Characterisation of chlorinated fatty acid metabolites in human cells after uptake of dichlorostearic acid, as determined by a halogen-specific detector (XSD)

Licentiate Thesis

by

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Abstract

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Recently a new halogen specific detector (XSD) has been introduced. It is described to be a sensitive, selective, and robust detector in the determination of halogenated pesticides. It has been declared to be more stable and easier to maintain than other halogen selective detectors. The XSD operates in an oxidative pyrolysis mode and the sample compounds are converted into their oxidation products. When halogen-containing compounds enter the hot detector, the detector current will increase. ClFAs have not earlier been studied by an XSD.

The aim of this work was to study i) the XSD, connected to a gas chromatograph, in the determination of ClFA methyl esters (ClFAMEs), and ii) the incorporation and metabolism of ClFAs in human cell lines, as determined by XSD detection.

It was shown that the XSD provides a detection limit (0.2 ng of dichlorostearic acid methyl ester) and a selectivity ((response ClFAMEs)/(response unchlorinated fatty acid methyl esters)) >> 10^4, similar to that of the electrolytic conductivity detector (ELCD), commonly used in determination of ClFAMEs. Furthermore, the XSD was found to be very easy and stable to maintain in the analysis of ClFAMEs.

In the determination of ClFAs obtained from human cell lines, it was established that human cells can incorporate dichlorostearic acid and degrade it to dichloropalmitic acid and dichloromyristic acid, probably through β-oxidation. ClFAs were found both in the neutral lipid and in the phospholipid fraction of the cultured cells. Dichloromyristic acid was the shortest ClFAs detected and was found to be released to the culture medium to a higher extent than the other ClFAs.

The XSD was found to be a good alternative to the ELCD in the determination of ClFAs and the XSD is suitable for use in continued studies of the turnover of ClFAs in human cells.

Keywords: halogenated fatty acids, halogen-selective detection, phospholipids, neutral lipids, dichloropalmitic acid, dichloromyristic acid, β-oxidation

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Table of Contents

List of Papers

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Åkesson-Nilsson, G., Nilsson, O., Odenbrand, I., and Wesén, C. A new halogen-specific detector, XSD, used for the analysis of chlorinated fatty acids. *J. Chromatogr. A*, under revision.

II. Gustafson-Svärd, C., Åkesson-Nilsson, G., Mattsson, M., Sundin, P., and Wesén, C. Removal of xenobiotic dichlorostearic acid from phospholipids and neutral lipids in cultured human cell lines by β-oxidation and secretion of dichloromyristic acid. Submitted to *Pharmacology & Toxicology.*

Abbreviations

ClFAs	chlorinated fatty acids
ClFAMEs	chlorinated fatty acid methyl esters
ECD	electron capture detector
ELCD	electrolytic conductivity detector
EOCl	extractable organically bound chlorine
FAMEs	fatty acid methyl esters
GC	gas chromatography
INT 407	the cell line Intestine 407
MS	mass spectrometry
SPE	solid phase extraction
XSD	halogen specific detector

Objectives

The purpose of this work was to study if the Halogen Specific Detector (XSD), connected to a gas chromatograph, is a suitable detector in the determination of chlorinated fatty acid methyl esters. The detection limit of dichlorostearic acid methyl ester and the selectivity, in terms of the response ratio between the chlorinated and the unchlorinated species, were studied.

The XSD was further used with the objective to study the incorporation, metabolism and distribution of chlorinated fatty acids in human cell lines, cultured in an medium containing dichlorostearic acid.

Introduction

Occurrence of chlorinated fatty acids

Chlorinated fatty acids (ClFAs) have been found to account for a considerable portion (up to 90%) of the extractable organically bound chlorine (EOCl) in aquatic animals (Wesén *et al.*, 1990; 1992a; 1995; Wesén, 1995; Mu, 1996; Milley *et al.*, 1997). ClFAs are bound both in storage lipids and in membrane lipids (Björn *et al.*, 1998a; Vereskuns, 1999). Well-known chlorinated environmental pollutants, such as PCBs, dioxins, and chlorinated pesticides normally contribute to only a minor portion (< 5%) of EOCl in aquatic species (Lunde *et al.*, 1976; Södergren *et al.*, 1988; Newsome *et al.*, 1993).

