RESEARCH ARTICLE

Hepatic Metabolomic Profile of Atorvastatin and Fenofibrate in Hyperlipidemic Rats

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ABSTRACT

Background: Metabolomics is an international technique used in clinical practice to predict or evaluate diseases by studying the endogenous level of small molecule metabolites. The lipid-lowering effects of atorvastatin (ATO), fenofibrate (FEN), and ATO+FEN in hyperlipidemia induced by high cholesterol diet (HCD) were studied by metabolomics technology using Gas chromatography–mass spectrometry (GC-MS) as an analytical tool.

Objective: The aim of this research is to study the metabolomic profile of ATO, FEN, and ATO + FEN in hyperlipidemic rats.

Material and methods: Male adult Wistar rats (30) allocated into five groups (6 animals each): were fed with HCD for 8 weeks to produce a model of hyperlipidemia, then animals treated with 1-normal control (saline); 2- Model control (saline); 3-ATO group (10 mg/kg/day); 4-FEN (150 mg/kg/day); 5- ATO+ FEN (10,150 mg/kg/day respectively) for an extra two weeks orally. Fasting blood samples were gathered at the end of the experiment (week 10) from each rat via retro-orbital center used in the metabolomic analysis.

Results: Principal component analysis (PCA) score plot analysis resulted in distinguished metabolic profiles in relation to both the HCD and the treatments given. In the hyperlipidemia rats, the major metabolites altered were: cholesterol, acetoacetate, 3-hydroxybutyric acid, linoleic acid, ornithine, phenylalanine, galactose, D-glucose and pyruvic acid. The plasma phenylalanine level dropped after consumption of HCD and recovered by FEN, while in ATO and ATO + FEN groups were not recovered. The plasma linoleic acid level dropped after consumption of HCD and recovered by ATO, FEN, and ATO+FEN, but by ATO reversed to the highest concentration.

Conclusion: The present study identified new series of potential biomarkers (metabolites) to describe the metabolic consequences of hyperlipidemia during the progression of the disease and the course of treatment such as: acetoacetate, 3-hydroxybutyric acid, linoleic acid, ornithine, phenylalanine, galactose, D-glucose and pyruvic acid; which will help in discussing the predicted effects and side effects.

Keywords: Atorvastatin, Fenofibrate, High cholesterol diet, Hyperlipidemic, Metabolomics.

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INTRODUCTION

Hyperlipidemia is a chronic condition that affects the body in a typically invisible, incremental, progressive and systemic manner. Hyperlipidemia is characterized as a lipid metabolism disorder that results in an irregular rise in total cholesterol, low-density lipoprotein or triglycerides level (TC, LDL, or TG, respectively), and reduction in high-density lipoprotein level (HDL).[1] Systemic arteriosclerosis may be accelerated by the overt harm of hyperlipidemia and is an important risk factor for multiple disorders, such as stroke, coronary heart disease, myocardial infarction, and sudden cardiac death.[2] One of the core challenges doctors and researchers face surrounding hyperlipidemia is early diagnosis. Metabolomics methods provide further information about the altered pathways and diseases' causes and consequences.[3]

FEN (PPAR-α agonists) and ATO (HMG-CoA reductase inhibitor) are the most commonly chosen drugs for lowering plasma lipid levels as mono or combination therapy, despite the pharmacological mechanisms extensively studied. Still, their efficacy and side effects in patients with hyperlipidemia throughout long-term treatment seem confused. Traditional pharmacological techniques could not successfully illuminate these changes until now. Metabolomics is new methodologies for the study of pharmacological effectiveness, pathways, and metabolome have grown in recent years to be the focal point for research into pharmacology and pharmacodynamics.[4]

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This study aims to investigate the metabolic map using Gas chromatography–mass spectrometry (GC-MS) based metabolomics approach to assess lipid-regulating effects and adverse reactions of ATO, FEN, and ATO+FEN in HCD-induced hyperlipidemia rats. In order to achieve this goal, rats were used as an experimental model treated with ATO, FEN, and (ATO+FEN), and the metabolomics profile of the plasma samples (level of lipids, amino acids, sugars, and other metabolites) was studied by using GC-MS.

