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ABSTRACT

OXIDIZED STEROLS IN PATIENTS WITH DIABETIC HEART DISEASE AND MICROWAVE-MEDIATED STEREOSELECTIVE SYNTHESIS OF 3-KETO CHOLESTEROL AND CHOLESTEROL EPOXIDE, CHOLESTERYL ESTER EPOXIDES

by

Meenakshi N. Kothavale

Oxidized low density lipoproteins (ox-LDL) may play important role in mediating hypercholesterolemic endothelial dysfunction. The uptake of modified LDL by macrophages to form foam cells has been implicated in the enhancement of atherosclerosis. Recent reports have suggested that when LDL undergoes oxidation in vitro , it is accompanied by a substantial loss of free and esterified cholesterol leading to the formation of oxidation products of cholesterol (oxysterols). These compounds are present in human atherosclerotic plaques and in human foam cells.

In this regard we have recently studied the plasma sterol composition in diabetic heart patients . We have found cholesterol α- and β-epoxides in addition to 7-ketocholesterol and 7α- and 7β-hydroxycholesterol. In order to characterize the structure and stereochemistry of cholesterol epoxides in diabetic patients, they were chemically synthesized via a rapid and convenient microwave irradiation technique. The structures of these compounds were determined by NMR and a combination of TLC-fast atom bombardment mass spectrometry. FAB-MS for Oxysterols: 5,6-epoxycholesterol, [M^+= 402, $(M+H+Na)^+$= 425, $(2M+H+Na)^+$= 827]; 5,6-epoxy cholesteryl palmitate $(M+Na)^+$= 663; 5,6-epoxy 3-ketocholesterol $(M+H)^+$= 401.

OXIDIZED STEROLS IN PATIENTS WITH DIABETIC HEART DISEASE AND MICROWAVE-MEDIATED STEREOSELECTIVE SYNTHESIS OF 3-KETO CHOLESTEROL AND CHOLESTEROL EPOXIDE, CHOLESTERYL ESTER EPOXIDES

by
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APPROVAL PAGE

OXIDIZED STEROLS IN PATIENTS WITH DIABETIC HEART DISEASE AND MICROWAVE-MEDIATED STEREOSELECTIVE SYNTHESIS OF 3-KETO CHOLESTEROL AND CHOLESTEROL EPOXIDE, CH0LESTERYL ESTER EPOXIDES

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This thesis is dedicated to
my beloved family

ACKNOWLEDGMENT

In the name of god, I wish to express my grateful thanks for the interest and assistance during this thesis by Dr. Bishambar Dayal, Ph.D., Associate Professor of Medicine and Adj. Associate Professor of Biochemistry and Molecular Biology , University of Medicine and Dentistry of New Jersey, under whose guidance this work is carried out.

A very special and particular debt of gratitude is due to my parents, Mr. Sakharam and Mrs. Sushila Badkundri, my husband Mr. Nitin Kothavale , who brought me up to this stage, my in-laws Mr. Moreshwar and Mrs. Sujata Kothavale and my brother, sister and brother-in-law for their constant love, care, encouragement, and immeasurable co-operation for my educational achievements. I can never cast up their accounts in this regard.

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I would also like to record my thanks to Dr. Anjali Asagaokar, V.A. Medical Center for her invaluable help and to all my friends for their love and co-operation.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Overview

Diabetes Mellitus has been identified as an independent risk factor in the development of atherosclerosis. The accelerated atherosclerosis of diabetes is undoubtedly the result of a number of different processes, but as yet they are poorly understood. The role of cholesterol oxidation products in atherogenesis has long been a controversial topic. Some products of cholesterol are cytotoxic and may be responsible for initial arterial cell injury and eventually results in atherosclerosis. On the other hand some oxidized cholesterol products are markedly less atherogenic than purified cholesterol (1).

Although high cholesterol levels have been strongly implicated in the epidemiology of coronary heart disease afflicting this society and other western nations, the majority of individuals who suffer myocardial infarctions have modest increases of both serum cholesterol and low density lipoprotein (LDL- "Bad cholesterol") (2). Dr. Steinberg and his colleagues have suggested that cholesterol by itself is not a risk factor, but becomes a risk when it is oxidized (3). This oxidized cholesterol serves as a "foreign substance" and is taken up by the macrophages of the scavenger cells in the lining of the coronary vessels. These fat-laden macrophages become the fatty streaks that later close off the vessels and eventually lead to heart attacks (3).

1.2 Oxidative Modification of Low Density Lipoproteins

Low density lipoproteins are thought to undergo oxidative modification by intracellular reactive oxygen species (ROS), probably generated by cellular lipoxygenases (4). There is an initial peroxidation of polysaturated fatty acids in the LDL lipids. The resulting fragmented fatty acids result in the accumulation of the LDL particles of highly reactive products include malonaldehyde, lipoic peroxides, and lysophosphatides (phosphates from which one molecule of a fatty acid has been split off). Thus the LDL becomes modified and highly atherogenic and immunogenic. Also, the LDL which is enriched in lipid peroxidation products is more electronegative than normal LDL based on its anion exchange high pressure liquid chromatography (HPLC) and gel electrophoresis characteristics (5). The increased oxidation is accompanied by increased levels of fatty and hydroperoxy derivatives and other oxysterols (unusual cholesterol oxidation products) that are largely associated with LDL and particularly enriched in the LDL fraction.(6)

Recent studies suggest that much of the cytotoxicity of oxidized LDL is associated with the peroxidation of oxysterols (7) which appears to be formed by gradual oxidation of lipoproteins in vivo. This is suggested by several studies in vitro demonstrating the cytotoxicity of these compounds found in LDL and their role in the process of atherosclerosis type lesion formation. The hypothesis that oxidized LDL is involved in the process of atherosclerosis is strengthened by the demonstration that probucol, an inhibitor of LDL oxidation , significantly reduces atherosclerosis in the Watanabe heritable hyperlipidemic rabbits (8).

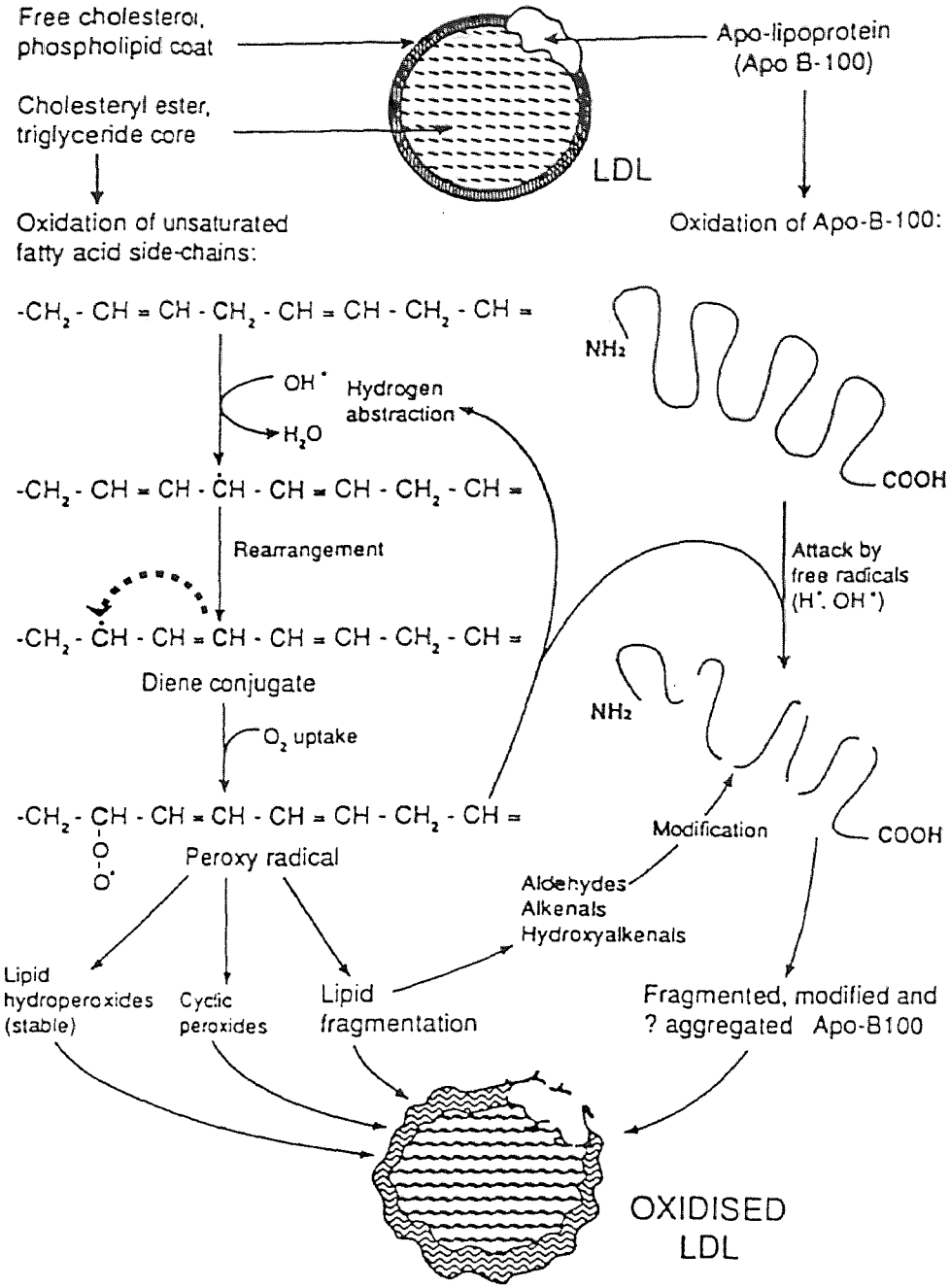


Figure 1.1 Oxidative damage to LDL affects both its lipid and apoprotein components. Peroxidation of unsaturated fatty acid side chains is illustrated on the left. The dot (•) represents an unpaired electron, introduced on a reactive hydroxyl radical (OH•). Rearrangement of double bonds results in a diene conjugate configuration, and subsequent oxygen uptake leads to the formation of reactive peroxy radicals. Peroxy radicals may cause lipid fragmentation, and are themselves capable of hydrogen abstraction from unsaturated lipids, thus initiating a free radical chain reaction. Oxidation of apolipoprotein-B is illustrated on the right. Free radical damage may fragment the molecule, and the subsequent appearance of higher molecular weight forms may imply aggregation of entire LDL particles. Thus, oxidation of the lipid moiety of the particle renders it cyto-toxic, while oxidation of the apoprotein (and apoprotein modification by the products of lipid oxidation) alters its recognition by cell receptors

Figure 1.1 Oxidative damage to LDL

Cholesterol autoxidation produces a large number of products, the first of which are hydroperoxides from which other products are generated such as epoxides, alcohols and carbonyl compounds. Cholesterol hydroperoxides have been recovered from air aged cholesterol. These hydroperoxides are formed in the body as a result of 1) cholesterol autoxidation 2) lipid peroxidation 3) enzymatic oxidation 4) photochemical oxidation.

