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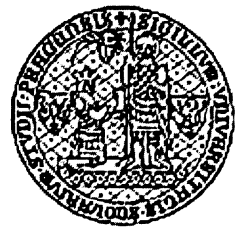

DIPLOMA THESIS

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Tandem mass of sphingolipids and its application in diagnosis of sulphatidosis and related diseases

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I declare that I have worked on this study independently under supervision of RNDr. Befekadu Asfaw, Ph.D. and that I have listed all references.

In Prague, 2nd of May 2006

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Content:

List of abbreviations

ARMS	Amplification refractory mutation system
amu	Atomic mass unit
ASA	Arylsulphatase A
AGM1	asialoGM1 ganglioside
	Gal(β1→3)GalNAc(β1→4)Gal(β1→4)Glc(β1→1′)Cer
BSA	Bovine serum albumin
BPS	Biphasic system
Cer	Ceramide
cps	Counts per second
CID	Collision induced decomposition
DNA	Deoxyribonucleic acid
EDC	N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
ES/MS/MS	Electrospray ionisation tandem mass spectrometry
Gal	Galactose
GalCer	Galactosylceramide
Gb_3Cer	Globotriaosylceramide, Gal(α1→4)Gal(β1→4)Glc(β1→1′)Cer
Glc	Glucose
GlcCer	Glucosylceramide
GM1	GM1 ganglioside
	Gal(β1→3)GalNAc(β1→4)[NeuAc(α2→3)]Gal(β1→4)Glc(β1→1′)Cer
GSLs	Glycosphingolipids
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
ISTD	Internal standard
kDa	KiloDalton
LacCer	Lactosylceramide

LSD	Lysosomal storage disease
MeOH	Methanol
MPS	Monophasic system
MLD	Metachromatic leukodystrophy
MRM	Multi reaction monitoring
m/z	Mass to charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
PCR	Polymerase chain reaction
RFLP	Restriction length polymorphism
SAP	Sphingolipids activator protein
SCDase	Sphingolipid ceramide N-deacylase (EC 3.5.1.69)
SFLs	Sphingolipids
SGalCer	Galactosylceramide I^3-sulphate
SM	Sphingomyelin
Sulpho-NHS	Sulphosuccinimide
Susp.	Suspected
TNFα	Tumor necrosis factor α
UDP	Uridine diphosphate

Glycosphingolipids and sulphatides

Glycosphingolipids posses a remarkable degree of structural diversity, and numerous enzymes are involved in their synthesis, recycling and turnover. Their composition is cell type specific and undergoes marked changes during cell growth, differentiation, viral transformation and oncogenesis [1].

Sulphatides belong to the group of glycosphingolipids (GSLs) [2], important components of eukaryotic plasma membranes. They are particularly abundant in brain tissue and the kidney, but are detectable in some other tissues too.

Structure and nomenclature of sphingolipids

Nomenclature of lipids used in this thesis follows the recommendation of IUPAC-IUB Commission on Biochemical Nomenclature [3].

Sphingolipids are complex molecules with hydrophobic ceramide and hydrophilic part, which consists of sugar or phosphorylcholine moiety. Ceramide is N-acylsphingoid, that is covalently attached by terminal hydroxyl group to reducing end of saccharide in carbohydrate moiety. The type of chemical bond between sphingoid and fatty acid is amide bond. Sphingoids (old trivial name sphingosines) are long chain aliphatic amino alcohols and their derivates. Structural variations in hydrophobic chains of sphingoids, fatty acids and carbohydrates result in great number of sphingolipid types (Fig.1, p.11).