Several forms and isomers of ClFAs have been identified, and 9,10-dichlorostearic acid (containing 18 carbon atoms and two chlorine atoms, on carbon 9 and 10) is one of those commonly found (Wesén *et al.*, 1992b; Wesén *et al.*, 1995).

ClFAs have been found to be assimilated and transferred in the food chain much in the same way as unchlorinated fatty acids (Cunningham & Lawrence, 1976, 1977abc; Vereskuns, 1999; Björn, 1999). The pattern of ClFAs in biota studied tends to differ between different types of samples and seems to depend on the area from which the samples are collected (Wesén, 1995). High concentrations of ClFAs in biota have been connected to anthropogenic inputs such as effluents from chlorine bleached pulp production (Wesén *et al.*, 1992a; Wesén, 1995; Björn *et al.*, 1998a), which is a major source of these compounds (Leach & Thakore, 1977). Dichloromyristic acid (containing 14 carbon atoms and two chlorine atoms) was found to be the major ClFA in lobster, and in fish from remote marine waters (Milley *et al.*, 1997; Wesén, 1995; Mu *et al.*, 1996b; Björn *et al*, 1998a). ClFAs have also been found in several foodstuffs in the USA (KAN-DO Office and Pesticides Team, 1995) probably originating from chlorinated flour (Komo-Suwelack *et al.*, 1983; Fukayama *et al.*, 1986; Heikes, 1992; 1993).

Determination of chlorinated fatty acids

Methods for the determination of trace amounts of ClFAs have relatively newly been developed, which may explain why these compounds did not come to attention until recently. ClFAs are not persistent to the clean-up steps employed in the determination of environmental pollutants such as PCBs and DDT, but are destroyed or removed when the samples are treated with concentrated sulphuric acid. Furthermore, the electron capture detector (ECD), which is the detector most commonly used for analysis of persistent halogenated organic pollutants, is not suitable for analysis of ClFAs (Remberger *et al.*, 1990; Sundin *et al.*, 1992; Wesén *et al.* 1992b). The analysis of ClFAs usually involves extraction of the lipids, transesterification of the fatty acids from glycerol esters to methyl esters, followed by separation by gas chromatography (GC) and detection by a halogen-selective electrolytic conductivity detector (ELCD) (Wesén *et al.*, 1992b; Mu, 1996; Heikes, 1993; Mu *et al.*, 1997a) or by mass spectrometry (MS), which also allows for identification (Mu *et al.*, 1996a). Consequently, MS has been an important technique in the identification of ClFAs (Wesén *et al.*, 1995, Mu *et al.*, 1996ab; Milley *et al.* 1997; Vereskuns, 1999).

Recently, a new halogen specific detector, XSD, has been introduced for the determination of halogenated compounds, such as pesticides and PCBs (Anonymous, 1998a; Cook & Engel, 1999). The Model 5360 Halogen Specific Detector (XSD) (OI Analytical, Texas, USA), is presented as comparable to the ELCD concerning sensitivity, and having several advantages over the ECD and the ELCD as a halogen detector (Anonymous, 1995; Anonymous, 1998a). It is claimed to be easier to maintain than the ELCD, and to have a superior stability. It is also claimed to have a higher selectivity, expressed as the chlorine/hydrocarbon ratio, than the ECD.

The XSD is made up of a temperature regulated reactor core, and a probe assembly mounted inside the core: The reactor core consists of an anodic platinum coil, wrapped around and in contact with an alkali glass ceramic rod and a cathodic platinum bead at the end of the rod (Figure 1 in Paper I). A potential is applied over the anode and cathode and a background current is obtained, probably caused by thermal electrons emitted from the cathode at high temperatures. The mechanism for the XSD response to halogenated compounds is not well known. The XSD reactor operates in an oxidative pyrolysis mode and the sample compounds are converted into their oxidation products. When halogen-containing compounds enter the hot detector, the detector current will increase (Anonymous, 1998a). Halogen containing compounds have been explained to remove alkali from the surface of the ceramic rod close to the anode, leading to an increased detector current (Rice, 1951; Roberts, 1957). The production of charged species in the reactor is explained by positive surface ionisation, negative surface ionisation and thermal electron emission (Anonymous, 1998ab). Negative halogen ions are formed when free halogen atoms hit the alkali activated platinum cathode and positive ions are explained to be emitted from the glass ceramic rod, which is in contact with the anodic platinum coil (Anonymous, 1998a; Paper I). The energy released per electron transfer is supposed to generate a local