There is extensive evidence that the autoxidation products of cholesterol and cholesteryl esters exert pronounced biological effects. The products of autoxidation of free cholesterol are well known inhibitors of the rate limiting enzymes in cholesterol and bile acid biosynthesis (9). In addition, it is also confirmed that the cholesterol oxides possess carcinogenic, atherogenic properties. Among the major products of cholesterol found in human plasma are the isomeric cholesterol 5α,6α- and 5β, 6β-epoxides. Similarly, oxidation products of cholesteryl esters are 5α,6α- and 5β, 6β-epoxides of cholesteryl ester.

There are several possible mechanisms for cytotoxic effects for oxidized Low Density Lipoprotein (LDL). It has been found that most of the cytotoxicity is attributable the lipid component of LDL and the oxidized LDL is taken up more readily than macrophages (any of the large phagocytic cells of the reticuloendothelial system whose function is to protect the body against infection and noxious substances) to create foam cells. Also, oxidized LDL is chemotactic (involving movement of an organism in relation to chemical agents) for circulating monocytes, and inhibits the motility of tissue macrophages. It may also be cytotoxic to endothelial cells and may increase vasoconstriction in arteries (2).

1.3 Objective

The purpose of this study is to measure chemically altered forms of cholesterol that may be more damaging than unchanged cholesterol to the heart in diabetic heart disease patients. This study will involve isolation, preparation and measurement of cholesterol products in oxidized LDL.

In this study, we have isolated, identified, cholesteryl epoxides as well as cholesteryl ester epoxides from human LDL in plasma of diabetic patients and also carried out the syntheses of these compounds using microwave irradiation technique for elucidating their structures. These studies then provided us with the quantitative measurements of cholesterol oxidation products in oxidized LDL. We report here, the results of several experiments in which organic reactions were carried out in Erlenmeyer flasks covered with a funnel and subjected to microwave irradiation. The high temperatures and pressures so readily obtained in these microwave-induced reactions have led to remarkable rate enhancement and dramatic savings in reaction times.

1.4 Microwave-Mediated Chemical Reactions of Sterols

Three different kind of reactions were studied :

(1) *Microwave-mediated Saponification of blood plasma of*

(i) Patients with diabetes (ii) Control subjects

(2) *Microwave-mediated syntheses of*

(i) Epoxidation of cholesterol using $KMnO_4$/ $CuSO_4 \cdot 5H_2O$ catalyst

(ii) Epoxidation of cholesteryl esters using $KMnO_4$/ $CuSO_4 \cdot 5H_2O$ catalyst

(iii) Epoxidation of cholesteryl esters using chloroperoxybenzoic acid.

Microwave irradiation using commercial domestic ovens has been used in large scale to accelerate organic reactions. The high heating efficiency of the oven gives rise to remarkable rate enhancements and dramatic reductions of reaction times. Highly accelerated rates of chemical reactions observed under these conditions have been ascribed to the high temperatures and pressures that are reached in minutes.

1.5 Materials and Methods

1.5.1 Microwave Irradiation

The microwave oven used in these experiments was a domestic Whirlpool commercial Microwave model number 3600XS operating at 2450 MHz (total cooking power of the mw 650 watts). The microwave's power was tested and the results showed that 100% of its power (total cooking power of the microwave oven = 650 W) was too much for the solvents and they would boil and evaporate. 10-40 % of the microwave's power was not enough to produce a definite result. After many experiments, it was determined that 60% of the microwave's power (260 W) generated the correct temperature to oxidize and hydrolyze cholesterol and cholesteryl esters to produce the necessary results. All experiments were performed for 69-90 seconds at 60% power.

The reactions described in this study were carried out in Erlenmeyer flasks or scintillation vials covered with a funnel or a watch glass. Each reaction was carried out both under traditional reflux conditions, as well as in a flask in a microwave oven. The quantities of reactants and their concentrations were the same under both sets of reaction conditions.

Reaction products were identified by Thin Layer Chromatography and by Fast Atom Bombardment Mass Spectroscopic studies (FAB-MS). This fast technique provides the oxycholesterols, cholesteryl epoxy-esters as pure compounds.

1.5.2 Thin Layer Chromatography

All epoxides were separated on Silica Gel G plates (Analtech, Uniplates, Newark, NJ, 0.25 mm thickness) in solvent system $CHCl_3$/ $(CH_3)_2CO$, 75:5 (v/v). The spots were detected with phosphomolybdic acid (3.5 % in isopropanol), sulfuric acid (10 %) and heated for 1 min at 110°C. Argentation TLC (solvent system: $CHCl_3$/$(CH_3)_2CO$, 57:2 (v/v) was used to separate saturated from unsaturated sterols for quantitative determination by GC.

1.5.3 Melting Points

Melting points (mp) were determined on a Thermolyne apparatus (Thermolyne Corp., Dubuque, IA) model MP-12600 and were uncorrected. All chemicals were purchased by Aldrich Chemical Co. and was used without purification.

1.5.4 Gas-Liquid Chromatography

Capillary GLC analysis performed on Hewlett-Packard model No. 4890 (equipped with flame ionization detector) and a split column injector using a Cpsil 5 (CB) WCOT capillary column (25 m * 0.22 mm with 0.13 mm film thickness). Helium was used as a carrier as at a flow rate of 20.2 mL/min (135 kPa). This is employed for the quantitative determination of the plasma oxidized sterols as their TMSi ether derivatives.

1.5.5 Mass Spectroscopy (MS)

Mass Spectra of the sterols were obtained with a JEOL JMS-HX 110A high resolution mass spectrometer (HR-MS). A SCIEX APT III triple quadrapole mass spectrometer equipped with a standard atmospheric pressure ionization source (SCIEX Inc., Thornhill, Ontario, Canada) was used. Fast Atom Bombardment mass spectroscopic studies (FAB-MS) were conducted on sterols using glycerol/ thioglycerol-NaCl and or glycerol/ thioglycerol-LiBr as a matrix and DMSO -d_6 as a solvent.

The interpretation of mass spectra is based on the chemistry of gaseous ions. As in many chemical reactions used for analysis, the basic purpose of the mass spectrometer is to convert the sample into measurable products that are indicative of the original molecule.

The types of ionization used fall into two general categories. "Hard" ionization such as with 70 ev electrons, produces substantial proportion of the ionized molecules, with such high internal energies that they fragment before leaving the ion source. The masses of these fragment ions are the basic substructure information used in interpretation. "Soft" ionization, on the other hand, minimizes such further fragmentation. This is particularly useful, for characterizing mixtures. Electron ionization (EI) is a "hard" ionization, the reagent producing the ionic products is a beam of energetic electrons.

With chemical ionization (CI) or Fast atom bombardment (FAB), a "soft" ionization is particularly suitable for polar molecules. In this technique, a solution of the sample in a low-volatility matrix, such as glycerol is bombarded with fast, heavy atoms/ ions(e.g. Xe, CS^+) in the ion source, producing a continuous ion beam necessary for scanning instruments.

CHAPTER 2

EXPERIMENTAL PROCEDURE

2.1 Oxidized Sterols in Patients with Diabetic Heart Disease

2.1.1 Preparation of Oxidized LDL

Whole blood was obtained by venipuncture from controlled diabetic patients who had fasted 12 h and diabetic patients. Blood samples were collected in tubes containing EDTA (1mg/mL). Plasma was separated by low speed centrifugation (1,500 ×g for 30 min), and butylated hydroxytoluene (20μM) was added. Plasma LDL (d =1.019 -1.063 g/mL) was prepared by sequential ultracentrifugation. LDL was dialyzed for 24 h at 4°C in the dark against vacuum degassed 0.01 M phosphate buffer, pH 7.4, containing 10μM EDTA, 0,15M NaCl and 0.1 mg/mL chloramphenicol.

The plasma separated from blood using ultra-centrifugation technique was stored in scintillation tubes.

2.2 Microwave-Mediated Saponification

About 1 mL of plasma (i) of diabetic patient (ii) of controlled diabetic patient was taken in 250 mL Erlenmeyer flasks. To this was added about 3 mL of 10 N NaOH and 25 mL of ethyl alcohol. The predigested mixture was irradiated in the microwave oven. The Erlenmeyer flask was covered with a funnel and the mixture was irradiated in the microwave oven for 75sec at 60 % power (390 W, the total cooking power of the microwave oven = 650 W).

In another 250 mL Erlenmeyer flask was placed the same volume of mixture. The flask was kept in a water bath. Hydrolysis of this solution was carried out for 2 h at minimum temperature of 65 degrees Celsius.

Excess solvent was evaporated under N_2 gas. The residue was then extracted three times with 15mL of hexane. The hexane layer was collected in a 500 mL clean, dry round bottom flask and evaporated invacuo roto-evaporator at a bath temperature of 35-40 ^{0}C. After extraction with hexane, the plasma was extracted three times using 15mL of ethyl acetate each time. The ethyl acetate layers were collected in another 500 mL clean, dry round bottom flask and evaporated invacuo using roto-evaporator.

A similar reaction was carried out using a saturated solution of LiOH in place of 10N NaOH.

2.2.1. Experimental

The solid obtained from above extracts was dissolved in minimum quantity of acetone. Sterols were analyzed using TLC (solvent systems: (i) $CHCl_3$ / $(CH_3)_2CO$, 3 : 0.2 (ii) Hexane / Diethyl ether, 9:1). The identity of the sterols was verified by column chromatography with authentic reference sterols and was confirmed by mass spectroscopy (FAB-MS). The mass of each sterol was corrected for the analytical losses by recovery of known quantities of reference standards.