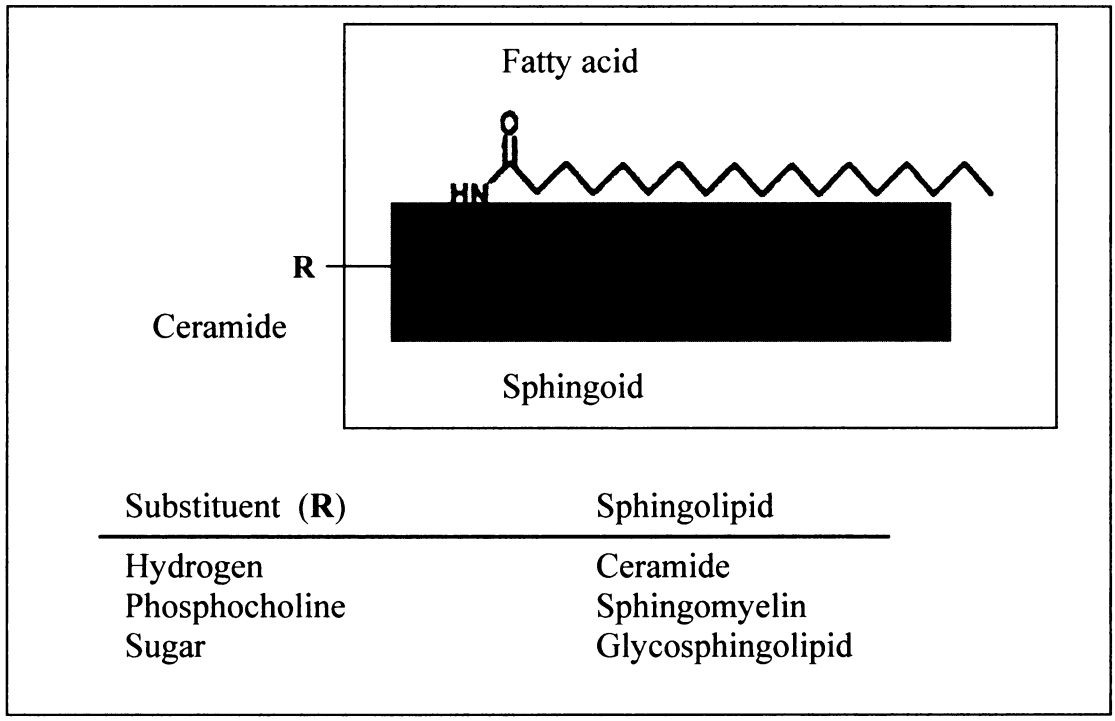
Glycosphingolipids (GSLs) are divided into two main groups:

Neutral

- mono- , oligo- and polyglycosylceramides

Acid

- sialoglycosphingolipids which contain sialic acid (N-acetylneuramic acid, 5-amino-3,5-dideoxy-D-glycerononulosidic acid and derivates)
- sulphoglycosphingolipids (sulphatides with esterically bonded sulphate moiety)
- phosphoglycosphingolipids (phospho mono- or diesters)



Substituent (**R**)	Sphingolipid
Hydrogen	Ceramide
Phosphocholine	Sphingomyelin
Sugar	Glycosphingolipid

Fig. 1: General sphingolipid structure

We may use systematic nomenclature for neutral glycosphingolipids e.g. β-D-galactosyl-(1→4)-β-D-glucosyl-(1→1)-ceramide. Trivial name for the structure above is lactosylceramide. In semisystematic names, we use trivial names (Table 1, p.12) for root structures as prefix (lacto-, neolacto-, globo-). These names consist of (root name)(root size)osylceramide, e.g. III^2-α-fucosylglobotriaosylceramide. Monosaccharide that carries the residue is designated by roman numerals (counting from the ceramide end) and arabic superscript indicating position of the residue to which is attached.

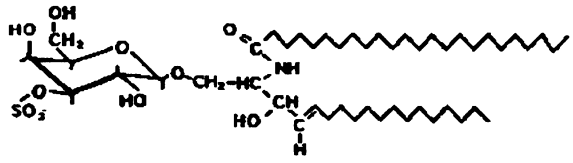


Fig. 2: Sulphatide - structure

Gangliosides are group of GSLs named as N-acetyl- (or N-glycoloyl) neuraminosyloligoglycosylceramides. Sulphoglycosphingolipids are sulphate esters of neutral GSLs, e.g. I^3-sulpho-GalCer or galactosylceramide I^3-sulphate or sulphatide by trivial name (Fig. 2).

Table 1: GSL nomenclature – symbol, structure and trivial name [3]

Glycolipid structure	*Oligosaccharide*		
	Trivial name	*Symbol*	*Short symbol*
Gal(α1-4)Gal(β1-4)GlcCer	Globotriaose	$GbOse_3$	Gb_3
GalNAc(β1-3)Gal(α1-4)Gal(β1-4)GlcCer	Globotetraose	$GbOse_4$	Gb_4
Gal(α1-3)Gal(β1-4)GlcCer	Isoglobotriaose	$iGbOse_3$	iGb_3
GalNAc(β1-3)Gal(α1-3)Gal(β1-4)GlcCer	Isoglobotetraose	$iGbOse_4$	iGb_4
Gal(β1-4)Gal(β1-4)GlcCer	Mucotriaose	$McOse_3$	Mc_3
Gal(β1-3)Gal(β1-4)Gal(β1-4)GlcCer	Mucotetraose	$McOse_4$	Mc_4
GlcNAc(β1-3)Gal(β1-4)GlcCer	Lactotriaose	$LcOse_3$	Lc_3
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcCer	Lactotetraose	$LcOse_4$	Lc_4
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcCer	Neolactotetraose	$nLcOse_4$	nLc
GalNAc(β1-4)Gal(β1-4)GlcCer	Ganghiotriaose	$GgOse_3$	Gg_3
Gal(β1-3)GalNAc(β1-4)Gal(β1-4)GlcCer	Gangliotetraose	$GgOse_4$	Gg_4
Gal(α1-4)GalCer	Galabiose	$GaOse_2$	Ga_2
Gal(1-4)Gal(α1-4)GalCer	Galatriaose	$GaOse_3$	Ga_3
GalNAc(1-3)Gal(1-4)Gal(α1-4)GalCer	*N*-Acetylgalactosaminylgalatriaose	$GalNAc1\text{-}3GaOse_3$	–

