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DOCTORAL (PhD) THESIS

INFLUENCE OF SOME MEDICINES USED IN THE TREATMENT OF ATHEROSCLEROSIS ON BLOOD FATTY ACIDS

- SUMMERY-

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T i m i ș o a r a
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SPECIAL PART

Keywords: Atherosclerosis, Rosuvastatin, Cholesterol homeostasis, Total fatty acids, Free fatty acid, Plasma lipids, GC-MS analysis.

GENERAL PART

The reason why this research topic was chosen was due the fact that cardiovascular disease generated by atherosclerosis is the origin of many deaths. In a ranking of causes of death in the USA in 2015, cardiovascular diseases are on the first place with 43.8%, followed by all types of cancer and respiratory diseases [1]. In Europe, cardiovascular disease increased in men from 1990 to 2015 by 33.57% [2]. This is the reason why the subjects selected for this study were men.

Atherosclerosis is a chronic inflammatory process, known for more than 500 years as one of the main causes of cardiovascular disease and implicitly a large number of deaths and people with disabilities.

The mechanism of atherosclerosis is complex and is generated by many factors. High blood lipid levels have been shown to promote atherosclerosis.

The most widely used drugs for lowering blood lipids are statins, niacin and fibrates or a combination of these [3]. Statins are most effective in treating people at high risk for atherosclerosis [4]. Statins reduce LDL (low density lipoprotein) cholesterol by up to 55% and triglycerides by up to 30% and increase HDL (high density lipoprotein) cholesterol by up to 14% depending on the dose and statin used [3].

The treatment with statins has shown substantial results in reducing the progression of coronary and carotid atheroma, as measured by intima media thickness, and may also lead to plaque regression [5]. Cholesterol-lowering statin therapy significantly reduces the possibility of cardiovascular disease [6, 7]. Studies in subjects with hypertriglyceridemia have shown that statins can also inhibit triglyceride synthesis [8]. Statins are associated with a decrease in the concentration of the entire set of free fatty acid (FFA) in plasma [9].

Large amounts of plasma FFA have contributed to an increase in the possibility of cardiovascular disease, which eventually leads to death [10, 11]. In addition, in some studies, associations have been made between type 2 diabetes and ischemic lesions with high levels of FFA [12, 13]. Recent studies have shown that each FFA has a different behavior in the pathologies of atherosclerosis. Thus, it was found that a high level of omega-3 acids leads to a decrease in blood pressure values [14]. Of the saturated fatty acids (FAs), only palmitic acid increased the chance of death in subjects with cardiovascular disease [15]. High level of trans FAs have been shown to be linked to inflammation and oxidative stress in

coronary heart disease. [16]. Many findings suggest that fatty acids with a high degree of unsaturation are of particular importance in all processes related to molecular recognition at the cell level as well as cellular metabolism. [17]. These results show the need to study each FA or class of FAs rather than analyze the entire set of FAs together.

SPECIFIC PART

This doctoral thesis had four main objectives. The experimental results show that all these objectives have been achieved.

OBJECTIVE 1

The first objective was to find and validate a new, simple and effective method to accurately determine the concentration of each fatty acid in overall fatty acids in blood lipids.

How this goal was achieved is presented in Chapter 4 [123]. In this chapter, we have developed a gas chromatographic method that can perform the simultaneous analysis of each fatty acid in total fatty acids along with the analysis of total cholesterol and neutral monosaccharides in blood, plasma or serum. The method was validated on whole blood and then was applied for plasma.

The method involves a step of quantitative release of fatty acids from all lipids by a basic hydrolysis with sodium hydroxide in a dimethyl sulfoxide medium, followed by the conversion of the carboxyl group from the fatty acid to a methyl ester by a reaction with methyl iodide.

This derivatization reaction converts all fatty acids to methyl esters in a short time. The novelty of the method is given by the realization in a single stage of hydrolysis and esterification. Also, the novelty is the esterification of fatty acids simultaneously with the per-O-etherification of neutral monosaccharides and that of cholesterol.

Therefore, a single chromatogram resulted in the separation of fatty acids, neutral monosaccharides and cholesterol. In a single analysis, each component of 3 different classes of substances is determined.