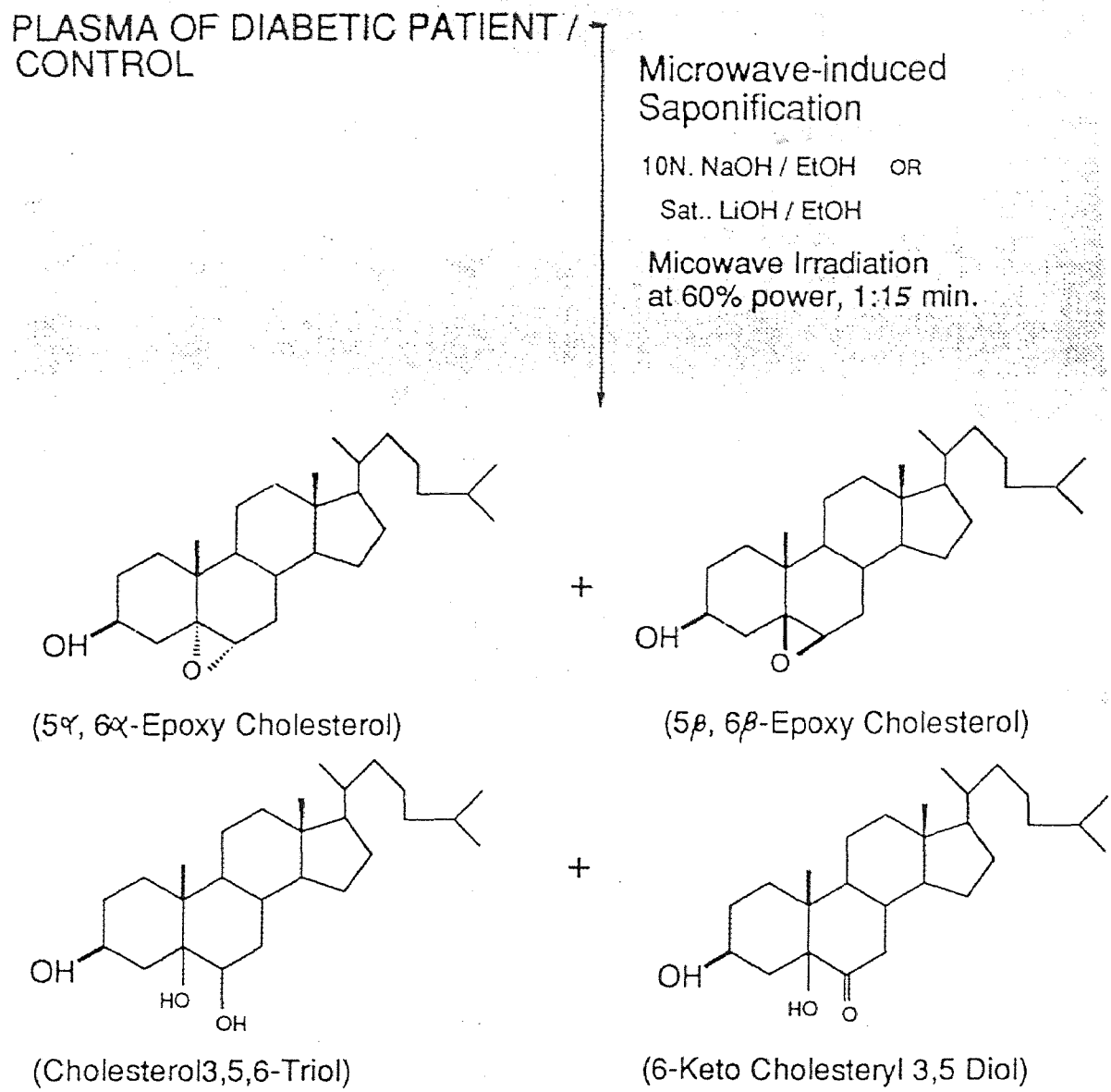


Figure 2.1 Microwave-mediated Saponification

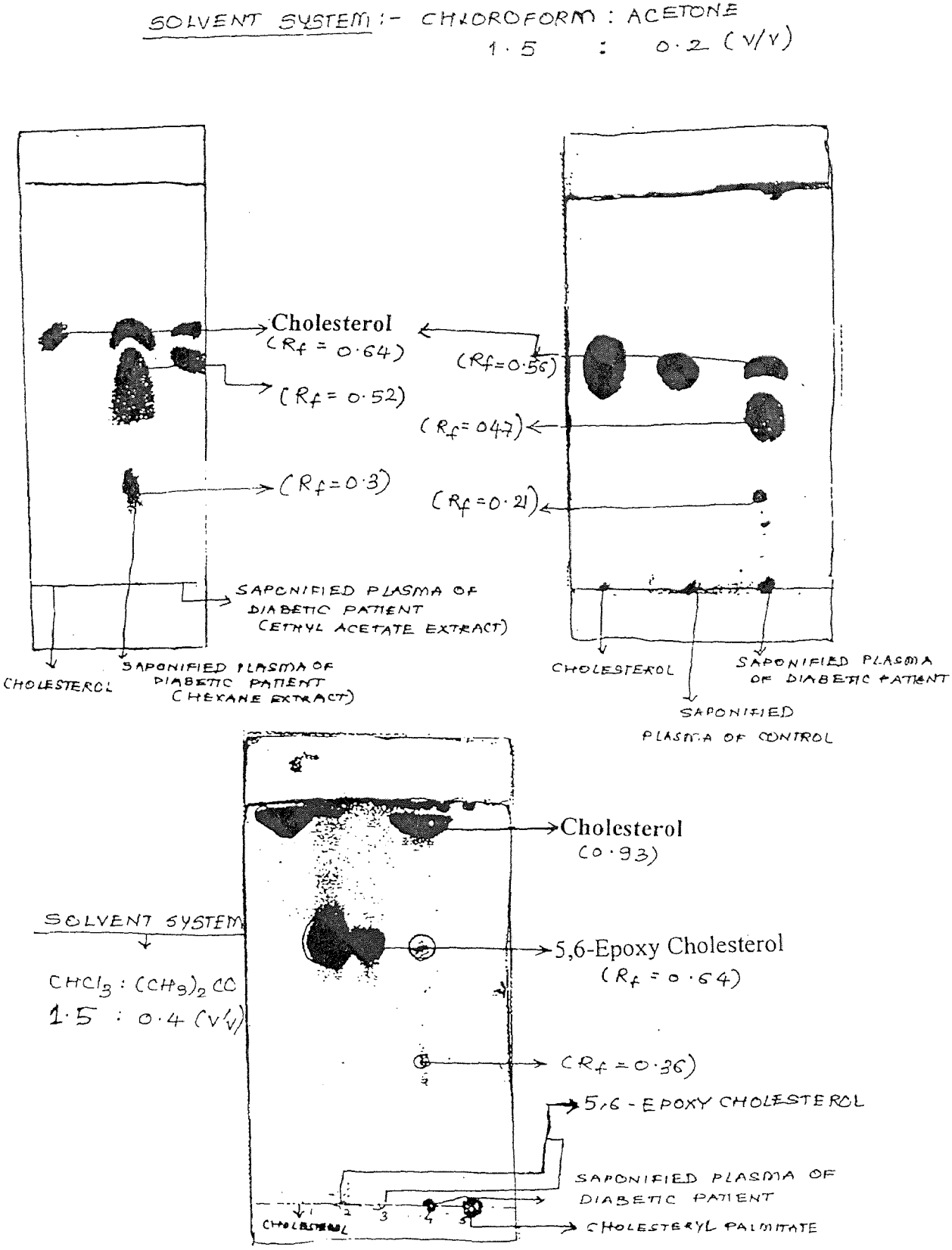
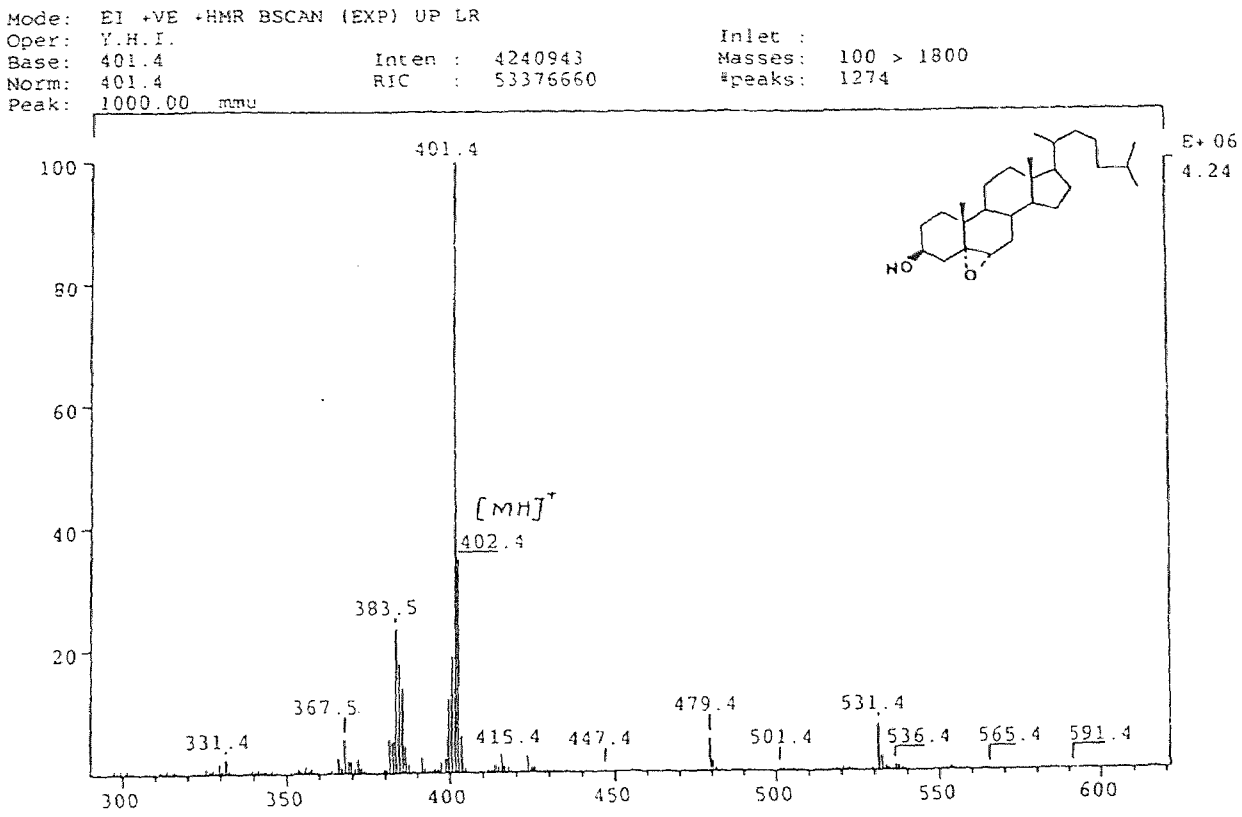


Figure 2.2 TLC Analysis: Microwave-mediated Saponification



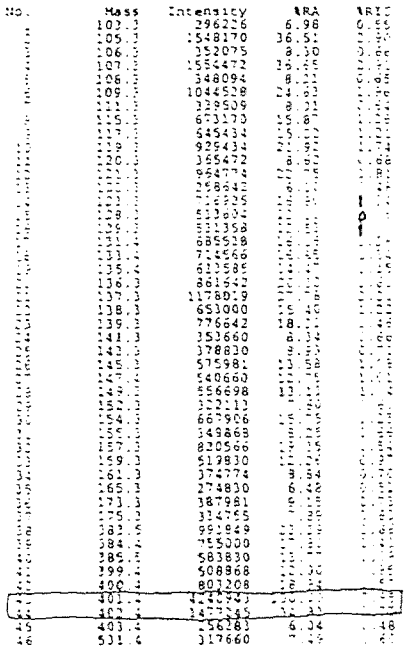


Figure 2.3 FAB-MS Analysis: Microwave-mediated Saponification

2.3 Microwave Mediated Stereoselective Synthesis

2.3.1 Using $KMnO_4$/ $CuSO_4.5H_2O$ Catalyst

The permanganate oxidations with KMnO4/ CuSO4•5H2O can be used effectively for the direct conversion of cholesterol and cholesteryl esters to a mixture of α- and β-epoxides under very mild conditions (8). Cholesterol and cholesteryl esters are treated with a well ground mixture of $KMnO_4/CuSO_4.5H_2O$ in dichloromethane containing catalytic amounts of tert-butyl alcohol/water at room temperature resulting in a stereoselective mixture of α- and β-epoxides (excess of β isomer over α isomer).