Chemical properties

Hydrophilic saccharide part together with hydrophobic lipid part give GSLs their amphiphilic properties. This results in the ability of creating micelar structures. Especially polar GSLs as gangliosides create micelar structures in range of concentrations from 10^{-9} M to 10^{-2} M [4, 5]. At higher concentrations, they create hexagonal cylindrical structures. Hydrophility increases with portion of saccharide moiety in molecule. This property is observable during separation and identification of GSLs by HPTLC, where different chromatographic mobility of GSLs due to polarity of their saccharide chains can be demonstrated.

GSLs do not create double layer structures spontaneously in aqueous solutions. Aggregation of gangliosides to bilayer structures requires presence of phospholipids.

GSLs are stable and well soluble in chloroform-methanol solutions at low temperatures for many years (-20°C). However, methanal fixation of tissue destructs polysialo gangliosides (loss of sialic acid) by influence of formic acid.

Resistence of GSLs to chloroform and methanol is used in Folch method for their extraction from other compounds in tissues [6].

Biosynthesis of GSLs

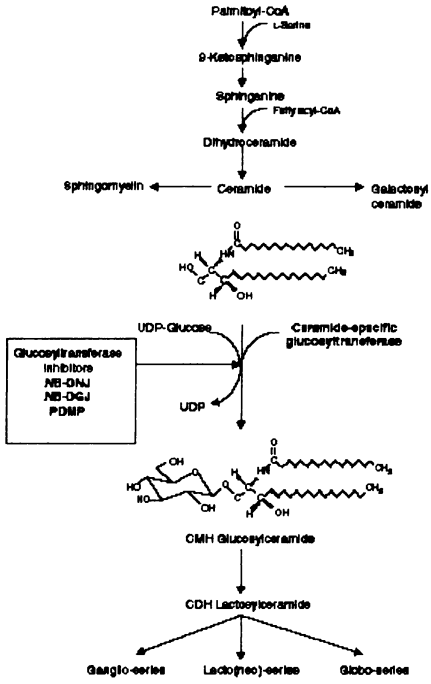


Fig. 3: Biosynthesis of GSLs [26]

There are differences in *de novo* synthesis and resynthesis of GSLs [7]. *De novo* biosynthesis of GSLs is located in endoplasmic reticulum (ER) and requires formation of membrane anchor [8]. First step is enzymic condensation of L-serine with acylCoA, usually palmitoylCoA, which forms 3-ketosphinganine. Enzyme participating on this reaction is pyridoxalphophate dependent serine palmitoyl transferase [9]. In the next step 3-ketosphinganine is reduced to D-*erythro*-sphinganine by 3-ketosphinganine reductase [10], which is NADPH dependent enzyme. Sphinganine (but not sphingosine) is acylated to dihydroceramide by N-acyltransferase [11]. Dihydroceramide is then desaturated to ceramide (Fig. 3) by specific desaturase enzyme [12]. All these reactions take place on cytosolic leaflet of the ER membrane [12, 13].

Ceramide is the precursor of complex GSLs [14], synthetised on luminal part of Golgi membrane [15].

Sulphatides are formed through sulphation of galactosylceramide. The reaction is catalysed by a microsomal sulphotransferase in presence of 3′-phosphoadenosine-5′-phosphosulphate (PASP) as sulphate donor [16].

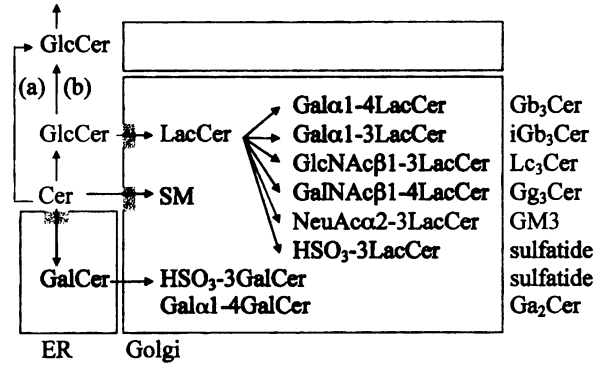


Fig. 4: Biosynthesis of LacCer and other GSLs [14]

Sphingomyelin is formed by the transfer of phosphorylcholine from phospholipid to the 1-hydroxyl group of ceramide [17].