temperature increase followed by a larger flow of "thermal" electrons (Anonymous, 1998ab). The process responsible for the transfer of alkali from the ceramic rod to the cathode (the activation of the cathode) is not explained.

The XSD has not before been used in analysis of chlorinated fatty acid methyl esters (ClFAMEs), and one purpose of the study was to use ClFAMEs to evaluate the performance of the detector in comparison to the ELCD. Furthermore, a theoretical chemical equilibrium study was made with the purpose to determine the oxidation products formed in the XSD reactor, and a scanning electron microscope was used to determine the elemental composition of the XSD.

Physiological effects of chlorinated fatty acids

Only a few studies of physiological effects of ClFAs are at hand. ClFAs have been indicated to affect in particular reproduction related processes (Cherr *et al.*, 1987; Håkansson *et al.*, 1990; Björn *et al.*, 1998b) but also more general adverse effects (impaired cell-growth, increased ATP release, and elevated mortality of fish) have been suggested (Høstmark *et al.*, 1998; Høstmark *et al.*, 1999; Ewald & Sundin, 1993; Leach & Thakore, 1977).

The presence of ClFAs in human tissues has not been reported, and it is not known if human cells can incorporate and metabolise these compounds. The exposure of ClFAs to humans is not known, but can be expected to be lower than to aquatic biota (Brandt & Lindbjerg, 1997). This means that the concentration of ClFAs is probably much lower in human tissues than in fish tissues. Determination of still lower concentrations of ClFAs than in fish needs further development of analytical methods. However, to tell if it is possible to find ClFAs in human tissues, it is important to determine if it is possible for human cells to incorporate and metabolise ClFAs. The other purposes of this study, was, therefore, to examine the uptake and metabolism of ClFAs in human cell lines.

Methods

XSD in the determination of chlorinated fatty acid methyl esters (Paper I)

To examine the suitability of the XSD in the determination of ClFAMEs, the detection limit of dichlorostearic acid methyl ester, and the selectivity, in terms of the (dichlorostearic acid methyl ester)/(oleic acid methyl ester) signal ratio was determined. ClFAMEs enriched from an earlier well-examined eel sample from Idefjord (Wesén *et al.*, 1992b; Mu *et al.*, 1996ab; Björn *et al.*, 1998a) were studied by an XSD and compared to earlier ELCD studies of this eel sample. ClFAMEs were enriched from the eel sample according to Mu *et al.* (1996a).

Dichlorostearic acid was prepared from *cis*-9-oleic acid according to Mu *et al.* (1996a) and the corresponding methyl ester was prepared by using 20% borontrifluoride in methanol (Morrison & Smith, 1964).

The XSD was connected to a GC equipped with an unpolar fused silica capillary column, and a manual wide bore injection technique was used. The XSD conditions in the determination of dichlorostearic acid methyl ester were optimised by selecting different detector temperatures and air flows.

In order to better understand the function of the XSD, a used XSD reactor was dismantled to study the elemental composition of the detector components. The reactor core, ceramic rod, and jet inlet were cut to pieces. The composition of these pieces was analysed by a scanning electron microscope. Furthermore, the theoretical chemical equilibrium in the XSD was studied for the combustion of a chlorine-containing sample.