A mixture of solid KMnO4 (1.0 g) and CuSO4.5H2O (0.5 g) was ground to a fine powder in a mortar and pestle. To this water (400 μL) was then added, and the slightly wet mixture was transferred to a 250 mL Erlenmeyer flask. To a stirred suspension of this mixture in dichloromethane (10mL) was added (i) Cholesterol-99% pure (100 mg) (ii) Cholesteryl esters (100mg) - Cholesteryl palmitate in dichloromethane (30 ml.) followed by addition of tert-butyl alcohol (6 mL).The flasks were then covered with a funnel and the reaction mixture was irradiated in microwave for 2 min at 60 % power. The same reaction was carried out at room temperature (25^0 C) with constant stirring for 4 h .The reaction mixture was allowed to cool and was filtered over a pad of celite and washed thoroughly with dichloromethane. The filtrate was collected, and the solvent was removed invacuo.

The solid was dissolved in acetone and analyzed by a TLC plate. The products were then isolated using TLC as well as flash column chromatography and analyzed by mass spectroscopy (FAB-MS).

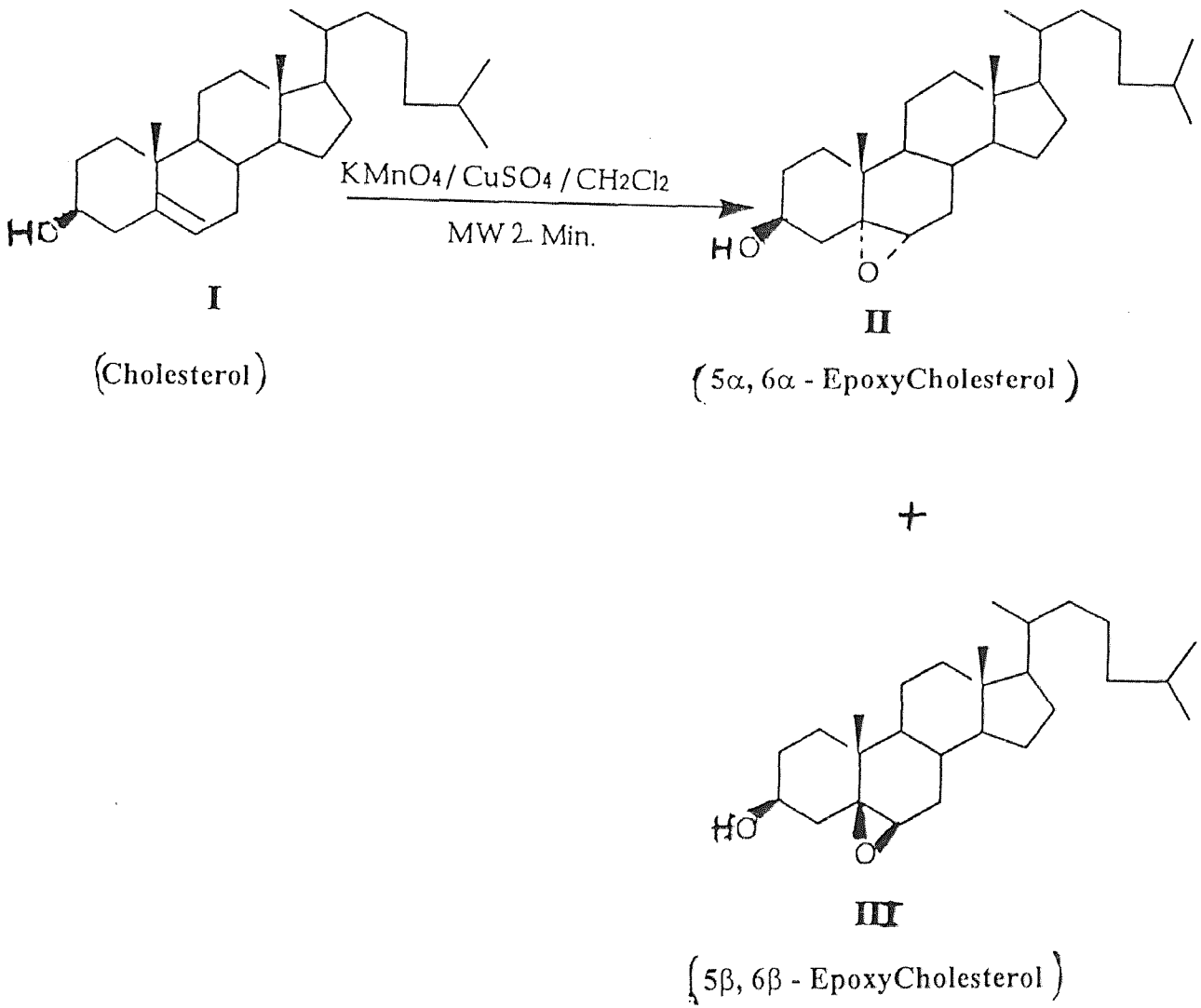
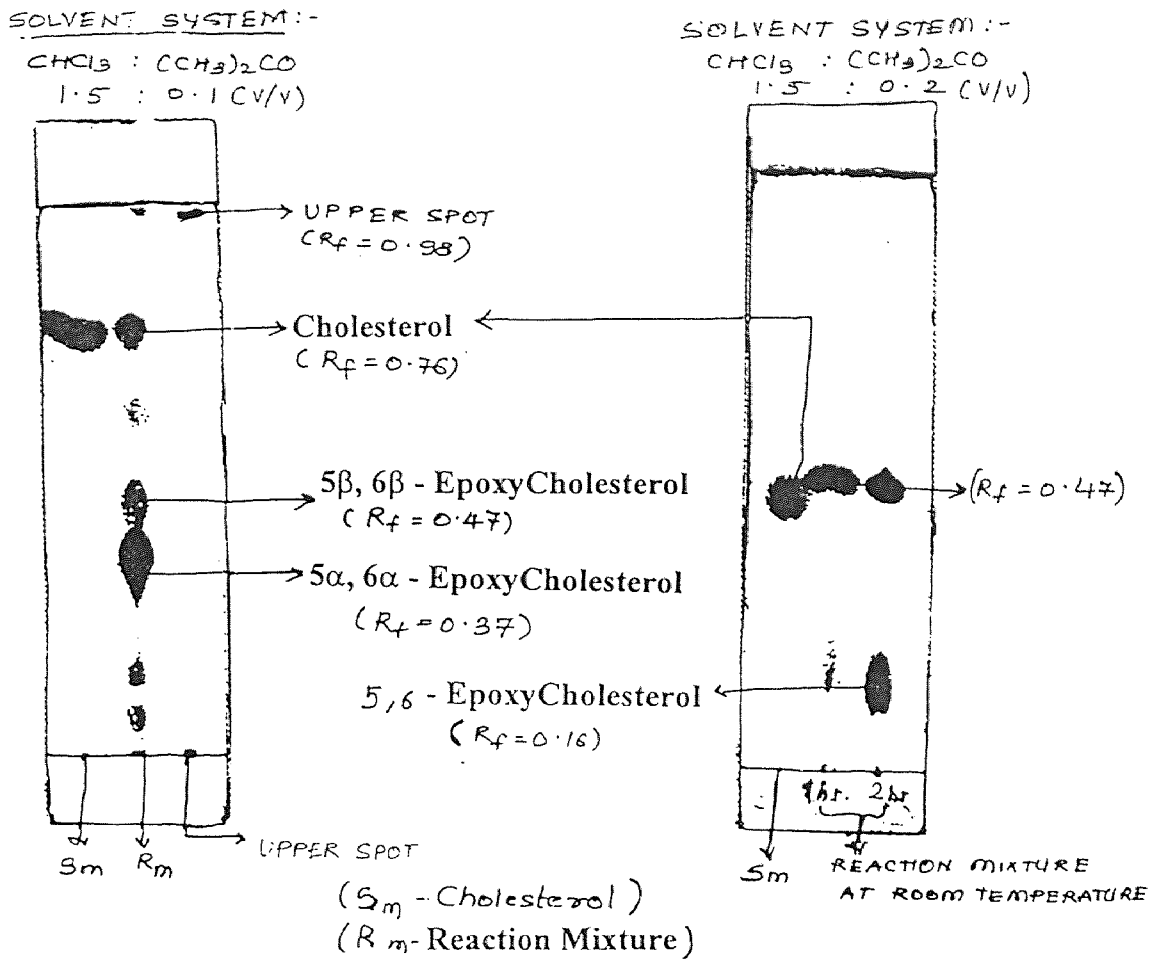


Figure 2.4 Microwave-induced Epoxidation of Cholesterol using $KMnO_4$/ $CuSO_4.5H_2O$ Catalyst



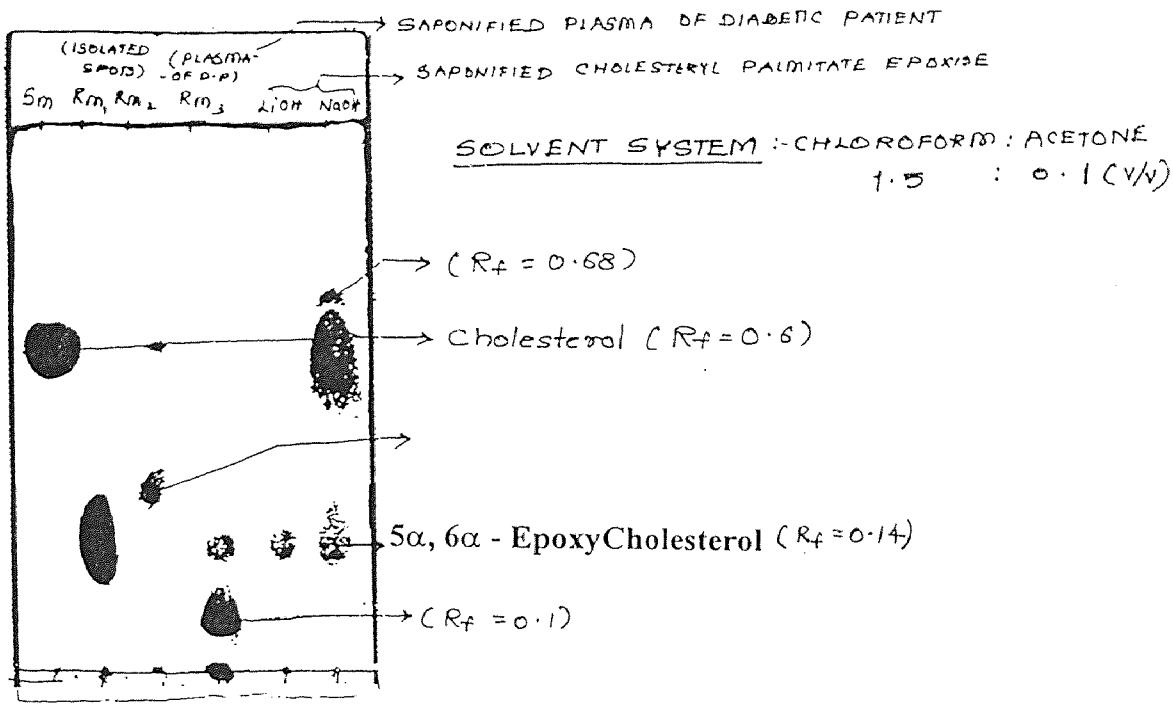


Figure 2.5 TLC Analysis: Microwave-induced Epoxidation of Cholesterol using $KMnO_4/CuSO_4.5H_2O$ Catalyst