Most of the animal GSL series are derived from lactosylceramide. Biosynthesis of lactosylceramide (Fig. 4) starts from GlcCer by addition of UDP-glucose to the 1-position of ceramide. The bond between ceramide and glucose is β-glycosidic [18-22]. Next

reaction is glycosylation of GlcCer by addition of UDP-Gal and is catalysed by galactosyltransferase I [15, 23].

Biosynthesis of gangliosides is a complex of processes. On these processes, different sialyltransferases with different substrate specificity are participating. The transferases that catalyse the first steps in ganglioside biosynthesis show high specificity toward their glycolipid substrates. Subsequent stepwise glycosylation is performed by only few glycosyltransferases of limited specificity [24].

Degradation of GSLs

The degradation of GSLs occurs in the acidic compartment of the cells, the endosomes and lysosomes. Parts of the plasma membrane are endocytosed and transported through the cytosol to the lysosomes. During the digestion of cellular membranes, cellular glycolipids are cleaved into their building blocks [24]. Lysosomal glycosidases sequentially cleave off the sugar residues from the non-reducing end of their saccharide chains (Fig. 5, p. 15) [24-26]. Plasma membrane components reach lysosomes by endocytic vesicular flow. GSLs are situated on small intra-endosomal and intra-lysosomal vesicles, which allows their digestion [27, 28]. GSLs, which are part of lysosome membrane, are protected from digestion by thick glycocalyx [29].

Another important fact is that GSLs are amphiphilic molecules and their catabolism must proceed on water-lipid interface. Enzymes are water soluble and GSLs are oriented by their saccharide chains into water environment. Sphingolipid hydrolases cleave substrates that are part of intralysosomal membrane structures. GSLs with carbohydrate chains of one to four residues are not sufficiently accessible to the hydrolases and their accessibility to these enzymes is mediated by SAPs (Sfingolipid Activator Proteins) or GM2-activator protein [24]. SAPs are derived from precursor protein called prosaposin (Table 2).

Table 2: Activator proteins and their role in degradation of GSLs

	stored GSL
SAP-A	GalCer (GM1, GM2, GlcCer)
SAP-B	Sulphatide, digalactosylceramide and Gb_3Cer
SAP-C	GlcCer, sphingomyelin and ceramide
SAP-D	Ceramide
GM2-AP	GM2

