–thaw-induced macrostructure degeneration in sera affecting sampling. A similar protocol was subsequently implemented for a further 36 serum specimens held at 4ºC for t=36 hr, matched against the t=0 hr specimens. Some multiple small differences in serum specimens stored for t = 36 hr at 4°C were detectable only by multivariate analysis, and were attributed to generalized alterations in proteins and protein fragments, and possibly trimethylamine-N-oxide. No other specific metabolite was implicated and the authors concluded that for the purposes of NMR-based analysis, storage of serum for up to t =24 hr at 4°C prior to freezing at -80°C or -196°C does not detectably affect the metabolic profile and the methodology is robust. Therefore, it is suggested that a robustly designed, observed and accurate protocol be adopted for the freezing of serum and plasma specimens, which includes rapid freezing of the sample to kinetically hinder the formation of ice crystals to avoid the rupture of lipoprotein outer bilayer (Rasmussen, Savorani et al. 2011).

In another study, spectral changes of human plasma stored at 4°C for as long as 6 days have been reported to be negligible (Asiago, Nagana Gowda et al. 2008). However, a decrease in methyl and methylene resonances of lipids in VLDL and chylomicrons and a broadening of all methyl and methylene resonances assigned to lipids has also been reported in human plasma after a prolonged storage (3-4 days) of samples at 4°C (Sitter, Sonnewald et al. 2002). Another study had reported the effects of storing rat plasma, at 4°C, on small metabolites and macromolecules by comparing between snap-frozen plasma analysed just after thawing and after storage for 1 week at the same temperature. 3-Hydroxybutyrate (δ 1.20), acetate (δ 1.91) and glycerol (δ 3.56 and 3.64) levels slightly increased while pyruvate levels (δ 2.36) decreased. The choline signal at δ 3.21ppm due to its methyl groups was also found to be increased in intensity. The reason behind this increase could be due to its release from phospholipids present in lipoprotein membrane. However, enzymatic lysis can also produce phosphocholine

(lecithin) instead of choline which would have the same chemical shift at δ 3.20 ppm. In a similar manner, the observed increase in glycerol may have resulted from the hydrolysis of some lipids by action of lipases. Since none of the changes were observed in deproteinised plasma samples stored at 4°C for three weeks therefore it was confirmed that enzymatic activity was majorly responsible for glycerol increase. An increase in concentrations of amino-acids were also observed in plasma stored at 4°C (Taylor, Wu et al. 2003; Lenz, Bright et al. 2004) Triglycerides in plasma are removed from chylomicrons and VLDL by the action of lipoprotein lipase which leads to the formation of glycerol and free fatty acids for their utilisation in adipose and muscle tissues (Howland 1990).

The effect of storage at -20° or -80°C for one month was investigated and was found to be insignificant. This observation is similar to previously reported results following storage of control human plasma for up to 10 days at -20°C (Moreno, Escrich et al. 1993). However, the results are different from earlier study in which the influence of storage conditions on the amino-acid concentrations in rat and human plasma was investigated. The study reported that changes in some amino-acid concentrations were observed in rat plasma stored at -20°C but these variations were not observed at -70 °C (Van Eijk, DeJong et al. 1994). The broadening of lipoprotein resonances has previously been described in human plasma stored at -20 8°C for 5 days (Bell, Sadler et al. 1987; Bell, Brown et al. 1988). The authors suggested that degree of enzymatic action varies from species to species and its inhibition is minimized but cannot be stopped. Therefore, different optimization techniques are required for storage and sample processing for different species. In a study, the effect of storage at -80°C for up to 9 months of storage had also been assessed. A plasma sample was divided into a number of separate aliquots which were frozen at -80°C. Three samples were thawed and analysed after 48 h, 3 months, 6 months and 9 months.

A study at storage effects at -70°C on lipid, apolipoprotein and lipoprotein measurements of unfractionated serum from normolipidaemic and hyperlipidaemic subjects for 10 days, 3 months and 6 months and reported that all metabolites showed minor and non-significant variations after six months of storage when compared with control (Koukoulaki, O'Donovan et al. 2008). A more recent study on effect of sample handling on quantitative lipid analysis had reinforced the similar findings and reported that the samples stored at -80°C for one week show significant variations in concentration of only three metabolites viz. diacylglycerol, phosphatidylcholine and triglyceride out of 786 macromolecules observed (Srivastava, Roy et al. 2011). Heparin is the recommended anticoagulant as EDTA gives additional signals in ^{1}H- NMR experiments.