Research Article

Biomedical Chromatography

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Lysophosphatidylcholine—biomarker of Metformin action: studied using UPLC/MS/MS

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ABSTRACT: An UPLC/MS/MS based metabonomic method was developed and applied to the elucidation of biomarker of metformin action. The plasma metabolite profiling in healthy volunteers before and after per os metformin was determined with UPLC/MS/MS and analyzed by using multivariate statistics. Significant difference in endogenous metabolite profiles was revealed before and after administration of metformin. Four biomarkers found were lysophosphatidylcholines (LPCs), and their structures were tentatively identified to be 16:0 LPC, 18:0 LPC, 18:1 LPC and 18:2 LPC according to the molecular ions information and corresponding fragments of product ion scan. Lysophosphatidylcholine in blood may be involved in metformin treatment. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: UPLC/MS/MS; metabonomics; metformin; biomarkers; lysophosphatidylcholine

Introduction

Metformin (Saenz *et al*., 2005) is a widely used drug in the therapy of patients suffered from diabetes mellitus. Despite the fact that its precise mechanism of action is not completely elucidated, long-term treatment with this drug in monotherapy improves glycaemic control (Kirpichnikov *et al*., 2002; Krentz *et al*., 2005) and reduces cardiovascular mortality (Lord *et al*., 2003) in overweight type 2 diabetic patients. Clinical evidence produced over the years suggests that metformin is useful in some clinical conditions other than diabetes mellitus (Cusi and deFronzo, 1998).

Metabonomics is a platform identifing and measuring metabolic profile dynamics of host changes as the result of exposure to a toxin or drug, to environmental effects, or to the onset of disease (Nicholson *et al*., 2002). Based upon the multivariate analysis of complex biological profiles, metabonomics has been used for toxicological screening, disease diagnosis and the mechanism of action (Robertson *et al*., 2000; Lindon *et al*., 2004; Bollard *et al*., 2005; Nicholson *et al*., 2005; Chen *et al*., 2006). Recently, high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC/ESI-MS), either alone or combined with other analytical devices, has been used for detecting and profiling endogenous metabolites in biofluids (Yang *et al*., 2004; Wang *et al*., 2005a; Castro-Perez *et al*., 2005; Williams *et al*., 2005). These approaches demonstrate that hyphenated LC/MS techniques are viable analytical techniques for metabonomic analysis, owing to the high chromatographic resolution, sensitivity and reproducibility.

In this paper, a method of ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS) was developed in combination with multivariate statistical analysis in order to elucidate the action mechanism of metformin.

Experimental

Chemicals and Reagents

Metformin hydrochloride tablets (Glucophage) were manufactured by Sino-American Shanghai Squibb Pharmaceuticals Ltd. Formic acid and acetonitrile of HPLC grade were obtained from Dikma (Richmond Hill, NY, USA). All other chemicals were of analytical grade. Water was purified by redistillation and filtered through 0.22 μm membrane filter before use.

Sample Collection and Preparation

After approval of the study protocol by local ethics committee, 20 healthy male volunteers aged between 18 and 45 years gave their written informed consent to participate in the study. At screening, a physical examination including ECG, clinical chemistry, hematology and serology testing for hepatitis A and B as well as HIV-1/-2, revealed no abnormal findings. This was a one-self control study with metformin tablet 500 mg twice daily and the dosing period was 7 days. Five-milliliter blood samples were obtained before dosing and 24 h after last dosing. Samples were collected by venipuncture or indwelling catheter into heparinized evacuated tubes. Plasma was removed by centrifugation at 4°C and stored in glass vials at −70°C until analysis.

To a 0.4 mL aliquot of serum samples, 0.8 mL acetonitrile was added for protein precipitation. After centrifugation at 13,000 rpm for 10 min, the supernatant was transferred and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dried residue was then reconstituted in 0.1 mL of acetonitrile–water (10:90, v/v). The contents were transferred to 2 mL glass vials and an aliquot of 5 μL was injected into the UPLC/MS/MS system for analysis.

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Abbreviations used: BPI, base peak intensity; ESI, electrospray ionization; PCA, principal components analysis.