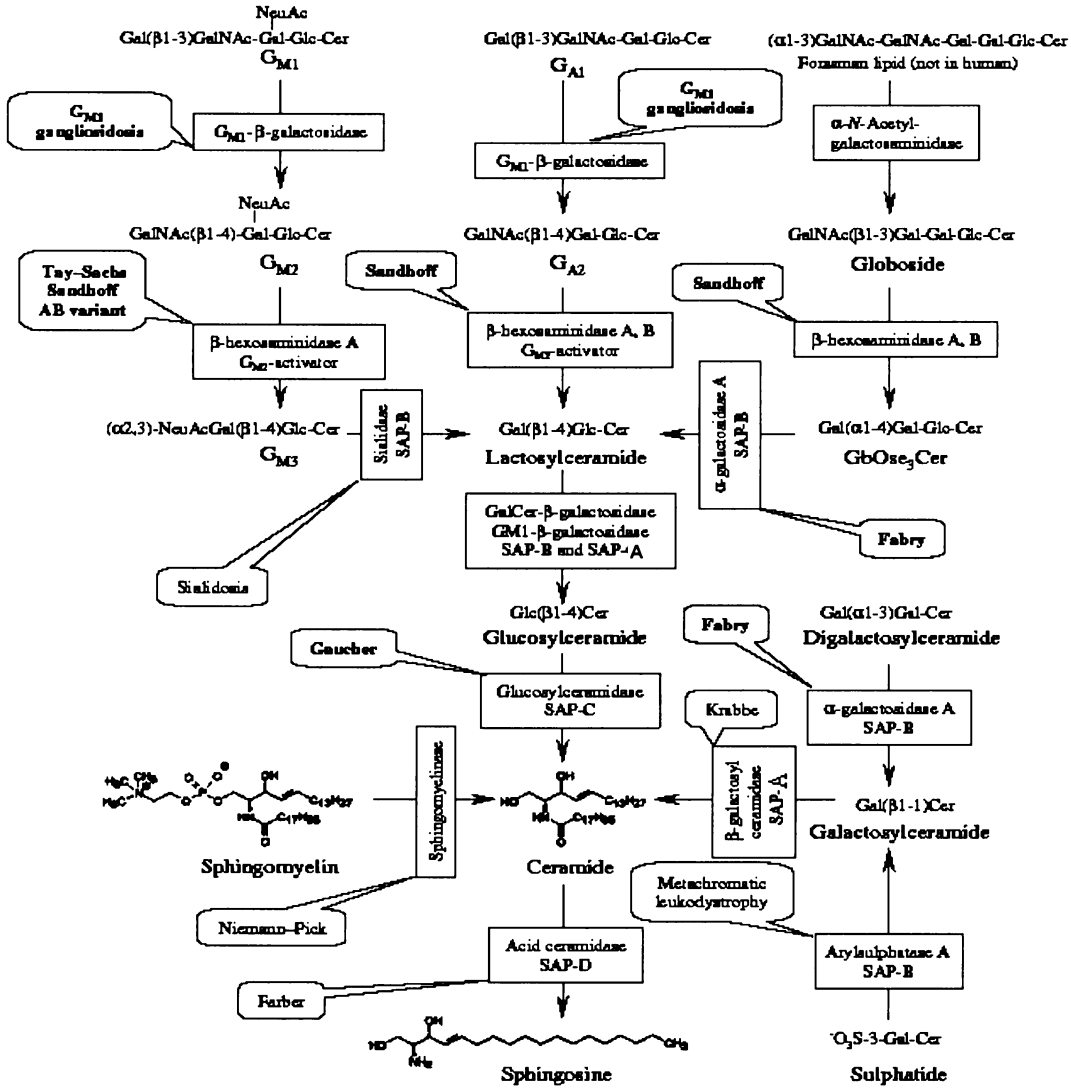


Fig. 5: Scheme of sphingolipid degradation [26]

Inherited defects of hydrolases or SAPs result in storage of corresponding sphingolipids in lysosomes. Deficiency of prosaposin is characterized by massive accumulation of several simple sphingolipids under clinical picture of complex sphingolipidosis [24, 27, 30-37].

Biochemical role of GSLs in organism

GSLs play important role in increasing of membrane rigidity. Conformation of complex GSL (e.g. gangliosides) in the region of inner galactose restricts region rich in oxygen, which is strongly nucleophilic and suitable for cation binding. Especially binding of Ca^{2+}

changes conformation and plays role in creation of ganglioside cluster on membrane surface.

Creation and decomposition of inner esters are the regulatory mechanisms of negative charge and are responsible for the formation of new antigen structures.

In specific conditions gangliosides together with other sphingolipids comprise special microdomaines called clusters (rafts). The clusters are responsible for the interactions between GSLs and ligand.

Further roles of GSLs in organisms [1, 38-45] are following:

- protection of cell surface from chemical and mechanical damage
- cell adhesion, growth, differentiation and regulation
- cell-cell interaction, cell recognition
- interaction with signal molecules and toxins
- changes in their carbohydrate chains may reflect oncogenic transformation and they are important as tumor antigens

Sphingolipidoses

Sphingolipidoses belong to the inherited metabolic disorders with affected lysosomal function. They are caused by functional defects either of sphingolipid hydrolases or their activator proteins. This results in storage of undegraded sphingolipid substrates in lysosomes of different cells of the body [26, 46, 47].

The frequency of individual diseases is not high but as a group they are relatively common and represent [48] an important health problem with combined frequency of 1:9000 live births in the Czech population (H. Poupětová – personal comm.). Since GSLs and in particular gangliosides are abundantly expressed in the nervous system, the brain is often affected by the storage and these disorders are the commonest cause of pediatric neurodegenerative disease [48].

Most sphingolipidoses are inherited in an autosomal recessive trait with the exception of Fabry disease, which is X-linked.

Theoretically, the level of residual enzyme activity has an impact on the prognosis of the age of disease onset, severity and life expectancy of affected individual. The higher the

residual activity the later onset and milder course of disease could be expected [49, 50]. Usually there are three clinical forms of the disease: infantile, juvenile and adult.

Early diagnosis is very important especially for prevention in afflicted families (prenatal diagnosis) and for possibility of treatment which is now available for some of these disorders. There are different laboratory diagnostic approaches to the analysis according to the clinical description of a case, material available and laboratory facilities. Generally, there are five major approaches to the confirmation of the diagnosis:

- *Analysis of sphingolipids (proof of substrate storage)* – in urine (important first tests in SAPs deficiencies, in Fabry disease and sulphatidosis), in cultured cells, bioptic and autoptic tissues etc.
- *Enzymic activity determination* – necessary for a confirmation of the diagnosis in living patients (recommended materials are leukocytes of peripheral blood, skin fibroblasts, amniocytes and chorionic villi biopsies, blood plasma etc.)
- *Loading experiments* – useful in defects of SAPs, assays performed with labelled natural substrates in cultivated skin fibroblasts
- *Mutation analysis* – confirmation of the defect using methods of molecular genetics: gene sequencing, PCR/RFLP, ARMS etc.
- *Tandem mass spectrometry* - new sophisticated method, changed instrumental approach in two first points above: analysis of accumulated sphingolipids and determination of activities of different lysosomal hydrolases using specific substrates [51]. In general it has a broad range of applications in both diagnosis and research of inherited metabolic disorders [52-54].