METHODS

Animals

Thirty adult male Wistar rats were used in this study, aged 4 months (weighing 200–220 g). Animals were reached and put in the animal house of College of Pharmacy/, Mustansiriyah University; they received water and normal pellets for 10 day to be acclimatized. Before the start of the protocol study, the animals were located for 10 days under controlled room temperature conditions (21 ± 1°C) and the light cycle of 12 hours light: 12 hours dark. The animals easily reached pellets and water. Approval of the Ethical Comity of the College of Pharmacy/ Mustansiriyah University was attained.

Study Design

The rats were allocated to five groups at random, each with six rats. Normal control group, fed with a normal diet from weeks 1–10. Model control and treated groups, fed with an HCD (2% (w/w) cholesterol, 28% (w/w) beef tallow, 70% (w/w) normal diet and vanilla powder 3 teaspoonful/kg)[5] during the first eight weeks, followed by a normal diet in weeks 9–10 without treatment in case of a model control group, with ATO (10 mg/kg daily) in case of ATO group, with FEN (150 mg/kg daily) in case of FEN group and with ATO+FEN (10, 150 mg/kg daily respectively) in case of ATO+FEN group, orally with gavage.

Sample Collection and Preparation

At the end of week 10 of the experiment (at 8:00 AM), samples of blood collected directly through the retro-orbital center into K3EDTA tubes and plasma samples were obtained by centrifugation of the blood samples at 2000×g for 10 minutes at 4°C; and then used for the metabolomic analysis, where all of the plasma samples were stored at -80°C until analysis.

Extraction of Metabolomics

Protein precipitation and plasma metabolites extraction were achieved according to the technique described by Karim LZA *et al*. (2019) using a chloroform-methanol procedure.[6] In order to avoid an enzymatic operation, plasma samples (100 µL) were held at room temperature for thawing (for 5 minutes), then immediately added 500 µL methanol (99.7%). Add 20 µL of (0.2 mg/mL) sorbitol stock solution as an internal standard (IS) to the mixture, then shacked in a water bath shaker (at 40 rpm at 70ºC for 15 minutes); (centrifugation at 4000 rpm for 10 minutes). The supernatant (250 µL) was mixed with 500 µL of pure water (HPLC grade) and 250 µL of chloroform and centrifuge (at 4,000 rpm for 15 minutes). The upper (polar) phase separates and then evaporate to dryness under a stream of N2 gas in a thermostatically controlled water bath (60ºC).

Derivatization of Plasma Metabolomics Extracts

Derivatization is achieved according to the technique described by Fiehn and Kind *et al*. (2005).[7] 30 µL of (15 mg/mL) methoxyamine HCL in pyridine add to each tube, and immediately sealed the tubes. Shake extracts in water bath shaker at (40 rpm at 30°C for 90 min). Add 90 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (MSTFA + 1% TMCS) to each tube, and immediately sealed the tubes. Shake samples in a water bath shaker at (40 rpm at 37°C for 30 minutes). Wait two hours before injecting the first sample into the GC-MS.

The GC-MS Operation Procedure

2 µL of each sample was injected in split/splitless injector manually by a 10 µL syringe. The GC oven heated as 10°C/min for 60°C to 325°C, 1-minute initial time and 10 minutes final time, running for 37.5 minutes and cooling down to 60°C. The ion source heat was adjusted to 220°C. Helium is being used as a flushed out flow carrier gas of 10.5 mL/min for 1-minute; a saver operated for 3 minutes at a rate of 20 mL/min. Mass selective detector (MSD) was put at 20 Hz signal data rate and set at 290°C for transfer line of the MSD. MS was operated on after 5.90 minutes of delay time of solvent.[8]

Statistical Analysis

Multivariate statistical analysis (MVSA) was performed using SIMCA-P v14.1 software (Umetrics, Umea, Sweden). PCA was used to calculate a basic model and overview the data, where data was visualized using PC scores and loadings plots.[9] The results were expressed as the mean of concentration in PPM ± standard error of the mean (SEM). One way ANOVA test, followed by a post hoc Tukey's multiple comparisons test, was used to detect the significant difference between groups. SPSS v25 software was used in the statistical analysis.[10]