A series of laborious steps of sample preparation used in other methods of analysis were eliminated. The countless substances in the blood that could interfere during the analysis were largely eliminated as a result of the derivatization and extraction processes.

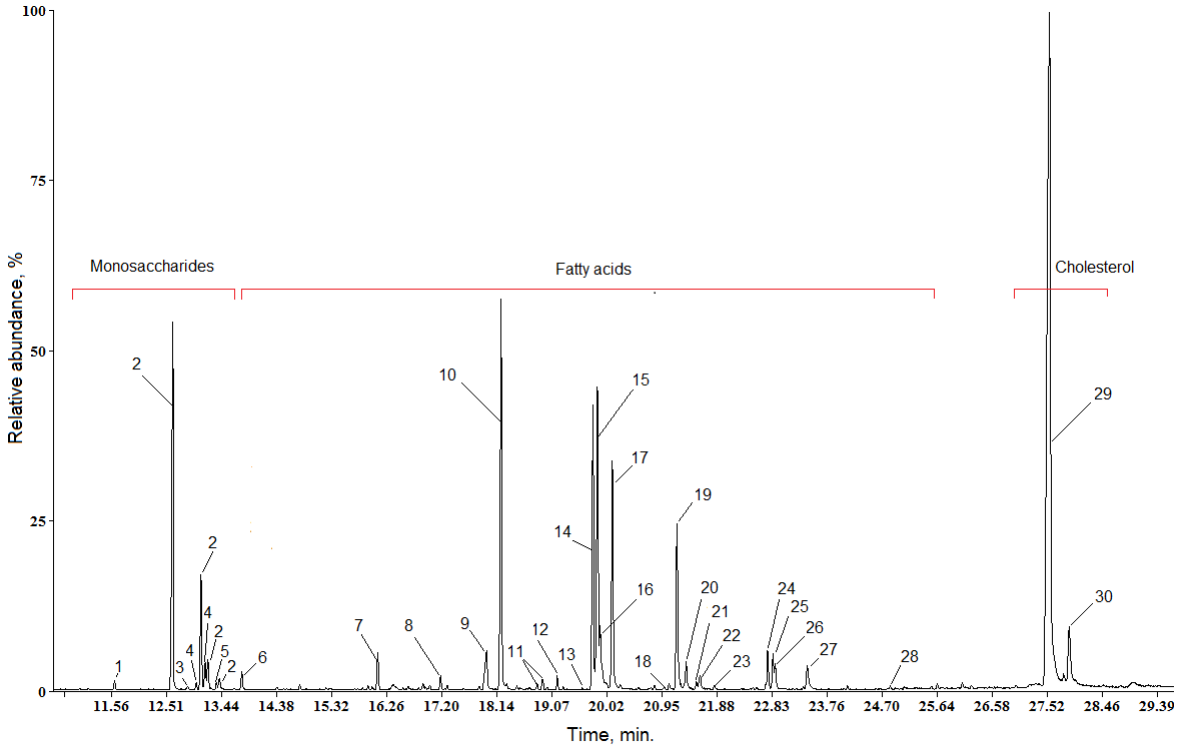

Figure 6. GC- total mass spectrometry ion chromatogram of cholesterol, fatty acids, and neutral monosaccharides, from blood after derivatization by methylation. Peaks: (1) methylated-1,5-anhydro-D-glucitol; (2) methylated-D-glucose; (3) methylated-D-fructose; (4) methylated-D-mannose; (5) methylated-myo-inositol; Peaks from 6 to 28 are fatty acids methyl esters and their identity is given in the Table 1. Peaks: (29) cholesteryl methyl ether; (30) cholesterol.

OBJECTIVE 2

The second objective was to find a technique for analyzing each fatty acid only in the fraction of free fatty acids in plasma and was developed in Chapter 5 [124]. The procedure described in this chapter provides a very fast and simple method for the selective methylation of free fatty acids with methyl iodide in dimethyl sulfoxide and in the presence of potassium carbonate at room temperature in a single step directly in plasma.