In therapy of sphingolipidoses symptomatic approach prevails, but there are also several targeted therapies. One of them is bone marrow transplantation, which aim is to repopulate blood with cells with normal level of enzymic activity. The same principle is applied in stem cell therapy. Substrate reduction therapy has its goal in balance of synthesis of sphingolipids with their affected degradation [26, 55-62]. Recently, enzyme replacement therapy has been established to supply a functional enzyme into organism (e.g. in Fabry disease, Gaucher disease, mucopolysaccharidosis type I) [63].

Future potential lays in the gene therapy which is still investigated on experimental level only [64-68].

The exact mechanism, which leads to the pathology in sphingolipidoses still remains unclear. The pathogenesis involves both the innate defect directly resulting from the

storage of sphingolipids and secondary responses to these cellular events [26]. There are some examples of events in pathology of sphingolipidoses we can speculate about [26, 69]:

- toxicity of some stored sphingolipids for the cell (e.g. ceramide and its lysoderivative in neurons)
- different trafficking of sphingolipids from membrane structures to late endosomes during recyclation
- defect in signalling processes in cells due to the changes in membrane microdomain composition (cholesterol and GSLs are sequestered in lysosome)
- dysfunction of microglial cells in brain tissue leading to massive neuronal cell death
- increasing of inflammatory factors such as TNFα, etc...

Sulphatidoses: MLD and SAP-B deficiency

Leukodystrophies are severe and progressive diseases of myelin or myelinating cells, which result in defect of white brain matter. There are four main types of leukodystrophies: dysmyelinative (metachromatic leukodystrophy), hypomyelinative, spongiform and miscellaneous [70].

Metachromatic leukodystrophy (MLD, OMIM 250100) is caused by the deficiency of arylsulphatase A with subsequent lysosomal storage of sulphatides [71]. Prevalence of metachromatic leukodystrophy is 1:145 000 newborns in the Czech population (H. Poupětová – personal comm.). Inheritance is autosomal recessive [72].

Arylsulphatase A (ASA; cerebroside-3-sulphate 3-sulphohydrolase, EC 3.1.6.8) is a lysosomal enzyme found in all body tissues and fluids. Its gene *ARSA* is mapped to chromosome 22q13, covers 3.2kb of genomic DNA and includes eight exons (GenBank NM_000487.3). There are more than 100 known mutations in *ARSA* gene leading to the disease (HGMD, http://www.hgmd.org/).

ASA is synthesized as 62 kDa polypeptide, than receives three N-linked oligosaccharide chains and undergoes conversion of a critical cysteine to Cα-formylglycine (FGly69) and forms dimers. The dimers are transported to the Golgi, where they acquire mannose-6-phosphate recognition markers and bind to receptors. The ASA-receptor complex is transported to endosomes where the complex dissociates. Receptor returns to Golgi and

ASA is delivered to lysosomes. At the acidic pH of lysosomes ASA oligomerises to form homo-octamer $(\alpha_2)_4$ and starts catabolism of sulphatides (desulphation) [57, 73, 74].

The disease has its specific symptoms: quadriparesis, peripheral neuropathy, seizures, blindness and dementia. In patients sulphatides are stored in lysosomes, affecting oligodendrocytes and Schwann cells (i.e. myelin forming glial cells), which leads to a severe loss of myelin sheaths. Sulphatides are also stored in bile duct, kidney and liver and are excreted in urine [72].

Three subtypes of MLD can be distinguished by clinical manifestation: infantile (age of 2 years), juvenile (3-16 years – 40% of cases) and adult (>16 years 20% of cases).

The diagnosis of MLD should base on combination of clinical, histological, biochemical and molecular examinations, but it is the enzyme analysis that confirms the primary defect, i.e. deficient activity of ASA (in fibroblasts, leukocytes of peripheral blood etc.). Important parameter for the diagnosis is the determination of sulphatides in urine or tissues (e.g. HPTLC or tandem MS). The diagnosis is then completed by DNA analysis and eventually sulphatide loading experiments in cell culture. Combination of these methods enables to determine reliably the diagnosis especially of atypical cases (ASA pseudodeficiency, SAP deficiency, prosaposin deficiency) [72, 75].

Currently there is no specific treatment that would halt the progression of MLD. The only definitive therapeutic option is bone marrow transplantation or stem cell therapy, but they have limited efficacy. Some other methods are in development (e.g. gene therapy and enzyme replacement therapy but with limits of the blood-brain barrier) [55, 56, 63, 66].

At present prenatal diagnosis, i.e. prevention of the disease, represents the only way of helping the afflicted families.