RESULTS

All of the data from the normal control (C), model control (M), ATO (A), FEN (F), and ATO+FEN (D) groups at the 10-week experiment were processed using SIMCA-P v14.1; pattern recognition analysis was conducted by employing PCA to calculate the basic model and an overview of the data. Samples were scattered clearly for the first and third components. These two components clarified the R^2 value of 80.4% for all GC-MS response variables based on the statistical parameters from the PCA model. They projected a Q^2 value of 68.1% for all observation per sample variables.

All of the samples in group C (C1–6) assembled in the downright quadrant of the plot, suggesting a stable status of the metabolome during the 10 weeks of the experiment. While samples in group M (M1–6) assembled in the top and down left quadrant of the plot in different regions from group C indicated that the rat model of hyperlipidemia exhibited a distinct metabolic profile compared with normal ones. Samples in group A (A1–6) assembled in the downright quadrant of

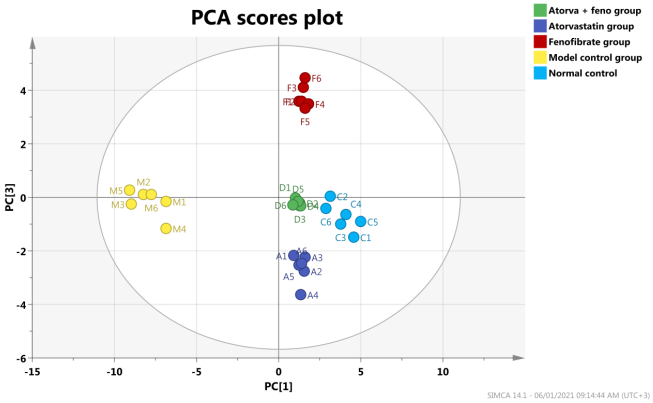

Figure 1: PCA scores plot of the five groups**.** The letters C, M, A, F and D denote the following different treatment groups: C (light blue) the normal control group; M (yellow) the model control group; A (dark blue) the ATO treatment group and F (red) the FEN treatment group; D (green) the ATO+FEN treatment group. The numbers 1–6 represent the numbers of the different samples at the 10-week experiment.

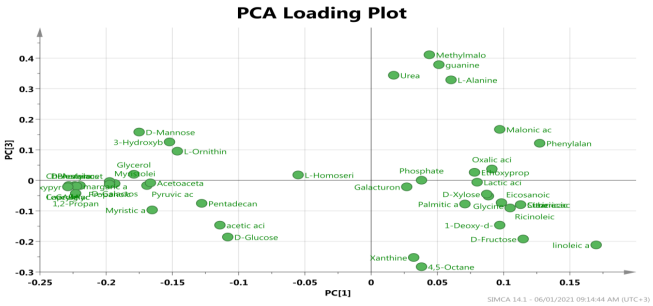

Figure 2: PCA loadings plot of the five groups**,** described variables accountable for the classification in the PCA scores plot.

the plot, and Samples in group F (F1–6) assembled in the top right quadrant of the plot, between C and M groups, indicating that ATO and FEN exhibited a therapeutic effect against hyperlipidemia and regular revert to normal status. Samples in group D (D1–6) assembled in the downright quadrant of the plot, between C, M, A, and F groups, indicating that ATO+FEN had a metabolic status between those of the other four groups and ATO + FEN exhibited a therapeutic effect against hyperlipidemia and regular revert to normal status (Figure 1).

Interestingly, samples of A, F, and D assembled in three different regions between C and M groups indicate that the rats had different metabolic responses to ATO, FEN, and ATO+FEN (Figure 1). The PCA scores plot of the five groups established significant effects of ATO, FEN, and ATO + FEN in lowering lipid profile in a rat model of hyperlipidemia. Variables accountable for the classification in the PCA scores plot were described in the PCA loadings plot (Figure 2).

