Eva Lenz:

Metabonomics, dietary influences and cultural differences:

a ^{1}H NMR-based study of urine samples obtained from healthy British and Swedish subjects

E.M. Lenz[1], J. Bright[2], I.D. Wilson[1], A. Hughes[3], J. Morrisson[3], H. Lindberg[4] and A. Lockton[3]

AstraZeneca Pharmaceuticals, Alderley Park, Macclesfield SK10 4TG, UK

[1]Drug Metabolism and Pharmacokinetics, [2]Global Enabling Science and Technology, [3]Experimental Medicine

[4]Research and Development, AstraZeneca, Lund, Sweden.

OBJECTIVES

The aim of this study was to assess the feasibility of metabonomic data in clinical studies.

Of particular interest were

a) the application of PCA as a preliminary screen in clinical studies to identify outliers and/or subjects who don't conform to the protocol.
b) cultural and dietary influences between subjects from Sweden and the UK.

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 two investigations on healthy subjects designed to evaluate the variability in metabonomic data, in view of dietary influences and cultural trends.

Study 1: Healthy British volunteers

120 human urines – set up as a blind study
No information on sex or age was disclosed initially.
NMR and PCA were performed separately.
Results were compared at the end.

Aim: Do volunteers conform to protocols?
How variable is the data?

Study 2: British *vs.* Swedish volunteers

Urines from 20 Swedish and 10 British healthy subjects

Aim: Are there cultural/dietary differences?

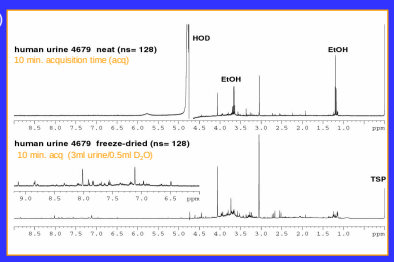
METHODS and MATERIALS

Samples and Protocols:
- All urine samples were collected at a single time point (first void urine)
- All subjects were from the AZ healthy volunteer panel
- Both sexes were employed for the studies.
- Age range: Study 1: 18-65 years (39±9 mean±SD)
  Study 2: 21-65 years (53±12 mean±SD)

^{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.
- 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.).

Study 1: 120 British volunteers. How compliant are human volunteers to clinical protocols?

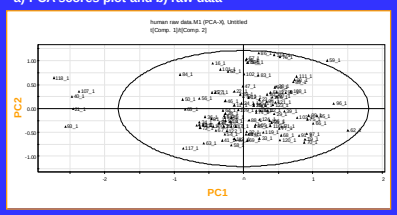
Figures 1a+b: The effect of freeze-drying to reduce analysis time.
Neat urine vs. freeze-dried



Freeze-drying causes loss of volatiles (e.g.EtOH) throughout.

Freeze-drying causes a shift of histidine signals, loss of urea, and collapse of the hippurate glycine signal into a singlet.

Figures 2a+b: Urines from 120 healthy volunteers
a) PCA scores plot and b) raw data

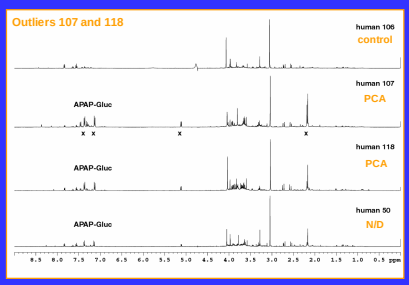


Q1: Can we distinguish between gender?
A1: No

Q2: Can we identify urine no. 50 as APAP-glucuronide containing?
A2: Not obviously

Outliers: 118= APAP-glucuronide
107= APAP-glucuronide
40= unknown triplet(aliphatic)
21, 93= creatinine-deuteration

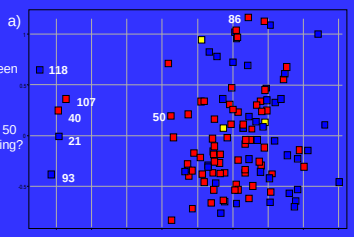
where is 50= APAP-glucuronide?
...and other outliers?



Key: x= paracetamol-glucuronide; PCA= successfully detected and N/D= not detected by PCA. Urine 106 serves as 'control'.

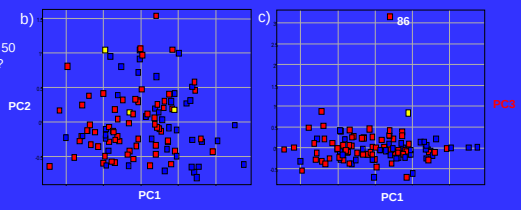
Q: Can we find urine no. 50 by excluding the outliers?
A: No

Figures 3a-c: The PCA scores plots exported into Spotfire for colour coding

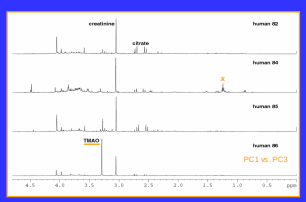


Key:
Red= female
Blue= male
Yellow= gender not disclosed

PCA scores plots 'excluding outliers' : re-submission of 'residual data-set' to PCA to 'find urine no. 50'