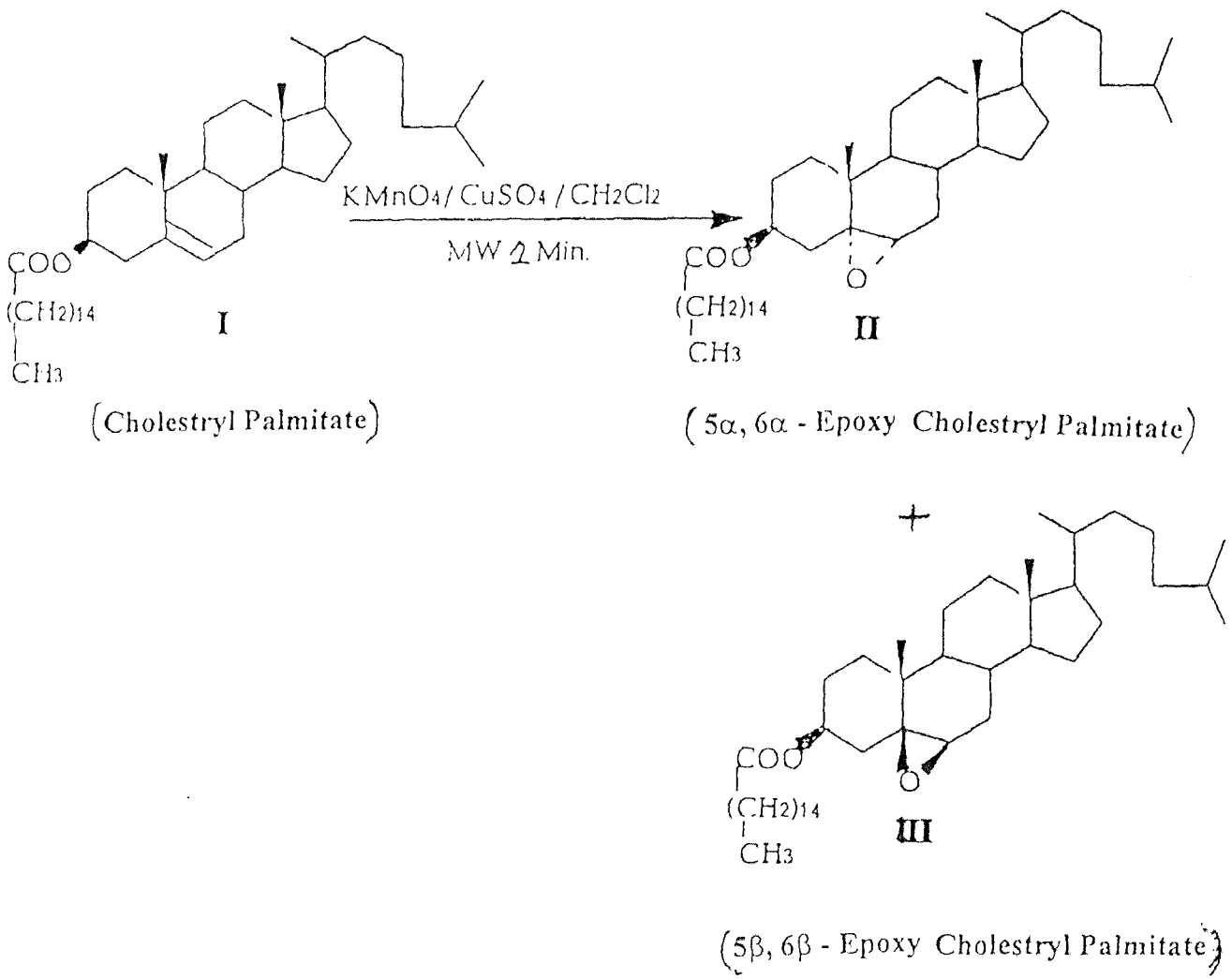


Figure 2.6 Microwave-induced Epoxidation of Cholestryl Palmitate using $KMnO_4$/ $CuSO_4.5H_2O$ Catalyst

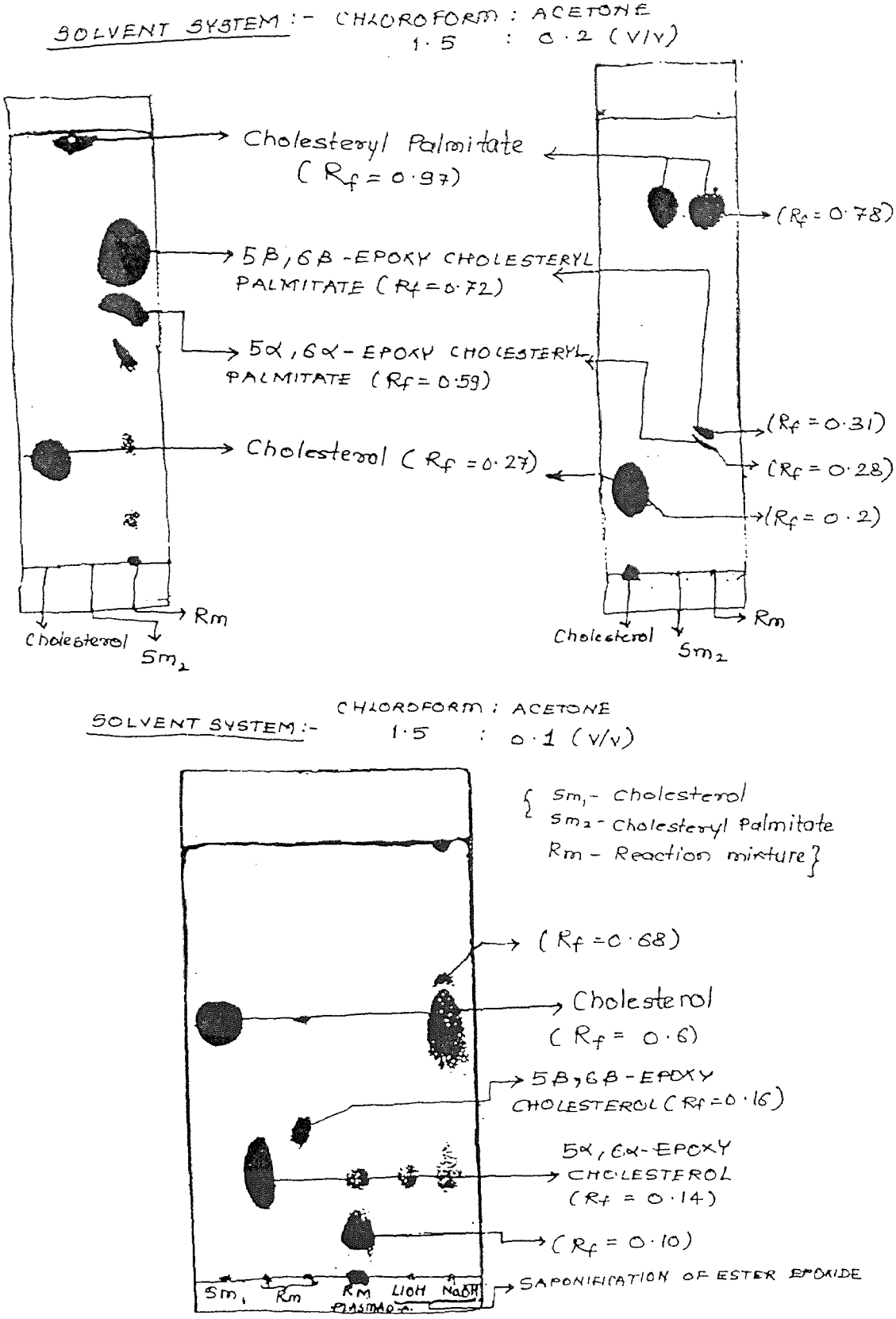


Figure 2.7 TLC Analysis: Microwave-induced Epoxidation of Cholestryl Palmitate using $KMnO_4/CuSO_4.5H_2O$ Catalyst