Determination of chlorinated fatty acids in cultured human cell lines (Paper II)

To examine the possibility that ClFAs may be incorporated in human tissues, human cell lines were cultured in media supplemented with 9,10-dichlorostearic acids, and the content of ClFAs in the cells were studied. Two different cell lines were used; Intestine 407 (INT 407), which is an epithelial cell line initiated in 1955 from jejunum and ileum of a human embryo of about 2 months of gestation (Henle & Deinhardt, 1957), and SH-SY5Y, a subclone of a human bone marrow metastases-derived neuroblastoma cell line SK-N-SH (Biedler *et al.* 1973). The cells were grown in monolayers in a culture medium containing 250 mg of 9,10-dichlorostearic acid/L. The culture medium was removed after 24 hours of incubation and new medium, not containing any ClFAs, was added. The culture cell medium was then changed every fourth day. The lipids in the cells and in the culture medium were extracted according to the method of Bligh and Dyer (1959), modified by addition of KCl to ensure the complete extraction of polar lipids (Folch *et al.*, 1957). The content of ClFAs in the cells and in the medium was determined every second day by GC/XSD of the corresponding methyl esters. In order to determine the distribution of ClFAs in cells, the chloroform/methanol-extracted lipids from the cells were separated by solid phase extraction (SPE), using an aminopropyl bonded phase (Bond Elut) (Kalunzy *et al.*, 1985). By using elution media of different characters, neutral lipids (storage lipids), free fatty acids and phospholipids (membrane lipids) can be selectively isolated in high yields and purities.

The incorporation of ClFAs was also studied with a radiolabelled tracer, 1-^{14}C-dichlorostearic acid, followed by thin-layer chromatography with autoradiographic detection, to confirm the GC/XSD and SPE results.

Results and Discussion

XSD in the determination of chlorinated fatty acid methyl esters (Paper I)

Factors influencing the XSD response

The XSD response is dependent on a) the flow of the combustion gas, being either air or oxygen, through the reactor, b) the reactor temperature, and c) the injected amount of sample (Anonymous, 1998a, Paper I).

In the experiment where different air flows through the detector were studied, it was found that the response of ClFAMEs was constant in the flow range of 10-20 ml/min, but when the air flow exceeded 20 ml/min or was below 10 ml/min the response was reduced (Paper I). An explanation to the reduced response at higher airflows might be that the compound will remain a shorter time in the detector (Anonymous, 1998a). The reason why the response also was decreased when the airflow became lower than 10 ml/min is not known (Paper I).

In the experiment where different detector temperatures were studied, it was found that the reactor temperature was an important factor for the detector response. The highest response was obtained at 1100 °C (the highest temperature that could be selected), and it was decreased by 50-55% and 80-85%, as the temperature was decreased from 1100 °C to 1000 °C and 900 °C, respectively. In the theoretical study of the chemical equilibria obtained on combustion of chlorine containing compounds, free gaseous chlorine Cl (g) and HCl (g) were found as the major components (Paper I). HCl (g) is the major compound in the temperature range 600-900 °C (Paper I) and Cl (g) is the major compound at temperatures above 930 °C, close to the temperature selected for the XSD analysis (Paper I). The calculated increment of Cl (g) formation as the temperature increases agrees with the experimentally observed increment of the XSD response with increasing temperature. Furthermore, Cl (g) is supposed to generate the increased current according to Anonymous (1998a).

The detector response to dichlorostearic acid methyl ester was shown to be linear in the range of 0.2-8 ng, but when larger amounts were injected, the detector response became non-linear, and lower than expected (Paper I). The non-linear phenomenon is possibly caused by an insufficient access to alkali atom-activated surfaces (Paper I).

Except for the sensitivity, another important property of a halogen selective detector is the chlorine/hydrocarbon selectivity. The selectivity in terms of the (dichlorostearic acid methyl ester)/(oleic acid methyl ester) signal ratio was decreased when the temperature increased. The selectivity was 3×10^3 at 1100 °C. At 1000 °C the selectivity was 10^4, which was the same value as determined for chlorine/hydrocarbon response (Anonymous, 1998a). At 900 °C the selectivity was $>>10^4$ (no signal was recorded from 5 µg of injected methyl oleic acid methyl ester). Thus, the highest absolute response of dichlorostearic acid methyl ester was obtained at 1100 °C at airflow of 10-20 ml/min, but the highest relative

response was obtained at 900 °C. The apparent advantage of performing the analyses at 1100 °C is refuted by the reduced selectivity at that temperature.