Outlier 'no. 86' in PC1 vs. PC3 displays high TMAO (fish-diet)

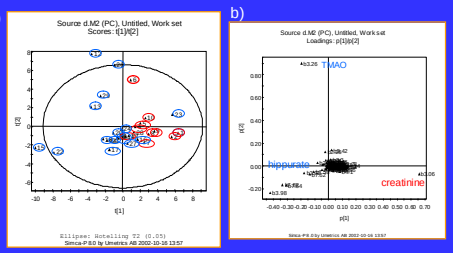


CONCLUSIONS :

1) Freeze-drying results in loss of volatiles (e.g. alcohol), throughout.
2) Based on the urinary profiles, a sex difference could not be detected.
3) PCA does not reliably identify non-compliant individuals (e.g. urine no. 50).

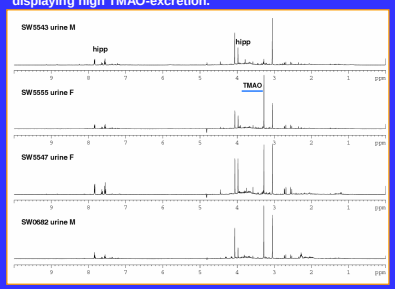
Study 2: Swedish *versus* British volunteers. Are there cultural and dietary differences?

Figure 1: Urines from British vs. Swedish Volunteers
a) PCA scores plot and b) corresponding loadings plot



Key: SE= red, SW= blue

Figure 2: Representative ^{1}H NMR spectra of Swedish Urines displaying high TMAO-excretion.



Key: TMAO= Trimethylamine-*N*-oxide, associated with fish diets[4].

Figure 3: Representative ^{1}H NMR spectra of British Urines. Urine no. 6 displays unusually high taurine[5].

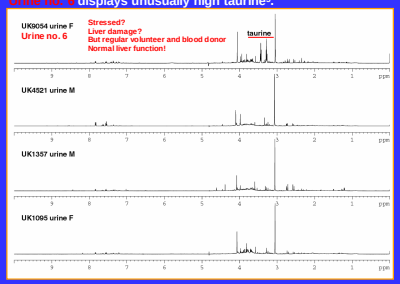
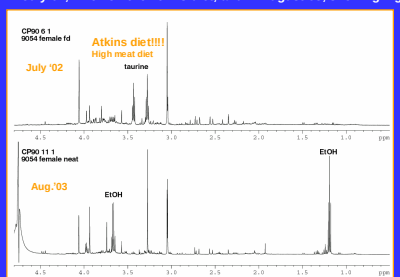


Figure 4: ^{1}H NMR spectra from British female volunteer 9054 (urine no. 6) acquired in July'02, when on the Atkins diet, and in August'03, showing high EtOH.



Note: The urine acquired in July'02 was freeze-dried (fd), while the urine in August'03 was neat.

CONCLUSIONS

1) The Swedish subjects excreted higher TMAO and hippurate.
2) TMAO is associated with fish-consumption[4].
3) The British subjects excreted more creatinine.
4) Taurine was unusually high in urine no. 6, from a female British volunteer, and has been associated with the Atkins Diet (high meat and shell-fish intake) – not liver damage[5].

Urinary profiles are governed by dietary preferences and life style effects!

Hence, great care needs to be taken in the interpretation of "biomarkers of disease and response to drug therapy" for diagnostic purposes.

REFERENCES

[1]Metabonomics, dietary influences and cultural differences: a ^{1}H NMR based study on urine samples Obtained from healthy British and Swedish Subjects.
E.M. Lenz, J. Bright, I.D. Wilson, A. Hughes, J. Morrisson, H. Lindberg, A. Lockton
J. Pharm. Biomed. Anal. (2004) 36, 841-849.

[2]A ^{1}H NMR based metabonomic study of urine and plasma samples obtained from healthy human subjects.
E.M. Lenz, J. Bright, I.D. Wilson, S.R. Morgan, A.F.P. Nash
J. Pharm. Biomed. Anal. (2003) 33, 1103-1115.

[3]Rapid and non-invasive diagnosis of the presence and severity of coronary heart disease using ^{1}H-NMR-based metabonomics.
Brindle J.T., Antti H., Holmes E., Tranter G., Nicholson J.K., Bethell H.W.L., Clarke S., Schofield P.M., McKilligin E., Mosedale D.E. & Grainger D.J.
Nat. Med. (2002) 8(12), 1439-1444.

[4]Influence of feeding on metabolite excretion evidenced by urine ^{1}H NMR spectral profiles: a comparison between subjects living in Rome and subjects living at arctic latitudes (Svalbard).
C. Zuppi, I. Messana,F. Forni, F. Ferrari, C. Rossi & B. Giardina (1998)
Clin. Chim. Acta 278, 75-79.

[5]An hypothesis for a mechanism underlying hepatotoxin-induced hypercreatinuria.
T.A. Clayton, J.C. Lindon, J.R. Everett, C. Charuel, G. Hanton, J-L. Le Net, J-P. Provost & J.K. Nicholson
Arch. Toxicol. (2003) 77, 208-217.