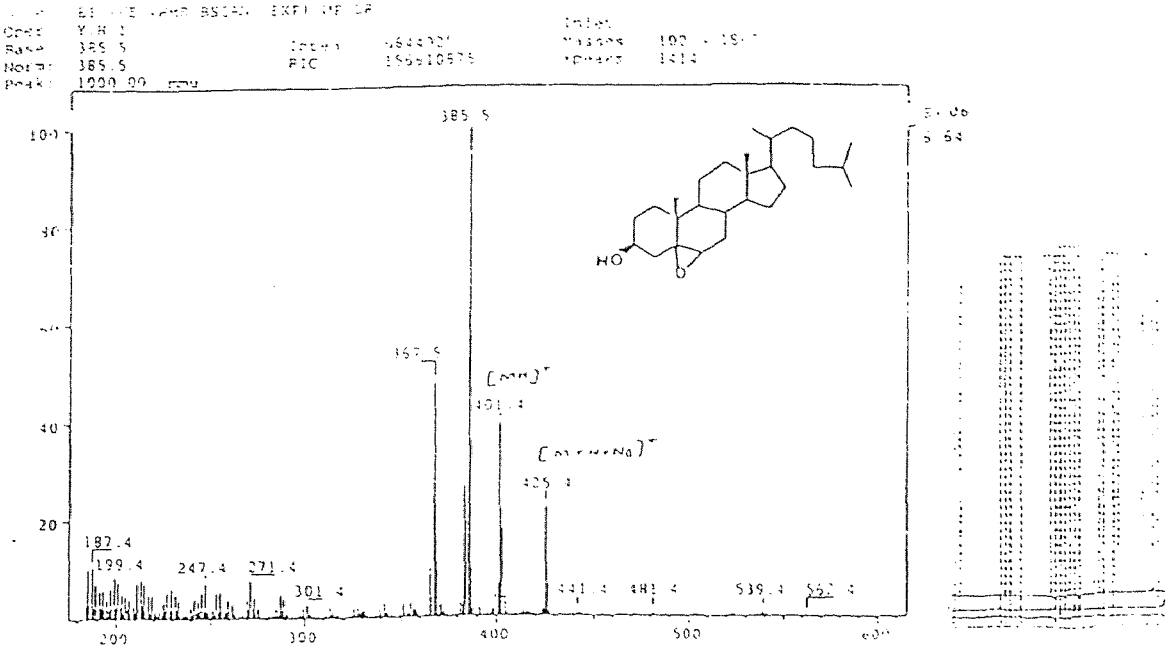


Figure 2.8 FAB-MS Analysis using Microwave-induced Epoxidation of Cholesterol

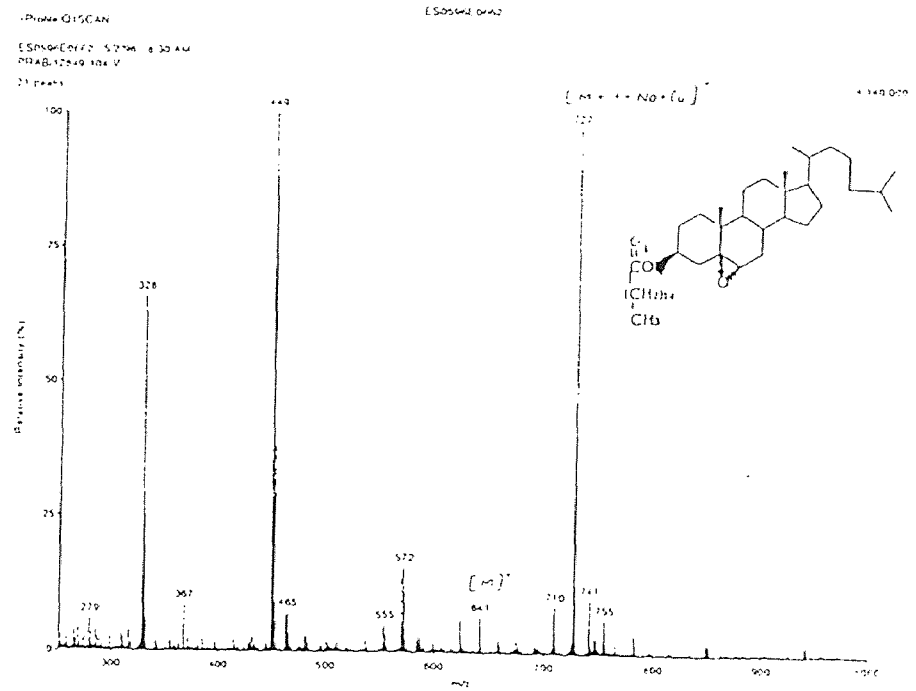


Figure 2.9 FAB-MS Analysis using Microwave-induced Epoxidation of Cholestryl Palmitate

2.3.2 Using Meta-Chloro Perbenzoic Acid

Meta-chloroperbenzoic acid is a strong oxidizing agent with superior selectivity than that obtained with perbenzoic or perphthalic acid, which oxidizes cholesterol and cholesterol esters to give α- and β-epoxy cholesterol and α- and β-epoxy cholesteryl esters. The reaction if carried out by microwaves is faster and gives stereoselectively α- and β-epoxide(excess of β-isomer over α-isomer).

In a 250 mL Erlenmeyer flask, 100 mg of 5-Cholesten-3β-ol palmitate was dissolved in 30 mL of dichloromethane. To this mixture, 200 mg (1.16 mmol) of m-chloro perbenzoic acid was added. A vortex mixer was used until all the compounds were completely dissolved. The flask was covered with a funnel and placed in a water bath. The mixture was irradiated with microwaves for 2 min at 60 % power. The reaction mixture was cooled down to room temperature. The reaction mixture was washed in a separatory funnel with saturated solutions of sodium sulfite and sodium bicarbonate respectively. The methylene chloride layer was retained, while the aqueous layers were discarded. The methylene chloride layer was collected and concentrated invacuo.

A similar reaction is carried out at room temperature with constant stirring for 4 h

.

2.3.3 Experimental

The dried reaction mixture was then dissolved in a small amount of chloroform and separated using flash column chromatography. The eluted product was filtered. The filtrate collected and analyzed by TLC plates. The products were then isolated using preparative TLC and flash column chromatography and analyzed using FAB-MS.

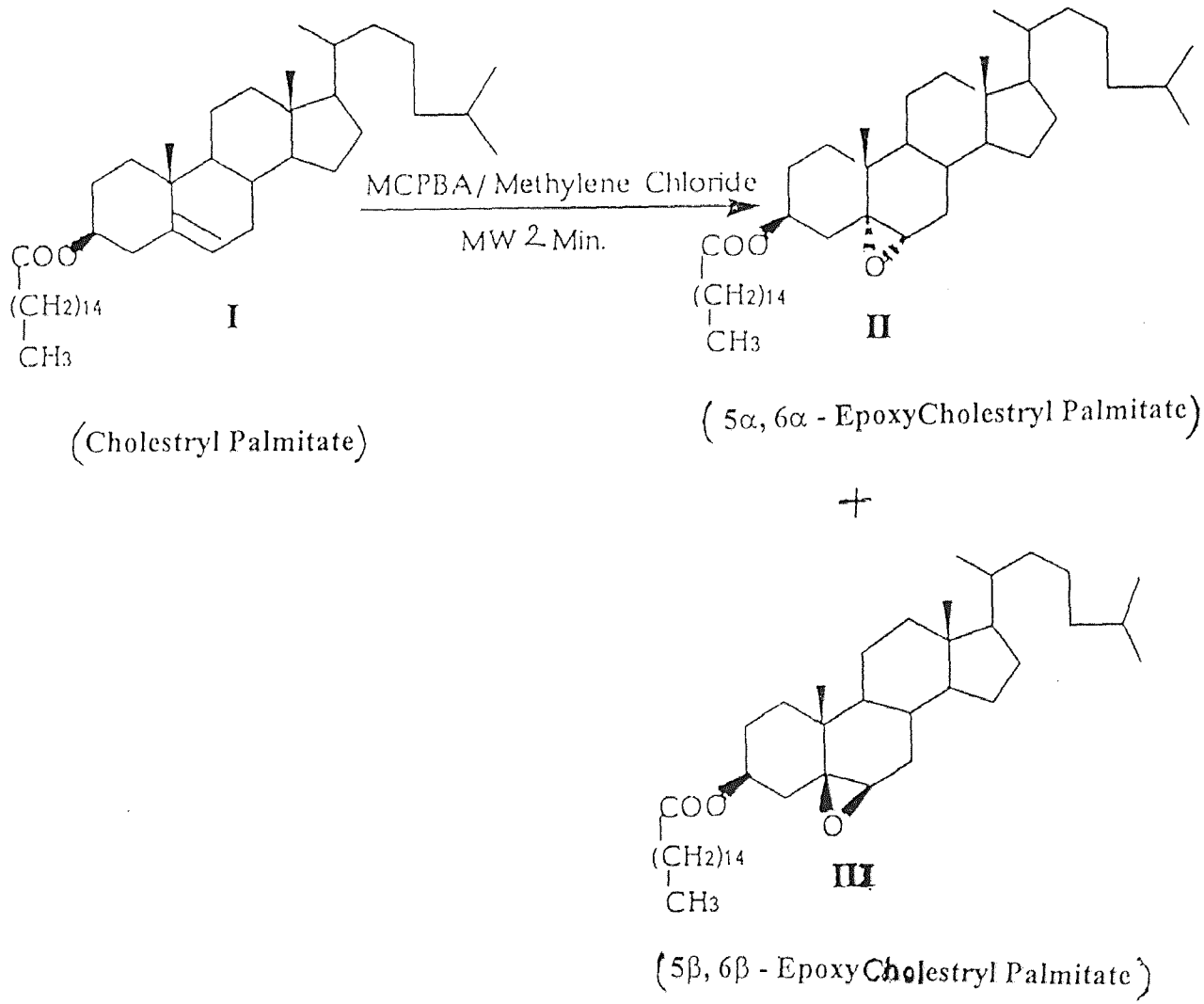


Figure 2.10 Microwave-induced Epoxidation of Cholesteryl Palmitate using Meta-Chloro Perbenzoic Acid

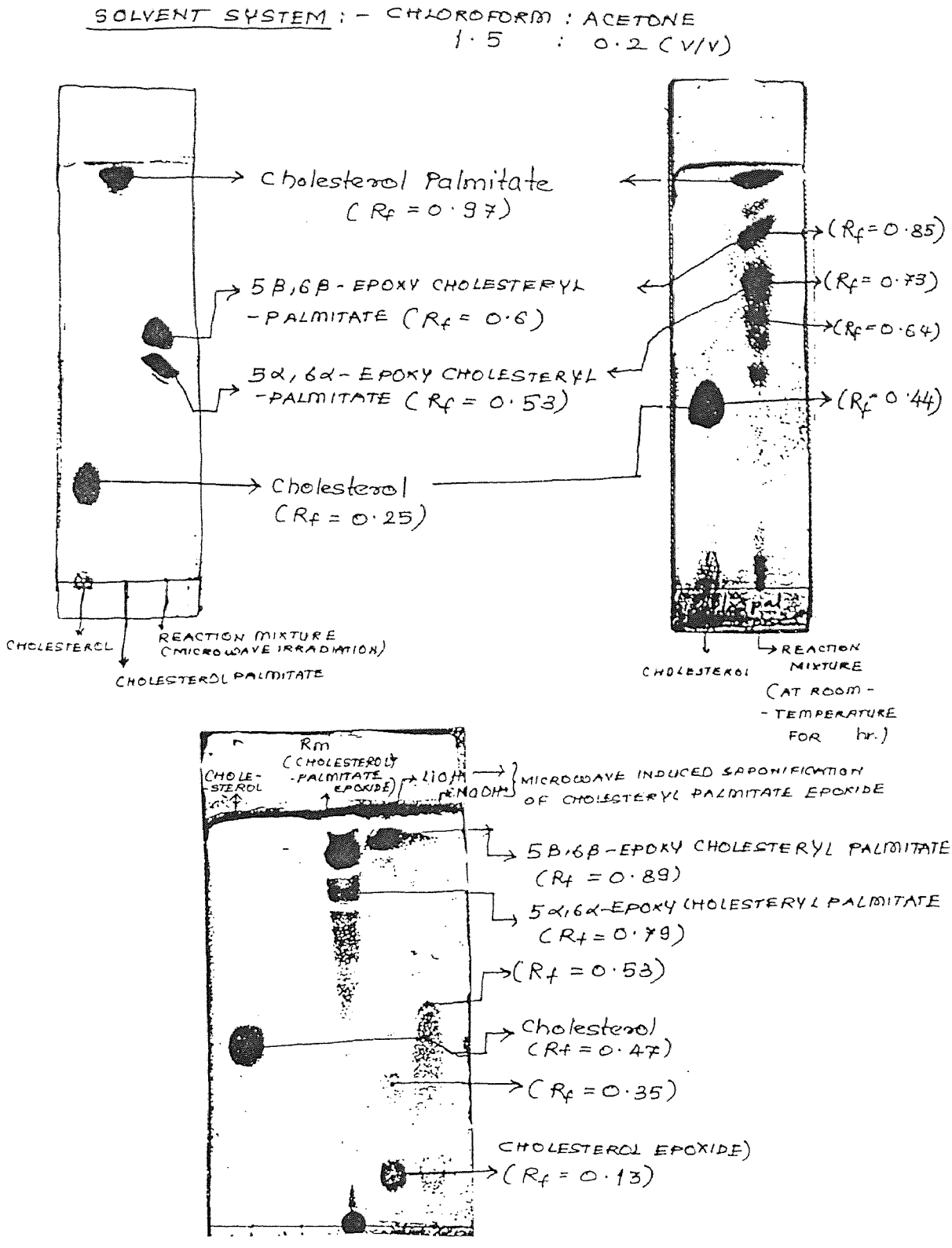


Figure 2.11 TLC Analysis: Microwave-induced Epoxidation of Cholestryl Palmitate using Meta-Chloro Perbenzoic Acid