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Liquid Chromatography

Liquid chromatography was performed on an Acquity™ UPLC system (Waters Corp., Milford, MA, USA) equipped with cooling autosampler and column oven enabling temperature control. Separation was achieved on an Acquity UPLC™ BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm) maintained at 40°C. A linear gradient was run at a flow rate of 0.25 mL/min with mobile phase consisting of 100% solvent A (0.1% formic acid in water) at the start (t = 0 min), linearly changing to 100% solvent B (0.1% formic acid in acetonitrile) at 37 min, followed by an equilibration of 100% A for 3 min. The autosampler was conditioned at 4°C.

Mass Spectrometry

Mass spectrometric detection was carried out on a Micromass Quattro micro™ API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The flow rate of desolvation gas was set at 400 L/h at a temperature of 300°C, the cone gas flow rate was set at 50 L/h and the source temperature at 120°C. The capillary voltage and the cone voltage were set at 3200 V and 38 V, respectively. Data were collected in centroid mode from *m/z* 100 to *m/z* 1000.

Data Collection and Analysis

For each analysis of plasma sample, a chromatogram that contained the information of retention times and related mass spectra of endogenous metabolites was obtained. The raw data were analyzed using the Mciromass MarkerLynx Applications Manage (version 4.0). MarkerLynx incorporating an Apex Track peak detection package allows detection and retention time alignment of the peaks eluted in chromatograms. The data were combined into a single matrix by aligning peaks with the same mass/retention time pair from each data file in the dataset, along with their associated intensity. The resulted three-dimensional data—mass/retention time, sample name and ion intensity—were analyzed by principal components analysis (PCA), a well-known pattern recognition method in which most of the variance within a dataset can be expressed by a small number of principal components (PCs). Each PC is a linear combination of the original data parameters and independent of the other PCs. As an unsupervised method, PCA was used to find out whether any intrinsic clustering exists within the data set of samples with score plot. The PC loading plot was employed to reveal the biomarkers represented by chromatographic peaks labeled with given retention time and mass/charge ratio (*m/z*).

Results and Discussion

UPLC/MS/MS Profiling of Plasma Samples

The analytical technique in metabonomics is used to provide comprehensive quantitative and qualitative information of endogenous metabolites in biological samples. The characters of UPLC/MS/MS enable the application of a fast gradient chromatographic separation without significant loss of information and make it a comprehensive and high-throughput analytical technique in metabonomics. The MS/MS detection provides additional selectivity for even co-eluted metabolites based on their difference in relative molecular masses and the ability to identify metabolites. In this study, full scan of human plasma samples was performed in both positive and negative modes, though positive ion mode gave more information-rich data than negative. Representative base peak intensity (BPI) chromatograms of plasma sample from a healthy male volunteer are presented in Fig.1. Extracted ion

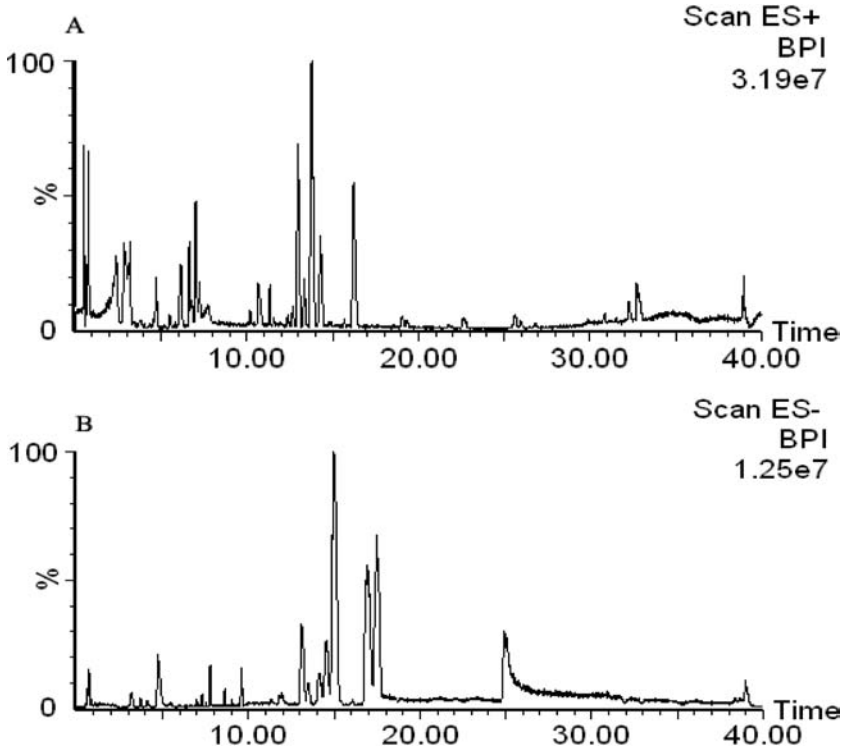

Figure 1. Positive (A) and negative ion (B) base peak intensity (BPI) chromatograms of plasma from a healthy volunteer.