The methylation reaction was investigated in dipolar aprotic solvents such as DMSO (dimethyl sulfoxide), DMAA (dimethylacetamide), DMFA (dimethylformamide), and ethyl acetate and in the presence of anhydrous powder such as K_2CO_3, Na_2CO_3, $KHCO_3$, $NaHCO_3$, KOH, and NaOH. The best reaction time for total methylation of free fatty acids without transmethylation of lipid-bound fatty acids was less than 1 min at room temperature

using methyl iodide and anhydrous potassium carbonate or sodium carbonate in dimethyl sulfoxide. A large excess of both methyl iodide and base favors the selective methylation reaction. DMSO is less toxic and can solubilize proteins better than other members of this class, such as DMFA and DMAA.

The main problem in the direct methylation of free fatty acids in plasma is the presence of fatty acids covalently bonded in triglycerides, cholesteryl esters, and phospholipids in the plasma sample. Saponification and transmethylation of covalently bonded fatty acids could occur in the presence of bases, altering the final results.

Optimal reaction conditions are mild for lipids and the transmethylation of covalently bonded fatty acids from plasma has been avoided. The resulting fatty acids methyl esters were introduced directly from the reaction medium into GC inlet or can be extracted with n-pentane for storage.

The limits of detection were around 0.1 ng/µL. Precision and accuracy were highlighted by low variations and good recovery. This method avoids laborious sample preparation such as thin layer chromatography or liquid-liquid extraction of free fatty acids from human blood.

Table 7 compares our method with other selective esterification of free fatty acids directly in plasma. Our proposed method requires the shortest derivatization time (1 min), avoids the transmethylation of covalently bonded fatty acids from lipids and performs a complete methylation of free fatty acids. The derivatization products can be injected without extraction.

Table 7. The main characteristics of the methods used for the direct methylation of free fatty acids in plasma.

Characteristic	Methylation method			
	Pace-Asciak [11]	Tserng and al. [12]	Lapage & Roy [13]	Proposed method
Reagent	Diazomethane-Methanol	2,2-Dimethoxypropane-Hydrochloric acid	Methanol- Acetyl chloride	Methyl iodide Solid K_2CO_3 or Na_2CO_3
Solvent	Diethyl ether	Water-Methanol	Methanol	Dimethyl sulfoxide
Temperature, °C	22	Room temperature	24-29	20
Reaction time, min.	10	15	45	1

The amount of derivatization reagents does not have to be very precise, being used in a large excess and without generating side products. These reagents are more accessible to researchers and do not require special conditions for handling.

OBJECTIVE 3

The third objective was to measure the change in the concentration of each fatty acid in the overall fatty acids and free fatty acids after rosuvastatin treatment and to determine the factors that cause these changes in plasma. This objective was developed in Chapter 6 [147].

Measurement of changes in the concentration of each acid in fatty acids as a whole and in all free fatty acids in plasma was studied in subjects over 65 years of age who did not have cardiovascular disease, hypertension, or diabetes, but had a high concentration of cholesterol. The process was placebo-controlled, double-blind, randomized, and crossover study. The subjects, data collectors, and outcome analysts were all unaware of the treatment received by subjects. The participating subjects were educated about lifestyle imposed during this study and they agreed to respect the lifestyle modification, regarding diet and exercise. They all ate the same habitual diet throughout the study period. Gas chromatography (GC) was used for individual analysis of the fatty acids. The technique needs the conversion of fatty acids into methyl esters.

The results from this chapter shows that rosuvastatin treatment significantly decreased the absolute concentrations of each saturated and monounsaturated FAs in the FFA fraction, as well as in the total FAs. Table 11 shows the change from baseline of absolute concentration and precentral change of each fatty acid in total plasma during rosuvastatin treatment. Fatty acids with more than two double bonds had small decreases. The exception is for polyunsaturated fatty acids with 20 - 22 carbons in the molecule, which had no significant change in the fraction of FFAs and a small increase in the total FAs.

The effects of rosuvastatin treatment on the level of each fatty acid in the fraction of FFAs and total plasma lipids may be a source of beneficial consequences, in addition to lowering LDL-cholesterol in cardiovascular diseases.

Our findings provide an explanation of the changes in the level of each plasma fatty acid based on the mechanisms of cholesterol homeostasis. From our experimental results, it can be assumed that by blocking the biosynthesis of cholesterol by rosuvastatin, the regulatory

mechanisms in cholesterol homeostasis will try to maintain the equilibrium with less cholesterol, changing the concentrations of lipids involved in cholesterol homeostasis.