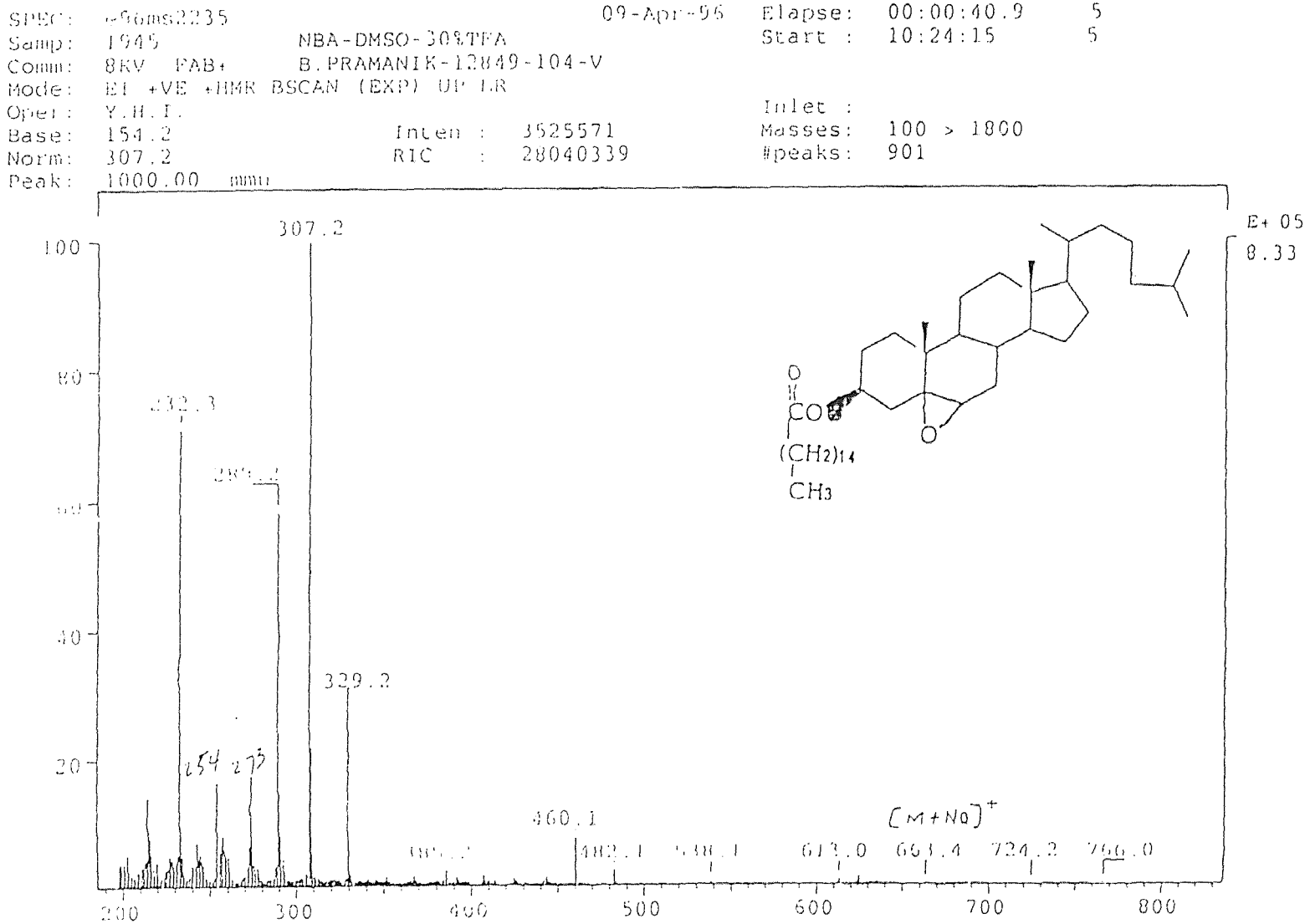


Figure 2.12 FAB-MS Analysis using Microwave-induced Epoxidation of Cholestryl Palmitate

2.4 Isolation and Purification

The isolation of the oxidized sterols found in the plasma of diabetic patients as well as those synthesized using oxidizing agents/catalyst was carried out by two different methods.

2.4.1 Preparative TLC

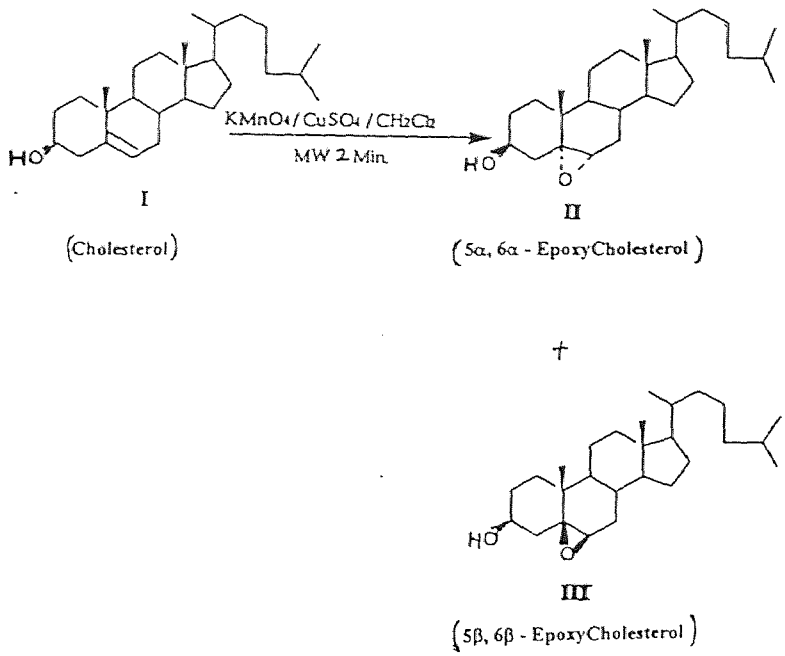
All the oxidized sterols obtained naturally or synthetically were dissolved in a minimum quantity of acetone. The solution was applied to TLC plate (20 × 20 cm). The plate was then developed using the appropriate solvent system ($CHCl_3/(CH_3)_2CO$, 75:5 (v/v). The plate was then sprayed with water and dried completely. The oxidized sterols were collected in a 50 mL beaker. The isolated oxidized sterols were then dissolved in a minimum quantity of acetone and filtered through a Buchner funnel. The eluted component was collected in a small vial and concentrated to 1/3 its volume while cooling in ice. The pure crystals were collected and analyzed using TLC and MS for the formation of epoxides.

2.4.2 Flash Column Chromatography

A column of 70 cm× 3 cm (length × diameter) was filled with a glasswool or cotton plug at the bottom of the column. The column was washed several time with a 1:1 (v/v) mixture of chloroform and petroleum ether.

About 30 g of silica gel (40-140 mesh size) is weighed in a 100 mL beaker. To it, added about 40 mL of 1:1(v/v) mixture of petroleum ether/ chloroform. The column is filled with the slurry of silica in petroleum ether / $CHCl_3$(1:1-v/v). The silica gel was allowed to settle and it measured up to 44 cm.

The reaction mixture that was dissolved in the small amount of chloroform was poured on top of the column. Another piece of cotton was placed on the reaction mixture. The column was then eluted with the solvent, chloroform, in 10 mL fractions. Thin layer chromatography was then used to detect the conversion of the starting material into epoxide.



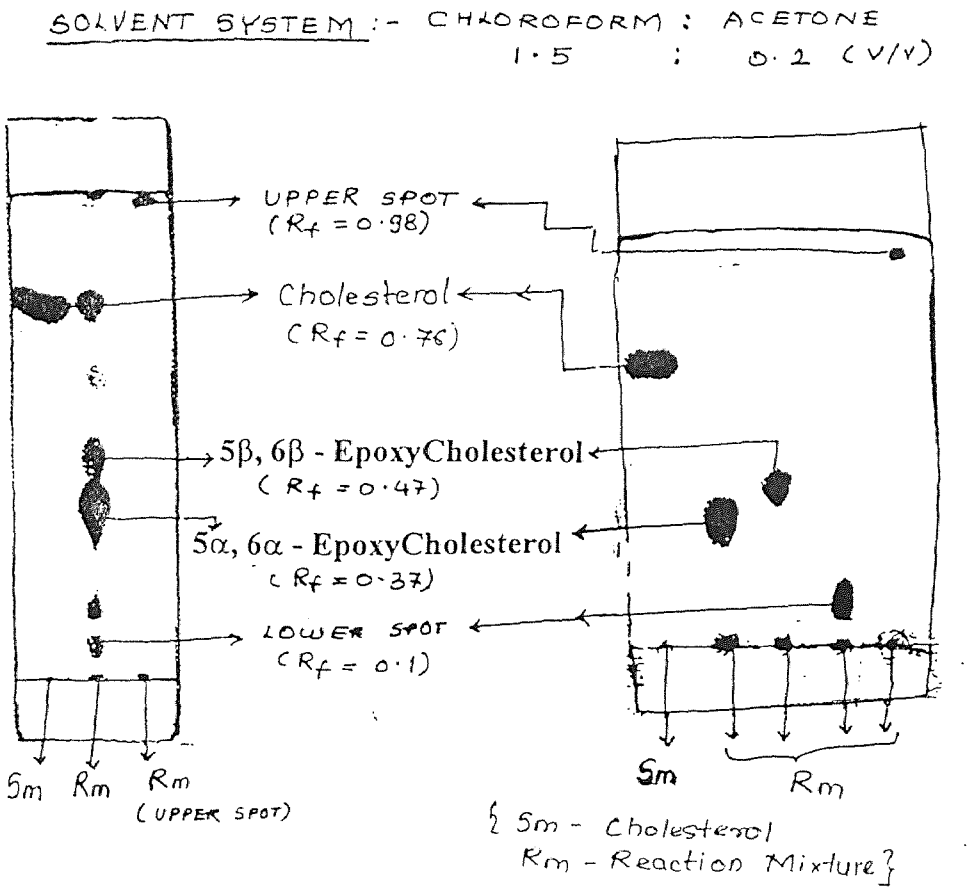
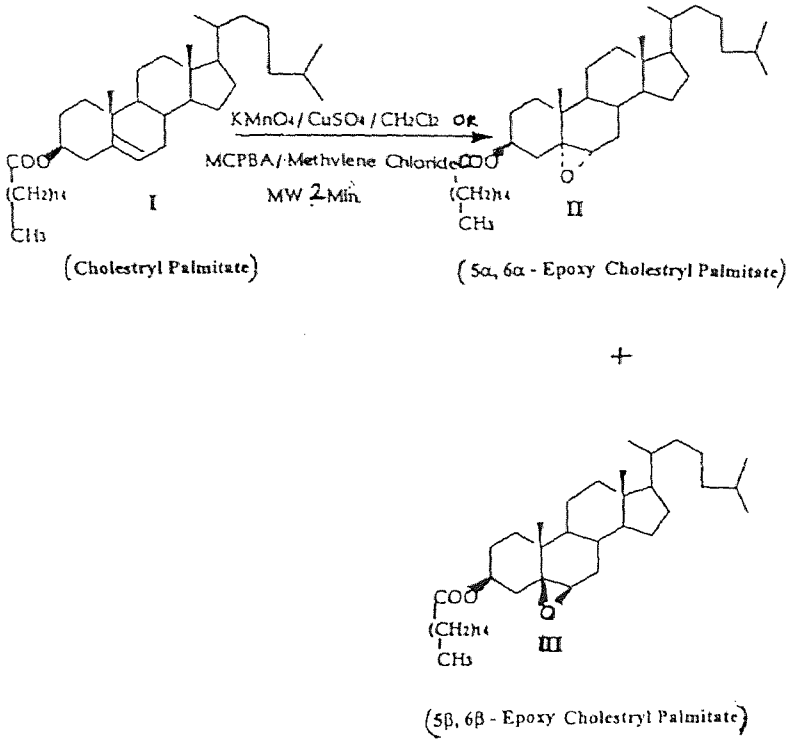


