A ^{1}H-NMR based metabonomics study of urine and plasma obtained from healthy human subjects

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OBJECTIVES

The aim of this study was to assess the variability of metabonomic data in clinical studies, with severe life-style and dietary restrictions.

Of particular interest were

a) the variability in urines
b) the optimum time-point representing least variability
c) the detection of diurnal variation
d) the variability in plasma
e) the application of PCA as a preliminary screen to identify outliers and/or subjects who don't conform to the protocol

INTRODUCTION

Metabonomics is well established as a means of disease and toxicity screening in experimental animals.

Metabonomics will find increased application in the study of healthy and diseased humans. However, one of the major obstacles in clinical investigations is the greater variability in a human population.

Here, we describe an investigation on the plasma and urine samples of healthy male volunteers de-signed to evaluate the variability in metabonomic data, when severe life-style and dietary restrictions are imposed.

12 *male* healthy volunteers

Collections on 2 study days, 14 days apart

Volunteers:	21+ years (mean 37±9) dietary restrictions* standard diet (CPU)
Urine collections:	first void (fasted) 0-12h (CPU, fixed diet) 12-24h (permitted foods)
Plasma collections:	9 am (CPU)

*Dietary Restrictions:	no alcohol, cherries, fish and cheese (Lindon *et al*. 1999 and personal observation)
Standard diet in CPU:	
Breakfast:	3 pieces of toast with tea/coffee
Lunch:	Vegetable soup, 2 packets of ham and salad sandwiches, banana.
Evening Meal:	Roast turkey with roast potatoes and vegetables Apple crumble and custard

METHODS and MATERIALS

Samples and Protocols:

- All subjects were from the AZ healthy volunteer panel.
- The volunteers were subject to a standard diet and exercise regime in the CPU unit on study days 1 and 2.

^{1}H NMR spectroscopy:

- 3ml aliquots of each urine sample were freeze-dried and reconstituted in 200µl D_2O for NMR analysis, in order to speed up analysis time.
- Plasma was analysed neat (200µl of plasma + 50µl D_2O)
- All spectra were referenced to TSP (δ_{1H} 0.0).
- NMR spectra were acquired out on a Bruker DRX500 NMR spectrometer using a 2.5 mm i.d. SEI microprobe.
- 64 scans were acquired into 64K data points over a spectral width of 9980Hz.
- Suppression of the water signal was achieved by applying the Noesypresat pulse sequence (Bruker Biospin Ltd.).
- ^{1}H CPMG spectra were acquired with 128 scans into 16k data points over a spectral width of 5995Hz, using a 96ms pulse train (τ= 0.5ms).

Human Urines - 3 time-points/day, 2 study days (Δ2 weeks): Is there diurnal variation?

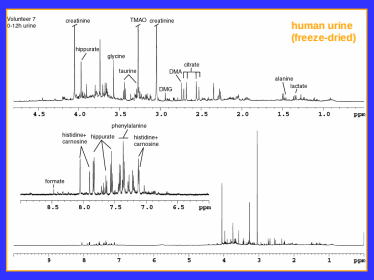


Figure 1: Representative ^{1}H NMR spectrum of freeze-dried urine

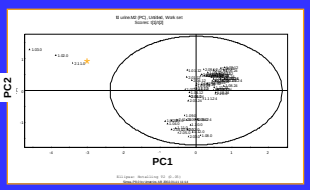
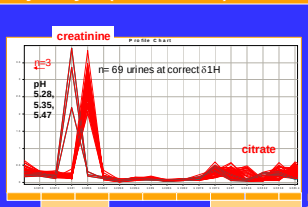


Figure 2: PCA scores plot of all urine samples (first void, 0-12h + 12-24h, Δ2 weeks, n= 72)

Outliers: first void, Vol. 2, SD1
first void, Vol. 3, SD1
first void, Vol. 11, SD2*

Key: SD= study day, Vol.= volunteer



Urinary pH values (mean ± SD)

Study day	first void	0-12 hours	12-24 hours
1	5.96±0.51	7.27±0.43	6.94±0.27
2	6.04±0.47	7.40±0.44	6.74±0.42

Figure 3: Urine spectra (first void, 0-12h, 12-24h, Δ2 weeks, n=72) reconstructed in Spotfire, highlighting pH shifts of creatinine + citrate. First void urines appear more acidic than subsequent time points.
(The integral bins are schematically represented. So is the merging of bins to produce superbins to avoid pH shifts)

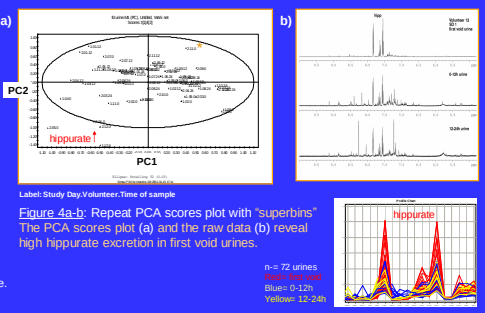
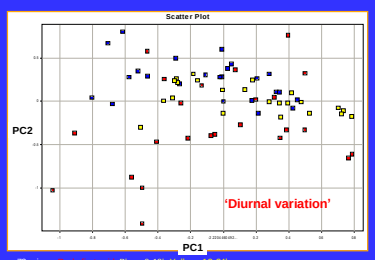
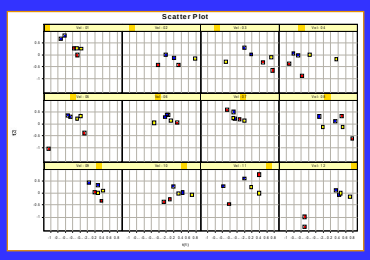