Rosuvastatin is directly involved only in the biosynthesis of cholesterol and indirectly by cholesterol homeostasis in the biosynthesis of fatty acids, cholesteryl esters, phospholipids apolipoprotein A-1, apolipoprotein B-100, and triglycerides.

Table 11. The change from baseline of absolute concentration and precentral change of each fatty acid in total plasma during rosuvastatin treatment.

Fatty acid	Change from baseline, µmol/L (Mean ± SD).	Mean change, %	Effect, *p* value
Dodecanoic	-62.33±5.58	-49.01	< 0.00001
Tetradecanoic	-148.93±19.64	-49.18	< 0.00001
Pentadecanoic	-2.69±2.23	-6.23	< 0.05
Hexadecanoic	-1311.53±101.87	-48.99	< 0.00001
Heptadecanoic	-4.57±2.32	-5.36	< 0.01
Octadecanoic	-548.86±68.66	-47.20	< 0.00001
Eicosanoic	-4.81±1.57	-10.80	< 0.001
Docosanoicacid	-13.33±4.43	-11.96	< 0.001
Hexadecenoic	-30.00±9.72	-10.59	< 0.001
Octadecenoic	-1432.17±160.47	-43.85	< 0.00001
Eicosenoic	-8.68±2.57	-10.42	<0.001
Octadecadienoic	-408.11±79.14	-14.40	< 0.0001
Eicosadienoic	0.30±1.49	0.93	0.65
Octadecatrienoic	-4.68±1.10	-6.26	<0.01
Eicosatrienoic	4.07±1.65	2.44	<0.01
Eicosatetraenoic	27.80±7.40	2.84	< 0.001
Docosatetraenoic	2.33±1.64	2.61	<0.05
Docosapentenoic	0.96±0.61	0.67	<0.05
Docosahexaenoic	3.05±1.35	1.13	<0.01
FA trace	-10.13±5.19	-3.02	<0.01

OBJECTIVE 4

The fourth objective was to determine the effect of increasing the dose of rosuvastatin on both total and free plasma fatty acids and to correlate these changes with the change in

the concentration of other lipids involved in cholesterol homeostasis. This objective was developed in Chapter 7 [148].

In this chapter was investigated for the first time the influence of four doses (5, 10, 20, and 40 mg) of rosuvastatin on the concentration of TFA and FFA (Figure 13) and the correlation of their changes in concentration with changes in the concentration of other lipids in men with hypercholesterolemia.