Figure 2.13 Isolation and Purification of α and β isomers of 5,6-Epoxy Cholesterol



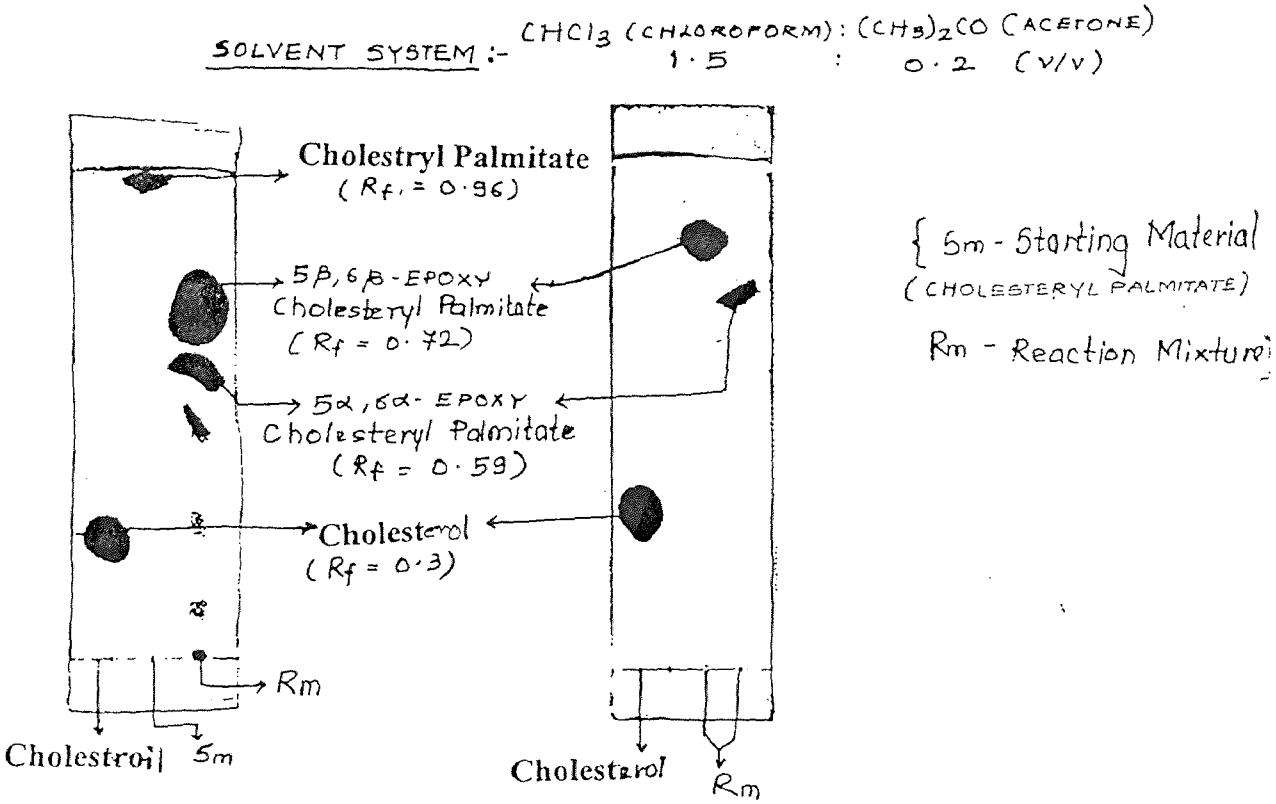


Figure 2.14 Isolation and Purification of α and β isomers of 5,6-Epoxy Cholestryl Palmitate

CHAPTER 3

RESULTS AND DISCUSSION

Despite widely accepted involvement of lipid peroxidation in pathophysiological modification of LDL, only a few specific oxidation products have been identified. Thus, Esterbauer et al. (3) identified a number of low molecular weight aldehydes, and Cazzolato et al. (4) have isolated several fatty and hydroperoxy derivatives.

It is our hypothesis that sterols, mainly cholesterol in lipoproteins, are highly susceptible to oxidative modification. When cholesterol crystals are stored in the laboratory for an extended period of time, autooxidation occurs with the production of several oxidized sterols including 7-ketocholesterol, 25-hydroxycholesterol and several others.

The purpose of our study was to measure chemically altered forms of cholesterol that may be more damaging to the heart disease patients. This study involved synthesis, isolation and measurement of cholesterol oxidation products in oxidized LDL. Since the experiments outlined are designed to investigate the individual compounds in blood, oxysterols and cholesterol hydroperoxides (oxygenated cholesterols) were isolated, characterized from LDL- cholesterol and some were verified by chemical syntheis via a facile microwave irradiation process.

In this study we have identified the 5,6- epoxycholesterol and 5,6- epoxy cholesteryl esters as high molecular weight lipid soluble peroxidation products of cholesteryl esters in LDL. Although the occurrence of these cholesterol oxidation products have been suspected specific epoxy- compounds have not been isolated or identified.

Our report describes here an efficient method for rapid synthesis and hydrolysis of cholesterol oxidation products as well as cholesteryl epoxyesters using Erlenmeyer flasks or scintillation vials in a commercial microwave oven. An Erlenmeyer flask or a scintillation vial covered with a watch glass or a funnel was adequate as a reaction vessel. Reaction products were purified by TLC, and identified by comparison of TLC mobilities, FAB-MS with authentic samples.

The Rf values of the sterols:-

Solvent System:- $CHCl_3$/ $(CH_3)_2CO$, 15:2

1] 5α,6α-epoxy-cholesterol = 0.37

2] 5β, 6β-epoxy-cholesterol = 0.47

3] 5α,6α-epoxy-cholesteryl palmitate = 0.72

4] 5β, 6β-epoxy-cholesteryl palmitate = 0.59

The FAB mass spectra of oxysterols:

Oxysterols extracted from plasma of diabetes patients

1] 5α,6α-epoxy-cholesterol and its corresponding 5β, 6β-epoxy-cholesterol provided molecular ions at m/z 402 = $[MH]^+$ and 827 = $[2M+H+Na]^+$

Oxysterols synthesized using $KMnO_4$/ $CuSO_4.5H_2O$ Catalyst

1] 5α,6α-epoxy-cholesterol and its corresponding 5β, 6β-epoxy-cholesterol provided molecular ions at m/z 402 = $[MH]^+$ and 425 = $[M+H+Na]^+$;827 = $[2M+H+Na]^+$

2] 5α,6α-epoxy-cholesteryl palmitate and its corresponding 5β, 6β-epoxy-cholesteryl palmitate provided molecular ions at m/z 663 = $(M+Na)^+$ and 641 = $[M]^+$

3] 5,6-epoxy 3-ketocholesterol provided molecular ions at m/z 401 =$[MH]^+$

Oxysterols synthesized using Meta-Chloro Perbenzoic Acid

1] 5α,6α-epoxy-cholesteryl palmitate and its corresponding 5β, 6β-epoxy-cholesteryl palmitate provided molecular ions at m/z 641 = $[M]^+$; 727 = $[M+H+ Na+Cu]^+$

In all microwave induced reactions, the synthesis of α- and β-isomers was stereoselective. In the microwave-induced reaction of cholesterol out using $KMnO_4$/ $CuSO_4.5H_2O$ Catalyst, the proportion of the epoxide isomers is: α : β, 80:20. The proportion for cholesterol palmitate epoxide isomers synthesized using: (i) $KMnO_4$/ $CuSO_4.5H_2O$ Catalyst: α : β, 10:90 (ii) Meta-Chloro Perbenzoic Acid: α : β, 25:75.

CHAPTER 4

CONCLUSION

There is no longer any doubt that high plasma levels of LDL are atherogenic and that lowering them can reduce the risk of coronary heart disease. Studies both in vitro and in vivo support the hypothesis that LDL undergoes oxidative modification that targets it for uptake by the macrophage through a specific receptor- the acetyl LDL or scavenger receptor. Many processes are still under investigation. Recent advances have brought us closer to being able to do this with regard to the uptake of LDL by macrophages and the development of the fatty streak, the earlier lesion in atherosclerosis. Much more remains to be done to establish the clinical relevance of these findings. Nevertheless, the experimental data available to date encourage aggressive additional research on the oxidative modification of LDL.

This study has emphasized the oxidative modification of LDL because the evidence for its occurrence in vivo and its role in atherogenesis is already persuasive. However, we recognize that with further study additional modifications may prove to be equally important or even more important. As we learn more about these modifications of LDL, we can hope to find ways of preventing them. To the extent that modifications of these kinds play an important part in atherogenesis, we may be able to intervene and obtain protection beyond that obtained by lowering LDL levels.

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