The metabolomics determined in the plasma of the rat based on the degree of metabolomics density in C, M, and treated groups, further analyzed by ANOVA. The metabolomics data represented as the mean of concentration in PPM is indicated as colored blocks in (Table 1). Schematic overview of the metabolomics pathways that change significantly in normal, model control, and treated groups exposed in (Figure 3).

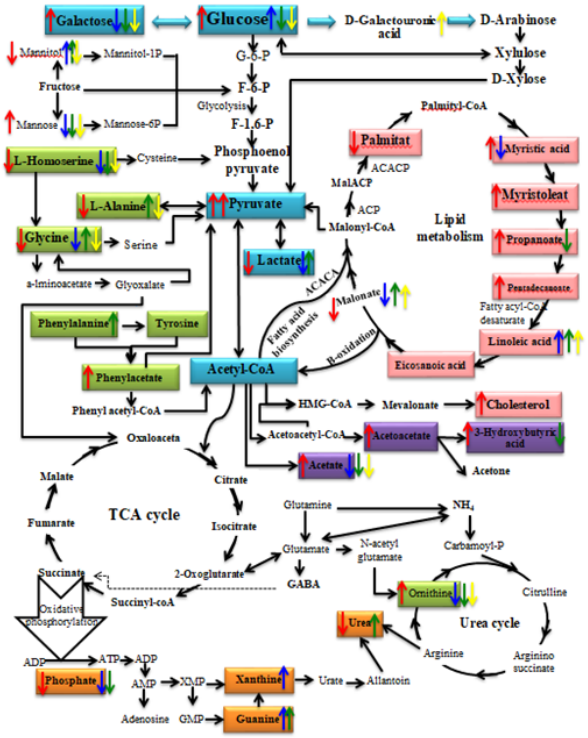

Figure 3: Schematic overview of the metabolomics pathways that change significantly in plasma: Model control group red arrows (↑) and (↓) compared with the normal control group. ATO group blue arrows (↑) and (↓); FEN group green arrows (↑) and (↓); ATO+FEN group yellow arrows (↑) and (↓); compared with the model control group. Where arrows represent metabolite up-regulation and down-regulation

DISCUSSION

Metabolomics is now considered an effective research field in studying plasma metabolism; there was also a great deal of attention in the use of metabolomics to recognize biomarkers in CVD.[11] In this study, a metabolomics approach based on GC-MS was used to present the metabolomics profile of rats plasma with hyperlipidemia induced by HCD. In the presence of ATO, FEN, and ATO + FEN as lipid-lowering agents resulted in clearly distinguished of metabolomics profiles of the C, M, A, F, and D groups from one another using a PCA score plot analysis, indicating: a stable rat model of hyperlipidemia; and ATO, FEN, and ATO + FEN exhibited a therapeutic effect against hyperlipidemia and regular revert to normal status (Figure 1). The present study identified potential biomarkers related to the plasma metabolism disturbance mainly involving glycolysis, metabolism of lipids, and metabolism of some amino acids by global metabolomics and may be used to identify the metabolic effects of hyperlipidemia during disease progression.

Cholesterol was found only in plasma samples of group M and was not appearing in C and treated groups. This established significant effects of ATO, FEN, and ATO + FEN in lowering lipid profile in a rat model of hyperlipidemia (Table 1), (Figure 3). The study demonstrated that the level of 3-hydroxybutyric acid and acetoacetate significant increased ($p < 0.01$) in model control compared with the

Table 1: Plasma metabolomics of normal control, model control, ATO, FEN, and ATO+FEN treated groups. Data presented as the mean of concentration in PPM ± SEM.