The choice of ceramic rod material is important for having a sensitive and long lasting detector, because the halogen mediated removal of alkali from the surface of the ceramic rod eventually results in a lack of alkali and the ceramic rod must be replaced by a new one. A material that seems to give excellent sensitivity and very long constant lifetime is synthetic Leucite ($K_3O/Al_2O_3/SiO_2$ =1/1/4) (Roberts, 1957). The content of potassium, aluminium, and silica that was found in the ceramic rod (Paper I) agrees with the composition of Leucite. It was not mentioned in Anonymous (1998a) which alkali metal was used, but in Anonymous (1998b) sodium is mentioned as the alkali metal present. Potassium, instead of sodium, probably makes the detector more sensitive, because potassium has a lower work function, 2.30 eV, than sodium, 2.75 eV (Handbook of Chemistry and Physics, 1980-1981), and consequently, the difference between the work function of the potassium activated platinum and the electron affinity of chlorine will be larger.

XSD, an alternative to the ELCD in the determination of chlorinated fatty acid methyl esters

The XSD was found suitable as a halogen selective detector in the determination of ClFAs (Paper I and II). The detection limit of dichlorostearic acid methyl ester measured at a reactor temperature of 900 °C (no response of unchlorinated FAMEs were observed at this temperature) and an air flow between 10 and 20 ml/min, was determined to be 0.2 ng (at two times the noise), which is similar to the detection limit in GC/ELCD (Wesén *et al.*, 1992b). The selectivity in terms of the (dichlorostearic acid methyl ester)/(oleic acid methyl ester) signal ratio was determined to be >> 10^4, which is comparable to that of the chlorine/hydrocarbon selectivity of 10^6 with the ELCD (Farwell *et al.*, 1981). The detection limit of dichlorostearic acid methyl ester was not notably different from that (0.1 ng) obtained by selected ion monitoring (Sundin *et al.*, 1992), a sensitive mass spectrometric technique used for the detection of ClFAMEs (Wesén *et al.*, 1995; Mu *et al.*, 1996ab). However, in such a mass spectrometric technique only known ions are monitored, unknown ClFAs may remain undetected.

Another decisive part of the work in Paper I was to compare the XSD response to the ELCD response, by studying an eel lipid extract containing different ClFAMEs, an extract which has earlier been well studied by ELCD (Mu *et al.*, 1996a). The pattern of these chromatograms and the relative intensities of the peaks were comfortingly equal (Figure 4 in Paper I), suggesting that the XSD is comparable to the ELCD also in analysis of ClFAMEs that appear in a complex sample matrix.

Determination of ClFAs in cultured human cell lines demands a halogen selective detector that has high sensitivity for ClFAMEs and high chlorine/hydrocarbon selectivity. Furthermore, good stability is important when a long series of samples is to be analysed. In order to obtain reproducible results it is also important to have a detector that is easy to maintain. The XSD meets these

demands in the determination of ClFAs in cultured human cell lines (Paper II). The results were reproducible, and the response of re-injected samples was almost the same even if it lasted 2-3 months between injections (Paper I). Furthermore, the stabilisation time proved to be less than two hours, which is much shorter than that of the ELCD and the ECD, which is in both cases is about 1 day.