SAP-B was the first discovered saposin in 1964 by Mehl and Jatzkewitz [76] and was called sulphatide activator protein. It is required for the degradation of sulphatide by arylsulphatase A. SAP-B is a small lysosomal glycoprotein with one N-glycosidically linked oligosaccharide chain and three disulphide bridges [31, 33, 34].

It behaves like physiological detergent and stimulates sulphatide degradation by solubilizing the membrane bound substrate. The inherited defect of SAP-B leads to the accumulation of sulphatides, digalactosylceramide and Gb_3Cer [24].

The phenotype of the patients resembles a variant form of MLD with late infantile or juvenile onset [24, 75].

The discovery of patients with SAP-B deficiency provided strong evidence for the *in vivo* function of this protein. The genetic defect of SAP-B leads to variant form of MLD but with a nearly normal ASA activity level. The degradation of externally added sulphatide or Gb_3Cer by cultured skin fibroblasts is almost totally blocked but can be raised by adding SAP-B to the culture medium [75].

Recent diagnostic approach lays on sulphatide loading tests, together with the determination of ASA activity *in vitro* (normal activity) and proof of sulphatide accumulation. Methods of molecular biology are used to confirm the diagnosis [75].

Tandem mass spectrometry

Principles of mass spectrometry

Mass spectrometry is an analytical method by which gaseous ions of sample material are selected by their mass-to-charge ratio (m/z or amu) [77]. Physical basis of mass spectrometry determines its instrumentation and essential analyte properties.

Mass spectrometry has broad general applications based on determination of masses of analysed molecules, fragmentation potential, determination of ionisation energy, ion reactivity, determination of some constants, isotopic measurements, kinetic study of processes, surface analysis etc. One of the most important fields is qualitative and quantitative analysis of species in complex samples [52, 53].

Mass spectrometer

Mass spectrometer consists of three fundamental parts, namely the ionisation device, the analyser and the detector.

1. Sample introduction and methods of ionisation

The sample can be directly injected into the ionisation device, or can undergo some type of chromatography (HPLC, gas chromatography, capillary electrophoresis).

There are examples of several different types of ionisation sources [77]:

- electrospray ionisation (ESI)

- matrix assisted laser desorption ionisation (MALDI) – ionisation and desorption of compounds from solid phase by laser pulses with help of matrix during the process
- fast atom bombardment (FAB) – energy for ionisation and desorption is created by impact of fast atom on solid phase matrix with studied compound
- electron ionisation (EI) – ionisation by electron impact with energy of about 70 eV, positively charged radical ions are produced
- chemical ionisation (CI) – ionisation of sample by reactions with ions of reaction gas, reaction gas is produced by EI in high pressure

With most ionisation methods there is the possibility of creation both positively and negatively charged sample ions depending on the proton affinity of the sample.

2. Analysis and separation of sample ions

Analyte then continues to the analyser, where charged particles are separated or resolved according to their m/z ratios. The separation can be achieved by different methods [78].

a) simple focusation

This method uses different radius of movement of a charged particle in homogenous magnetic field. Ions with the same charge and different mass move in homogenous magnetic field in circles with different radius. Ions fly through focusing device in different curves due to their m/z and drop on detector at different places creating electric signal. Signal is processed and recorded as a spectrum. Better resolution is performed in devices with double focussing.

b) quadrupole

Quadrupole consists of four parallel metal rods of hyperbolic shape in ideal condition. Separation is attained by application of radiofrequence voltage (RF) and direct current (DC). Radiofrequence voltage is responsible for transition of ions through quadrupole. Direct current influences amplitude of spiral movement of ions and is responsible for separation of ions according to their m/z ratio. Only ions that fulfil conditions of RF and DC, pass through quadrupole to the detector. Ratio of U/V determines resolving power and sensitivity of ion separation. The advantages of quadrupole are speed of spectra measurement and the possibility of working at higher pressure in the analyser.

c) ion traps

They consist of monopole (two parallel metal rods in circle). Ions are repulsed to the detector by creating nonstable conditions inside the ion trap.

d) time of flight

Time of flight devices use measurement of flight time of ions to detector to determine ions m/z.

e) Fourier Transform Ion Cyclotron Resonance (FTICR)

Nowadays FTICR is the most powerfull device. Ions are moving in circles with different radius and are non-destructively excited to measure their properties. This device has the highest resolving power among all mass spectrometers.

3) Detection of sample ions

Examples of detectors are following: Faraday cage, electron multiplyer, photographic and electro-optic detector.

Mass spectra

Mass spectra have its specific shape. Spectra consist of series of peaks with two most important: a basic peak and a molecular peak. Basic peak is the peak with 100% intensity in spectrum and intensities of other peaks are compared to this one. Molecular peak corresponds to molecular mass of the compound. Peaks can split into peak series according to the isotopic composition of an analyte. The equation for calculation of the mass for molecular type $C_XH_YN_ZO_W$ is:

$$M = 12x + y + 14z + 16w$$

Intensities of M+1 and M+2 peaks will depend on probabilities of abundance of isotopes in nature.