chromatographic peaks of five ions (*m/z* 468.5, 496.4, 520.4, 522.4 and 524.4 in positive ion mode) were used for method validation (Gika *et al*., 2007). The sample stability after preparation was tested by analyzing a sample left at autosampler (4°C) for 4, 8, 12 and 24 h. The relative errors (RE) of peak areas were from −0.4 to 7.4%. The developed UPLC/MS/MS method was evaluated for its intra- and inter-day precisions. The intra-day precision (RSD) of peak areas and retention times was 2.6–4.1% and within 0.80% determined by analyzing five replicates of a sample at different times during the same day. The inter-day precision (RSD) was determined as 10.6–12.6% for peak areas and 2.5–4.6% for retention times respectively by repeating the analysis on three consecutive days.

Identification of Endogenous Metabolites

Sixteen endogenous metabolites were tentatively identified in the metabolite profile of the human plasma based on their molecular ion information and corresponding fragments of product ion scan with reference to the literature (Wang *et al*., 2005b). Figure 2 shows the positive [Fig. 2(A)] and negative [Fig. 2(B)] product ion scan spectra of metabolite with *m/z* 522.4 ($[M + H]^+$). In the positive ion mode, three major fragment ions are observed. The ions at *m/z* 103.8 and 184.1 represent the fragments of $[HOCH_2CH_2N(CH_3)_3]^+$ and $[H_2O_3PO–CH_2CH_2N(CH_3)_3]^+$, respectively, which readily give the head group information of phosphatidylcholine (PC) class. Another fragment ion with *m/z* 504.3 $[M–H_2O + H]^+$ further indicates that the metabolite may belong to lysophosphatidylcholine. In negative ES scan spectrum [Fig. 2(C)], the parent ion of the metabolite at *m/z* 566.4 represents its $HCOO^-$ adduct ion. In Fig. 2(B), the negative product ion scan spectrum of this lysophosphatidylcholine shows a fragment ion at *m/z* 506.4 representing the demethylated lysophosphatidylcholine, and an abundant fatty acid fragment ($[C_{17}H_{33}COO]^-$) is observed at *m/z* 281.3. Accordingly, the metabolite with *m/z* 522.4 ($[M + H]^+$) was identified as C18:1 lysophosphatidylcholine with its structure shown in Fig. 2(D). Other endogenous compounds were similarly identified. Qualitative results of 16 endogenous metabolites are shown in Table 1. Twelve of them are lysophosphatidylcholines (LPCs) and four phosphatidylcholines (PCs).

Biomarkers Related to Metformin Treatment

In order to determine the difference in plasma metabolites between healthy volunteers without and with metformin treatment from the complex information provided by metabolite profiles, PCA model was applied to analyze these data. The variables of *m/z and* t_R from UPLC/MS/MS spectra were set as columns of a matrix and 48 samples were arranged in rows of the matrix. Then PCA analysis was performed on the matrix.

Figure 3(A) shows the PCA score plot, where each spot represents a sample. The samples are roughly classified into two groups, one is before and the other after metformin treated group. Figure 3(B) is the loading plot that indicates the most influential metabolites responsible for the separation between sample classes. The metabolites having the greatest influence on the PCA score plot are those furthest away from the main cluster labeled with their retention time and molecular ion *m/z*. These endogenous metabolites should be the biomarkers related to metformin treatment. The possible biomarkers as indicated in Fig. 3(B) are those with *m/z* $[M + H]^+$ 496, 520, 522 and 524 in positive-ion mode. They are 16:0 LPC, 18:2 LPC, 18:1 LPC and 18:0 LPC identified as described above. 16:0 LPC and 18:0 LPC have been reported to be the biomarkers of diabetes (Wang *et al*., 2005a), while 18:1 LPC and 18:2 LPC are biomarkers related to metformin action newly found in this study. Figure 4 shows that the levels of the four lysophosphatidylcholines in plasma of volunteers dosed with metformin for 7 days were obviously decreased compared with the control group. This result is similar to a report in which a reduction of LPC in hepatic cells by metformin was found (Wanninger *et al*.,