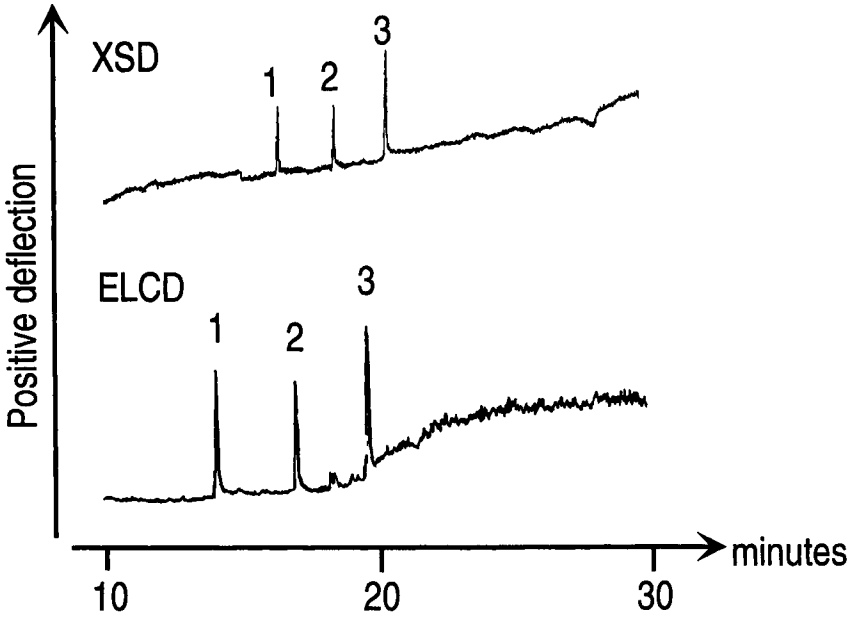

Figure 1. Comparison between gas chromatograms obtained by an XSD and an ELCD (using an unpolar column) of ClFAMEs derived from ClFAs released from INT 407 cells (after 6 days of metabolism). Similar amounts of FAMEs from the same extract were injected. The peaks correspond to the (1) dichloromyristic acid- (2) dichloropalmitic acid- and (3) dichlorostearic acid methyl esters.

The costs for running the XSD were acceptable, 10 m^3 of medical air, one reactor core, one reactor assembly, and one O-ring were consumed in one year of operation, and the cost for this was about 16 000 SEK. The corresponding cost for the ELCD is slightly less than 10 000 Swedish crowns. However, the ELCD is more laborious to handle compared to the XSD, because the ELCD is a more complex detector than the XSD. Except for mass transfer processes over gas-solid interfaces (occurring both in the ELCD and in the XSD), the function of an ELCD additionally is based on mass transfer over gas-liquid interfaces. Less transfer processes and media in the XSD makes this detector easier to handle, and thus cheaper compared to the ELCD to maintain, when also the labour needed is accounted for.

The XSD results from the cell study were validated by subjecting a number of samples from the study to ELCD analysis. The relative intensities of the peaks were comfortingly equal in the XSD and in the ELCD chromatograms (Figure 1).

Incorporation of dichlorostearic acid in INT 407 cells (Paper II)

In the study where INT 407 cells were grown in media containing dichlorostearic acid, it was found that INT 407 cells incorporated extracellular dichlorostearic acid (Figure 2a; Paper II). ClFAs were found both in the neutral lipid and the phospholipid fractions (Figure 2 in Paper II). Similar results have been obtained for fish by other researchers (Björn *et al.*, 1998a; Ewald *et al.*, 1996; Vereskuns, 1999). Dichlorostearic acid was the predominating ClFA being incorporated, representing about 80% in both lipid fractions (Paper II). The presence of minor amounts of dichloropalmitic acid (containing 16 carbon atoms and two chlorine atoms) and dichloromyristic acid in the cultured cells (Figure 2a), however, indicates that dichlorostearic acid to some extent was metabolised by β-oxidation before being incorporated in the cell lipids. Free ClFAs, supposed to appear in the free fatty acids SPE fraction, were not detected, or were present at very low levels compared to the amounts in neutral lipids and phospholipids. This result agrees with what is the case for normal, unchlorinated fatty acids, which seldom occur free in living cells (Voet & Voet, 1995). The result of thin-layer chromatography separating the radiolabelled tracer agreed with the SPE result, concerning incorporation of ClFAs in phospholipids (Paper II).

Metabolism of incorporated chlorinated fatty acids (Paper II)

In energy production, fatty acids are dismembered through β-oxidation (the fatty acid chain is degraded, from the carboxyl end, by the sequential removal of two-carbon units) to yield acetyl-CoA units, (Voet & Voet, 1995; Stryer, 1995). ClFAs may also undergo degradation by β-oxidation (Conacher *et al.*, 1984, Björn 1999, Mu *et al.* 1997b; Paper II). The time dependent decrease of dichlorostearic acid in the INT 407 cells (Figure 2a, 2b and 2c) and in the SH-SY5Y cells (Figure 7 in Paper II) together with the parallel increase of dichloropalmitic acid and dichloromyristic acid suggested that dichlorostearic acid was metabolised through β-oxidation (Paper II). Cellular metabolites shorter than dichloromyristic acid were not found (Paper II), which might indicate that further β-oxidation was hindered. These findings agree with previous studies where dichloromyristic acid was found to be the shortest ClFAs with an even-numbered carbon chain (Conacher *et al.*, 1984; Vereskuns, 1999; Wesén, 1995; Mu, 1996; Milley *et al.*, 1997).