Figure 4a-b: Repeat PCA scores plot with "superbins" The PCA scores plot (a) and the raw data (b) reveal high hippurate excretion in first void urines.



n-= 72 urines, SD1= first void, Blue= 0-12h, Yellow= 12-24h

Figure 5: Corresponding PCA scores plot (in Spotfire for colour-coding) showing 'diurnal variation'



n-= 72 urines, SD1= first void, Blue= 0-12h, Yellow= 12-24h

Figure 6: PCA ' trellis plot' (in Spotfire for colour-coding) comparing every volunteer with himself (Δ2weeks).
Despite high inter-subject variability, generally every volunteer at a given time-point appears relatively consistent over 2 weeks.

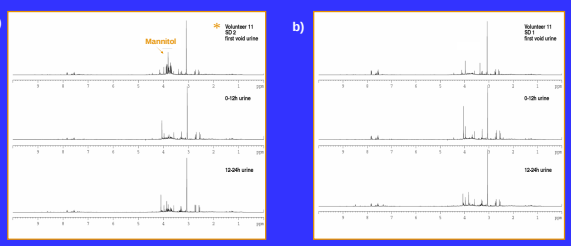


Figure 7 a-b: Urines from volunteer 11 displaying high mannitol excretion on study day 2 (first void urine), but not on study day 1.
This urine sample was not identified as an outlier by PCA despite the high concentration of mannitol (see Figure 4a). Mannitol is widely used as a dusting sugar and artificial sweetener.

Human plasma: 1 time-point/day, 2 study days (Δ2 weeks): How variable is the plasma data?

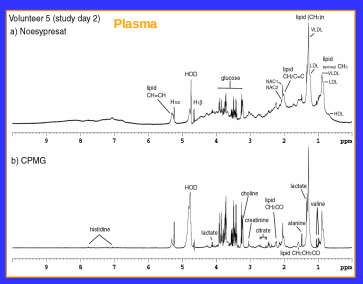


Figure 1: Representative ^{1}H NMR spectra of plasma.

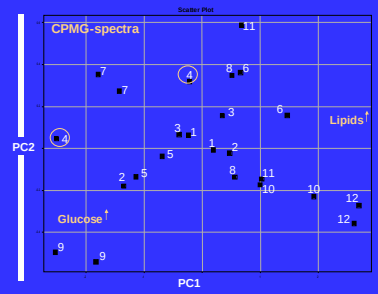


Figure 2: PCA scores plot (output into Spotfire) of plasma CPMG spectra (12 volunteers, single time-point, Δ2 weeks, n= 24) displaying variability mainly due to differences in the concentrations of glucose and lipid. Generally, despite inter-subject variability, intra-subject variability is "low" (with some exceptions, see Figure 3).

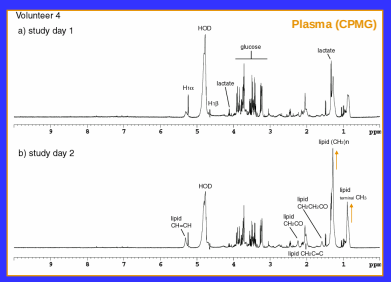


Figure 3: The CPMG NMR spectra of volunteer 4, displaying large differences in the glucose:lipid ratio between study days 1 and 2.

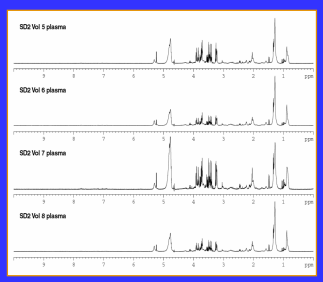


Figure 4: Representative CPMG NMR spectra of the plasma samples from 4 volunteers on study day 2, highlighting the natural variability in glucose:lipid ratio.

CONCLUSIONS

Urines:

1) Observation of distinct inter-individual variability (representing normal genetic variation), but generally low intra-individual variability over 2 weeks.
2) The first void urines were more variable than later time-points (may reflect differences in the subject's diets and life-style.
3) The urine samples showed some diurnal variation (high hippurate excretion in first void urines).
4) PCA didn't reliably highlight volunteer 11's urine sample (study day 2), containing high mannitol, as an outlier!

Plasmas:

1) Plasmas are prone to inter-individual variability due to differences in glucose:lipid concentration.
2) Hence, it may be difficult to assess 'biomarker effects' reliably *across a population.*
3) Generally, intra-individual variability was low over 2 weeks.
4) Blood sampling at the same time-point following dietary control may be advisable.

Urinary profiles are governed by dietary preferences and life style effects[2]!
Hence, standardisation of diet, life-style and time of sample collection appear to be highly advisable!

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