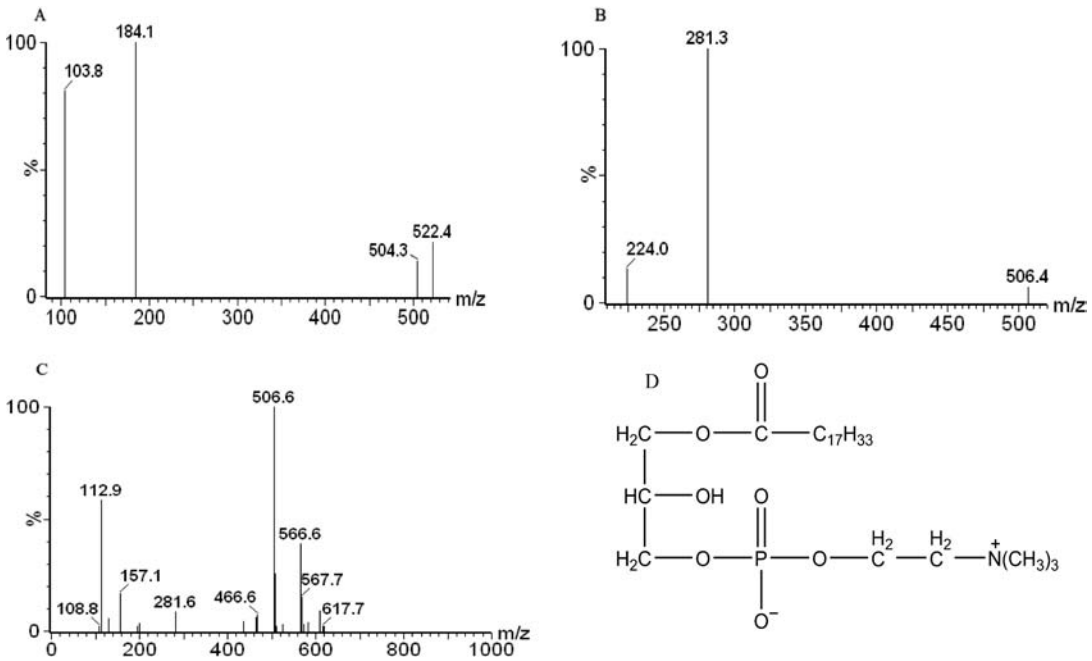

Figure 2. Product ion scan spectra of *m/z* 522.4 $[M + H]^+$ (A), *m/z* 566.6 $[M + HCOO]^-$ (B), negative ES scan spectrum of the biomarker at *m/z* 522.4 in positive ion mode (C) and the structure of identification (D).

Table 1. Qualitative identification results of plasma metabolite profiles

	Positive ion		Negative ion		
t_R (min)	Molecular ion ($[M + H]^+$)	Character fragments	Molecular ion ($[M + HCOO]^-$)	Character fragments	Identification
11.94	468.5	184.1, 450.3	512.5	227.3, 452.3	14:0 LPC
12.38	494.6	184.1, 466.2	438.6	253.3, 478.6	16:1 LPC
12.93	568.6	184.1, 550.3	612.7	327.1, 552.2	22:6 LPC
12.98	520.4	184.1, 502.3	564	279.4, 504.3	18:2 LPC
13.03	544.6	184.1, 526.4	588.6	303.2, 508.6	20:4 LPC
13.44	570.6	184.1, 552.3	614.8	329.4, 554.0	22:5 LPC
13.76	496.4	184.1, 478.3	540.4	255.3, 480.3	16:0 LPC
14.29	522.4	184.1, 504.3	566.6	281.3, 506.3	18:1 LPC
14.90	548.5	184.1, 530.5	592.3	307.4, 532.3	20:2 LPC
14.93	510.6	184.1, 492.4	554.6	269.5, 494.5	17:0 LPC
16.24	524.4	184.1, 506.6	568.6	508.4, 283.3	18:0 LPC
16.73	550.8	184.1, 532.8	594.8	534.1, 309.3	20:1 LPC
25.63	774.8	184.1	819	255.3, 295.2	36:0 PCp
30.81	782.9	184.1	827	279.1	36:4 PC
31.50	806.9	184.1	851	327.1, 283.2	38:6 PC
32.73	758.9	184.1	803	255.5, 279.2	34:2 PC