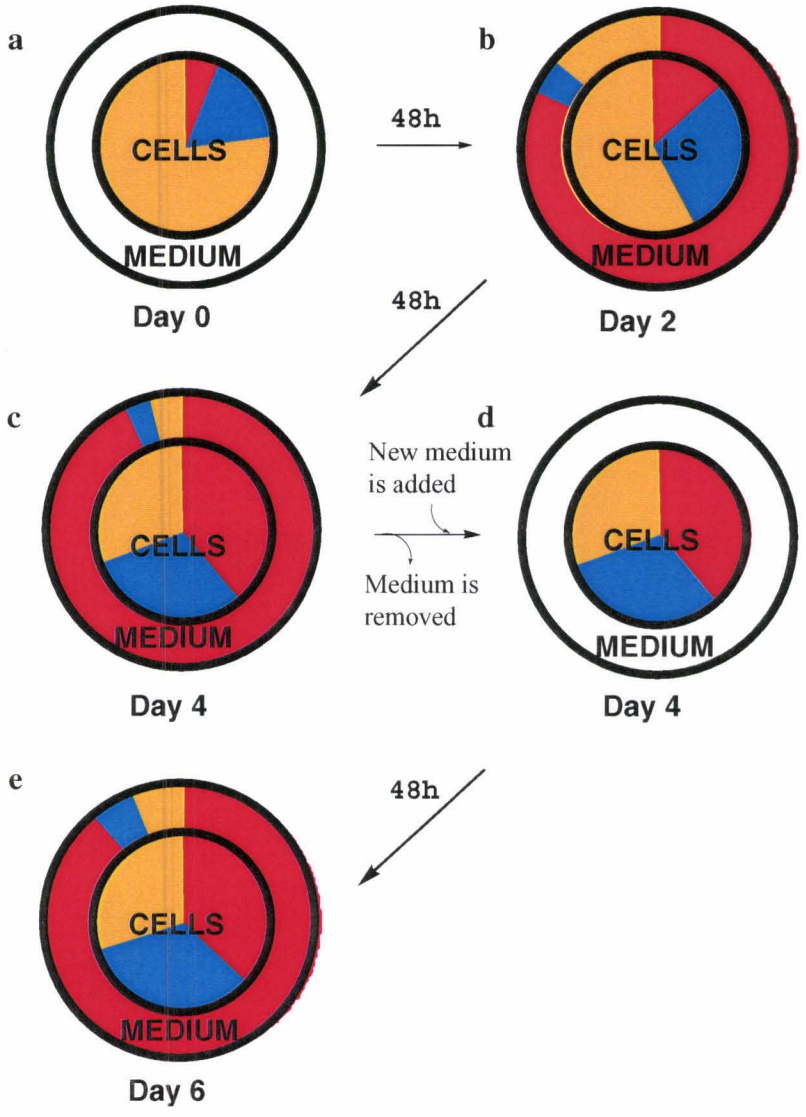

Figure 2. Relative distribution of ClFAs in INT 407 cells and their surrounding culture medium from day 0 to day 6. Day 0 shows the relative distribution of ClFAs in the cell after 24 hours of incubation. The culture medium was changed day 0 and day 4. This is a compilation of the results in figure 4 and figure 6 in Paper II.

Dichlorostearic acid

Dichloropalmitic acid

Dichloromyristic acid

Non chlorinated fatty acids

Fatty acids in storage lipids are normally metabolised completely by β-oxidation for energy production (Voet & Voet, 1995). The supposed β-oxidation of ClFAs might be hindered due to bulkiness of halogen atoms when these "come too close" to the carboxylic moiety of the molecule, (Figure 3; Ewald & Sundin, 1993), which might explain why dichloromyristic acid was found to be the major ClFAs in certain aquatic animals from remote areas (Mu, 1996b; Milley *et al.*, 1997) and the shortest ClFAs found in the incubated human cells (Paper II). Another degradation pathway of fatty acids is ω-oxidation (oxidation of the "last" carbon atom, at the terminal, methyl end), probably an unusual fatty acid oxidation pathway (Voet & Voet, 1995). The formed dicarboxylic can then be further dismembered by β-oxidation. Highly substituted molecules have been found to be initially catabolised through ω-oxidation (Gurr & Harwood, 1996).