Metabolomes	*Normal control*	*Model control*	*ATO*	*FEN*	*ATO + FEN*
Glycine	6849 ± 2447.4	533.6 ± 22.5**	495.1 ± 123.4**	1197.3 ± 116.6*	368.8 ± 293.9**
L-Homoserine	8478.04 ± 2619.2	7791.5 ± 905.4	566.2 ± 132.7$^{**\#\#}$	3181.4 ± 986.5	639.1 ± 88.6$^{**\#\#}$
L-Alanine	233.1 ± 43.2	80.1 ± 10.8*	—	320.5 ± 50.1$^{\#\#}$	19.19 ± 2.8**
L-Ornithine	63.94 ± 18.83	154.85 ± 35.18*	33.87 ± 12.96$^{\#\#}$	77.92 ± 14.86$^{\#\#}$	22.7 ± 5.6E-16$^{\#\#}$
Phenylalanine	383.08 ± 78.37	—	—	210.54 ± 2.58*	—
Phenylacetic acid	—	119.61 ± 18.02	—	—	—
Pyruvic acid	—	4123.1 ± 1471.3	—	—	—
Malonic acid	128.01 ± 16.44	44.8 ± 11.06**	19.37 ± 0.0**	89.8 ± 17.1	45.8 ± 3.6**
Lactic acid	1814.5 ± 451.5	434.1 ± 84.8**	402.3 ± 76.5**	612.3 ± 101.5**	—
Citric acid	320.1 ± 0.0	—	—	—	—
Galacturonic acid	—	—	—	—	313.66 ± 100.98
D-Galactose	422.8 ± 115.2	3469.9 ± 823.3**	150.1 ± 24.3$^{\#\#}$	281.7 ± 85.6$^{\#\#}$	423.8 ± 79.7$^{\#\#}$
D-Mannose	651.1 ± 27.3	4864.1 ± 888.6**	1685.3 ± 277.7$^{\#\#}$	3123.1 ± 313.8*	1553.9 ± 471.9$^{\#\#}$
D-Glucose	3558.5 ± 530.0	8141.2 ± 1204.5**	8926.5 ± 1030.6**	4299.4 ± 380.3$^{\#}$	3347.9 ± 629.6$^{\#\#}$
Glycerol	146.6 ± 42.3	580.1 ± 120.9**	—	69.5 ± 4.8$^{\#\#}$	—
Cholesterol	—	134.99 ± 5.7	—	—	—
acetic acid	—	333.07 ± 155.7	213.5 ± 44.8	19.43 ± 3.8$^{\#}$	24.37 ± 1.06$^{\#}$
Acetoacetate	177.1 ± 61.8	3351.4 ± 1423.2*	—	—	—
3-Hydroxybutyric acid	342.8 ± 59.2	746.7 ± 142.4**	—	312.8 ± 72.9$^{\#\#}$	—
Myristic acid	88.06 ± 23.6	181.5 ± 14.7**	27.5 ± 4.6$^{*\#\#}$	—	—
Myristoleic acid	—	151.09 ± 43.8	—	—	—
Pentadecanoic acid	96.9 ± 0.0	124.5 ± 2.3E-15**	—	—	—
Palmitic acid	1068.6 ± 220.1	168.4 ± 19.6**	—	—	—
margaric acid	—	83.6 ± 10.9	—	—	—
linoleic acid	117.5 ± 5.2	—	152.7 ± 11.1**	65.29 ± 4.1**	46.23 ± 7.4**
Eicosanoic acid	91.7 ± 24.2	—	—	—	—
Propanoic acid	228.6 ± 4.4E-15	1989.7 ± 445.1**	—	10.5 ± 0.7##	—
Urea	402.4 ± 23.7	265.4 ± 0.0**	—	554.4 ± 0.0$^{**\#\#}$	—
Xanthine	—	—	239.9 ± 72.9	—	—
Guanine	—	—	96.8 ± 2.2E-15	652.5 ± 0.0	—
Phosphate	1205.8 ± 451.1	465.3 ± 87.5	96.8 ± 2.2E-15**	329.2 ± 78.2*	—

The data are presented as mean of concentration in PPM ± SEM, (*: $p < 0.05$ significant, **: $p < 0.01$ highly significant) compare to normal control, ($^{\#}$: $p < 0.05$ significant, $^{\#\#}$: $p < 0.01$ highly significant) compared to model control.