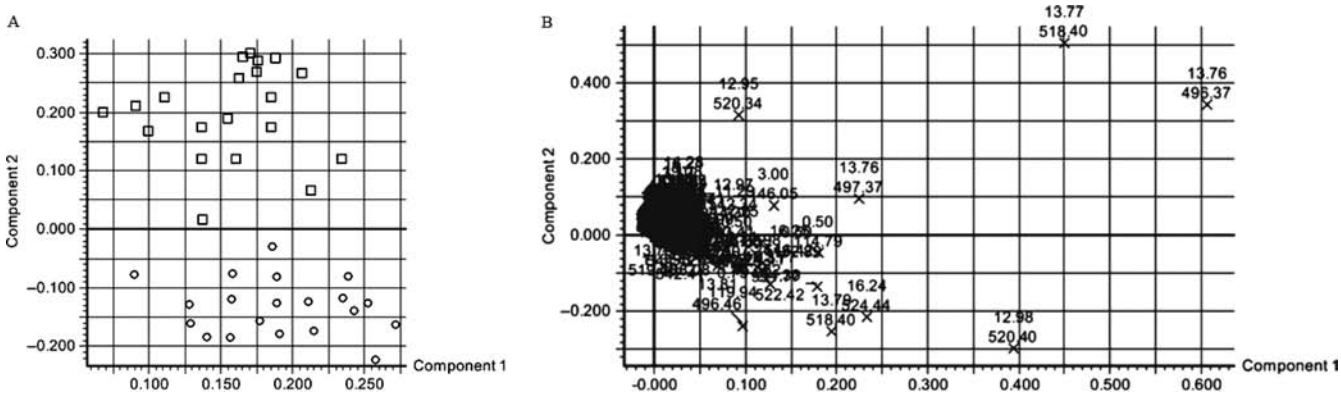

Figure 3. The result of principal components analysis derived from the UPLC/MS/MS profiles of plasma samples obtained from a healthy volunteer. (A) Score plot [PC1 *vs* PC2; control (○), treated with metformin (□)]; (B) loading plot with metabolites labeled with *m/z* $[M + H]^+$ and retention times.

2008). These results indicate that metformin regulates lysophosphatidylcholines level in plasma and improves lipid metabolism, which may be related to the therapy of metformin on diabetes mellitus.

Conclusion

In this paper, UPLC/MS/MS followed by multivariate statistical analysis was used to study the biomarkers of metformin action. Four potential biomarkers were discovered and all belong to LPC. Metformin treatment may involve the regulation of LPC level in plasma.

This work has illustrated the potential of metabonomics to elucidate the drug action and the combination of multivariate statistical analysis and UPLC/MS/MS method results in a very powerful tool for metabonomics research.

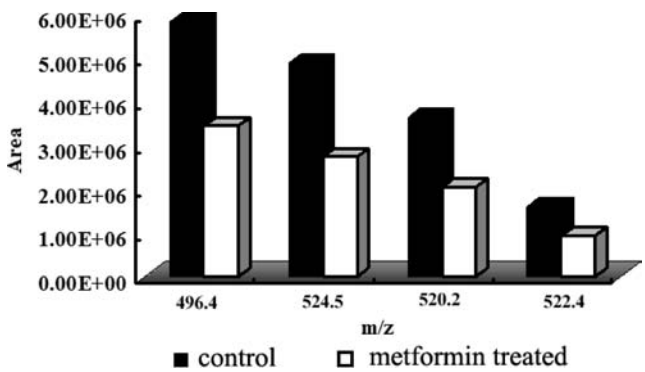

Figure 4. Mean peak areas of four biomarkers in metabolite profiles of control group and metformin treated group in positive ion mode.