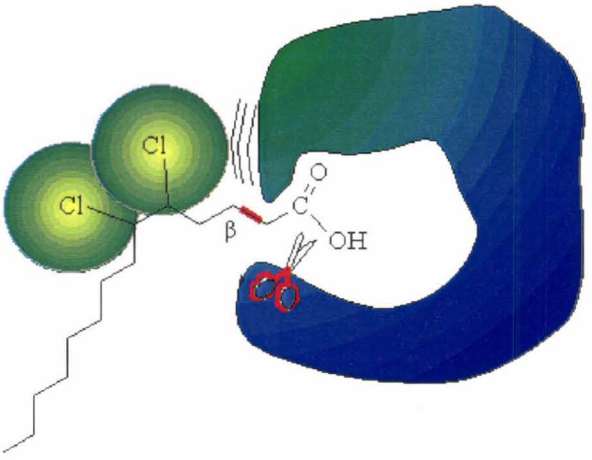

Figure 3. A supposed, sterically hindered β-oxidation of 5,6-dichloromyristic acid (a metabolite, which is supposed to be formed by β-oxidation of 9,10-dichlorostearic acid). The active site of the enzyme (marked as red scissors) cannot reach the β-carbon and cut the bond (marked in red).

The relative distribution of ClFAs in the phospholipid fraction and the neutral lipid fraction of the INT 407 cells was changed after further incubation (Figure 9 in Paper II). Initially, dichlorostearic acid was the predominating ClFAs in both membrane and storage lipids. After 6 days, the amount of dichloropalmitic acid was significantly higher (Two-sided paired Student's t-test) than the amounts of dichlorostearic acid and dichloromyristic acid in the phospholipid fraction, in opposite to the neutral lipid fraction, where the amount of dichloropalmitic acid was significantly lower than the amounts of dichlorostearic acid and dichloromyristic acid after 6 days (Figure 9 in Paper II). ClFA bound in storage lipids are indicated to have a turnover rate different from that of ClFAs bound in

membranes, which agrees with earlier studies of fish lipids (Mu, 1996; Björn, 1999). Changes in the fatty acid profiles of membranes can affect the maintenance of the normal cellular functions, because the ratio between e.g. saturated and unsaturated fatty acids in membrane phospholipids is an important factor in determining membrane fluidity and permeability (Stubbs & Smith, 1984). The incorporation of ClFAs in the membrane might be the explanation for the increased release of ATP observed in cells exposed to dichlorostearic acid (Ewald & Sundin, 1993).

ClFAs, especially dichloromyristic acid, were found to be secreted into the culture medium by the INT 407 cells (Figure 2b, 2c and 2e) and the SH-SY5Y cells (Figure 8 in Paper II). It seems reasonable to suspect that the presence of ClFAs above a certain level in a cell might hamper normal cellular functions (Cherr *et al.*, 1987; Håkansson *et al.*, 1990; Björn *et al.*, 1998b; Vereskuns, 1999). The release of dichloromyristic acid in both cell lines might suggest that the mechanisms responsible for the cellular removal of ClFAs is widely distributed among different cell types, and possibly of general importance in the cellular treatment of xenobiotic fatty acids (Paper II).

Conclusion

The XSD is a good alternative to the ELCD in determination of ClFAMEs released from complex samples. The XSD is stable, simple to handle and not too expensive to maintain. The XSD can be used e.g. in continued studies of the turnover of ClFAs in human cells.

Human cells can incorporate extracellular dichlorostearic acid, and degrade it to dichloropalmitic acid and dichloromyristic acid, probably though β-oxidation. Dichloromyristic acid tends to be the shortest ClFA formed, and is released to the culture medium.

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