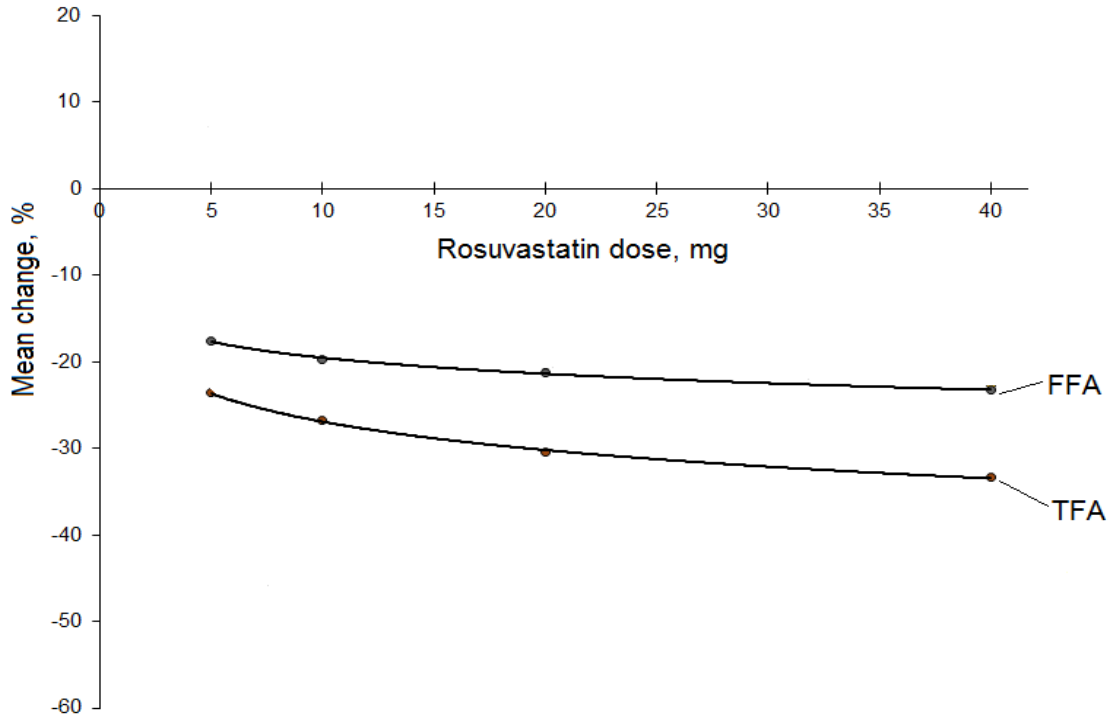

Figure 13. Variation of percentage changes in fatty acids in total lipids (TFA) and total free fatty acid (FFA) as a function of rosuvastatin dose.

The results of our experiments indicated that compared with placebo, all doses of rosuvastatin significantly reduced TFA, FFA, LDL- and total-cholesterol, apolipoprotein B-100, triglycerides, and phospholipids and increased apolipoprotein A-1 and HDL-cholesterol. The feedback effect of cholesterol homeostasis, which increases the endogenous synthesis of HMG-CoA reductase due to its inactivation by rosuvastatin, explains the logarithmic shape of the variation curves of the changing in the concentration of TFA, FFA and other lipids involved in cholesterol homeostasis.

The association between TFA and FFA changes with changes in LDL-, HDL-, total-cholesterol, apolipoprotein B-100 and A-1, phospholipids, and triglycerides was suggested by linear regression analysis. We demonstrate that TFA and FFA changes in concentration are strongly correlated with changes in the concentration of lipids involved in cholesterol homeostasis. Figure 18 illustrates the correlations for TFA. Due to this high correlation, we can use the linear regression functions to predict the TFA and FFA values from the values of other lipids.

Table 18. Correlation parameters of TFA with lipids and lipoproteins involved in cholesterol homeostasis.

Independent variable (X)	Dependent variable (Y)		
	TFA		
	Linear regression	Correlation coefficient (r)	*P*-Value
Total cholesterol	Y= 1.71X + 2.57	0.9997	< .0001
LDL-cholesterol	Y= 1.69X + 5.52	0.9983	< .0001
HDL-cholesterol	Y= -38.88X + 59.25	-0.9991	< .001
Triglycerides	Y= 20.59X - 6.25	0.9994	< .001
Phospholipids,	Y= 5.9X - 1.32	0.9979	< .001
Apolipoprotein B-100	Y= 0.027X + 4.47	0.9982	< .01
Apolipoprotein A-1	Y= -1.87X + 82.7	-0.9914	< .0001

CONCLUSIONS and ORIGINAL CONTRIBUTIONS

1. Chapter 4 presents for the first time a method that can simultaneously determine in a single gas chromatogram the composition of FAs, the composition of monosaccharides, and the level of cholesterol in a blood sample. The method is very specific and selective, if we consider that there are many substances in the blood. No other chromatographic or enzymatic method could achieve this.

   For the first time, the conditions for the methylation reaction allow the simultaneous realization of both fatty acid esterification reactions and monosaccharide and cholesterol etherification reactions.

2. In Chapter 5 we performed for the first time the analysis of FFA by GC, using a selective esterification of free fatty acids from plasma in the shortest time (1 min) compared to other methods. This new method avoided the transmethylation of covalently bonded fatty acids in lipids and performed a complete methylation of free fatty acids.
3. In Chapter 6 we measured for the first time the change in the concentration of each fatty acid in the overall fatty acids and free fatty acids after rosuvastatin treatment.
   An explanation of the changes in the level of each plasma fatty acid based on the mechanisms of cholesterol homeostasis has been proposed for the first time.
4. In Chapter 7 we investigated for the first time the influence of four doses (5, 10, 20, and 40 mg) of rosuvastatin on changes in the concentration of TFA and FFA. We demonstrated for the first time that TFA and FFA changes in concentration are strongly correlated with changes in the concentration of lipids involved in cholesterol homeostasis.

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