normal group and were not appearing in plasma samples of ATO and ATO+FEN groups, while the 3-hydroxybutyric acid significant reduced ($p < 0.01$) in FEN group compared with model control group (Table 1). This may indicate that endogenous cholesterol synthesis by acetyl-CoA could be inhibited with high exogenous levels of cholesterol. In the meantime, acetyl-CoA is also produced from fatty acids through a higher β-oxidation pathway. Therefore it is possible to transform excessive acetyl-CoA into ketone bodies, such as acetoacetate and 3-hydroxybutyric acid. Further, the reduction in 3-hydroxybutyric acid and acetoacetate after administration of ATO, FEN, and ATO+FEN indicate that changes in metabolic pathways of acetyl-CoA so less amount transform into ketone bodies. Xu *et al*. (2014) also demonstrated the high level of 3-hydroxybutyric acid in the model of hyperlipidemia and significant reduction after ATO and FEN administration.[3] The present study demonstrated that the level of saturated fatty acids (SFA) significantly rises ($p < 0.01$), myristic acid, pentadecanoic acid, and propanoic acid, in model control compared with the normal group. While the SFA such as pentadecanoic acid and palmitic acid were found only in plasma samples of a model control group and did not appear in treated groups' plasma samples (Table 1). This increasing related to excessive intake of SFA resulted in high levels of TC, TG, and LDL, which is the main reason for atherosclerosis and increased CVD risk.[12] While unsaturated fatty acids (USFA) such as eicosanoic acid was found only the C group, linoleic acid was found in C and treated groups (Table 1); (Figure 3).

This may indicate that there was excessive consumption of USFA during hyperlipidemia. These results indicate changing in metabolic pathways of SFA and USFA and also established significant effects of ATO, FEN, and ATO+FEN in lowering SFA and rising USFA in a rat model of hyperlipidemia. The process of transportation and metabolism of cholesterol must be combined with linoleic acid, and therefore lack of linoleic acid may lead to metabolic disorders and atherosclerosis gradually. Linoleic acids are mainly metabolized into arachidonic acid then to prostaglandins (PGs). PGs have many beneficial effects against CV risk, including hyperlipidemia and essential hypertension.[1,3]

The study demonstrated that the level of ornithine significantly rise ($p < 0.01$) in model control compared with the normal group and significantly reduce ($p < 0.01$) in treated groups compared with the model control group (Table 1). This finding indicates that lipid regulation and the pathological process might be predicting by ornithine (biomarker) during hyperlipidemia.[3] Phenylalanine was found only in C and F groups and did not appear in M, A, and D groups (Table 1), (Figure 3). This finding indicates that most amino acids in the body affected by hyperlipidemia and could result in higher protein catabolism,[13] and FEN has a significant effect while ATO and ATO + FEN have minimal effect on phenylalanine level recovery. Phenylalanine is a tyrosine precursor that converts then to catecholamines (epinephrine, norepinephrine, and dopamine) and plays a function in stimulating the metabolism of lipids.[14]

The study demonstrated a significant rise ($p < 0.01$) in the level of galactose, D-mannose, D-glucose, and glycerol, while the lactic acid significant ($p < 0.01$) decrease in model control compared with normal group. According to these results, there is crosslinking between hyperlipidemia and carbohydrate metabolism, where excessive HCD ingested lead to glycolysis inhibition and energy metabolism dysfunction.[15]

CONCLUSIONS

According to the results of this study, it can be concluded that HCD significantly induced hyperlipidemia. Metabolomics approach based on GCMS combined with MVSA, revealed the pathological changes in HCD-induced hyperlipidemia rats and the therapeutic processes associated with ATO, FEN, and ATO + FEN treatment, where oral administration to the rats led to regular revert to normal status with a synergistic effect in case of ATO + FEN. The present study identified a new series of potential biomarkers (metabolites) to describe the metabolic consequences of hyperlipidemia during the progression of the disease and the course of treatment: acetoacetate, 3-hydroxybutyric acid, linoleic acid, ornithine, phenylalanine, galactose, D-glucose and pyruvic acid.

ETHICAL CLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

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