Acknowledgements

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References

Bollard ME, Stanley EG, Lindon JC, Nicholson JK and Holmes E. NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. *NMR in Biomedicine* 2005; **18**: 143–162.

Castro-Perez J, Plumb R and Liang L. A high-throughput liquid chromatography/tandem mass spectrometry method for screening glutathione conjugates using exact mass neutral loss acquisition. *Rapid Communications in Mass Spectrometry* 2005; **19**: 798–804.

Chen M, Su M, Zhao L, Jiang J, Liu P, Cheng J, Lai Y, Liu Y and Jia W. Metabonomic Study of Aristolochic Acid-Induced Nephrotoxicity in Rats. *Journal of Proteome Research* 2006; **5**: 995–1002.

Cusi K and DeFronzo RA. Metformin: a review of its metabolic effects. *Diabetes Reviews* 1998; **6**: 89–131.

Gika HG, Theodoridis GA, Wingate JE and Wilson ID. Within-day reproducibility of an HPLC-MS-based method for metabonomic analysis: application to human urine. *Journal of Proteome Research* 2007; **6**: 3291–3303.

Kirpichnikov D, McFarlane SI and Sowers JR. Metformin: an update. *Annals of internal medicine* 2002; **137**: 25–33.

Krentz AJ and Bailey CJ. Oral antidiabetic agents: current role in type 2 diabetes mellitus. Drugs 2005; **65**: 385–411.

Lindon JC, Keun HC, Ebbels TM, Pearce JM, Holmes E and Nicholson JK. The Consortium for Metabonomic Toxicology (COMET): aims, activities and achievement. *Pharmacogenomics* 2005; **6**: 691–699.

Lord JM, Flight IH and Norman RJ. Metformin in polycystic ovary syndrome: systematic review and meta-analysis. *BMJ (Clinical Research)* 2003; **327**: 951–955.

Nicholson JK, Connelly J, Lindon JC and Holmes E. Metabonomics: a platform for studying drug toxicity and gene function. *Nature Review Drug Discovery* 2002; **1**: 153–161.

Nicholson JK, Holmes E and Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. *Nature Review Microbiology* 2005; **3**: 431–438.

Robertson DG, Reily MD, Sigler RE, Wells DF, Paterson DA and Braden TK. Metabonomics: evaluation of nuclear magnetic resonance (NMR) and pattern recognition technology for rapid in vivo screening of liver and kidney toxicants. *Toxicological Sciences* 2000; **57**: 326–337.

Saenz A, Fernandez-Esteban I, Mataix A, Ausejo M, Roque M and Moher D. Metformin monotherapy for type 2 diabetes mellitus. *Cochrane Database Systematic Reviews* 2005; **20**: CD002966.

Wang C, Kong H, Guan Y, Yang J, Gu J, Yang S and Xu G. Plasma phospholipid metabolic profiling and biomarkers of type 2 diabetes mellitus based on high-performance liquid chromatography/electrospray mass spectrometry and multivariate statistical analysis. *Analytical Chemisty* 2005a; **77**: 4108–4116.

Wang C, Xie S, Yang J, Yang Q and Xu G. Structural identification of human blood phospholipids using liquid chromatography/quadrupole-linear ion trap mass spectrometry. *Analytica Chimica Acta* 2005b; **525**: 1–10.

Wanninger J, Neumeier M, Weigert J, Liebisch G, Weiss TS, Schäffler A, Aslanidis C, Schmitz G, Schölmerich J and Buechler C. Metformin reduces cellular lysophosphatidylcholine and thereby may lower apolipoprotein B secretion in primary human hepatocytes. *Biochimica et Biophysica Acta* 2008; **1781**: 321–325.

Williams RE, Lenz EM, Evans JA, Wilson ID, Granger JH, Plumb RS and Stumpf CL. A combined (1)H NMR and HPLC-MS-based metabonomic study of urine from obese (fa/fa) Zucker and normal Wistar-derived rats. *Journal of Pharmaceutical and Biomedical Analysis* 2005; **38**: 465–471.

Yang J, Xu G, Zheng Y, Kong H, Pang T, Lv S and Yang Q. Diagnosis of liver cancer using HPLC-based metabonomics avoiding false-positive result form hepatitis and hepatocirrhosis diseases. *Journal of Chromatography B* 2004; **813**: 59–65.