Tandem mass techniques

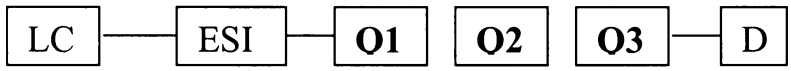


Fig. 6: Scheme of triple quadrupole tandem mass spectrometer
LC – liquid chromatography; ESI – electrospray ionisation;
Q1-3 – quadrupoles; D - detector

Tandem mass (MS) spectrometer is a device that combines two classic mass spectrometers connected in series (Fig. 6). The first mass spectrometer is used to select a single (precursor) mass that is characteristic of a given analyte in a mixture (Q1 quadrupole). The mass-selected ions then pass through a region where they are activated usually by colliding with a neutral gas in a process called collision-induced decomposition (CID) [79]. This collision causes them to fall apart to produce fragment (product) ions (Q2 quadrupole/collision cell working as RF mode only). The second mass spectrometer is used to separate the fragment ions according to mass (Q3 quadrupole).

Tandem mass spectrometry is capable of performing either classic mass or specific mass techniques.

Classic techniques use Q1 quadrupole as a mass separator and Q2 and Q3 work as transit quadrupoles with only application of RF voltage. In general there are two techniques [80]:

- *scanning* – mass spectrometer scans through range of m/z
- *selected ion monitoring* – mass spectrometer collects signal from specific m/z (useful for quantitation tasks)

Specific techniques are [80]:

- *product ion scan* – Q1 is set to specific mass; Q2 works as collision cell (to perform CID); Q3 – scans for product ions of analyte selected on Q1 after CID; this technique is useful in structural studies
- *precursor ion scan* – Q3 is set to specific mass; Q2 works as collision cell; Q1 – scans for ions which produce product ion of set mass
- *neutral loss scan* – this technique allows to scan for non-charged particles after CID; Q1 and Q3 simultaneously scans masses with mass difference corresponding to predicted mass of neutral particle lost during CID; Q2 – works as collision cell; this technique is useful for structural studies

- *multiple reaction monitoring (MRM)* – in this technique known pairs of precursor and product ions are used, those pairs are selected by Q1 and Q3 quadrupole by switching among these m/z pairs; Q2 – works as collision cell; overall signal of ion pairs is gathered by the detector; this technique is used for very specific quantitation of compounds from complex samples

Electrospray ionization

Electrospray ionisation (ES) uses electrohydrodynamic spraying of liquids. All processes occur at atmospheric pressure. In ES liquid containing a sample is passed through a metal capillary at flow rates of 1-20 μl/min. The capillary is raised to potential of 2-5kV (negative or positive) relative to a counter electrode. Under the influence of electric field the liquid emerging from the capillary is dispersed into an aerosol of fine charged droplets. Droplets leaving capillary have size about 1-2μm and charge about 50-70% of Rayleigh limit. Droplets travel through the ambient air for a few milliseconds. During this time their radius decreases due to the solvent evaporation and the droplets thus approach the point where the forces of surface tension and the Coulomb repulsion counterbalance each other (Rayleigh limit). Then it will suffer what is frequently called a Coulomb explosion or Rayleigh fission. For primary droplet this sequence repeats two or three times until its charge state becomes too low to cause another fission. Finally evaporation of the remaining solvent leads to a charge condensation on the analyte molecule and thus to a gaseous ion [77].

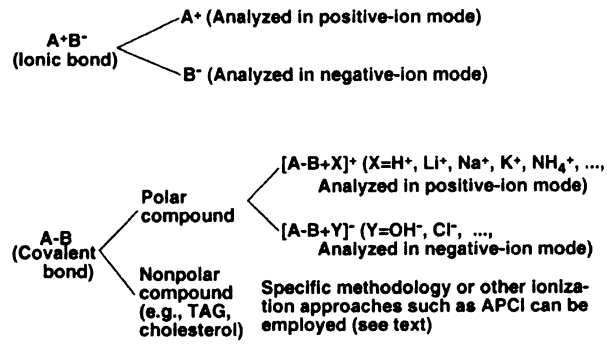


Fig. 7: ES of ionic or covalent compounds [52]

Tandem mass spectrometry of sphingolipids

The utility of selective ES ionization allows creating negatively or positively charged ions for tandem MS analysis (Fig. 7). This allows resolution of sphingolipids directly from

chloroform:methanol extracts. Particular molecular species are then analysed by tandem MS (Fig. 8).

Through appropriately prepared lipid sample ES/MS/MS allows [52]:

1. complete quantitative analysis of lipid classes, subclasses and individual molecular species in minutes without prior chromatographic separation or derivatization

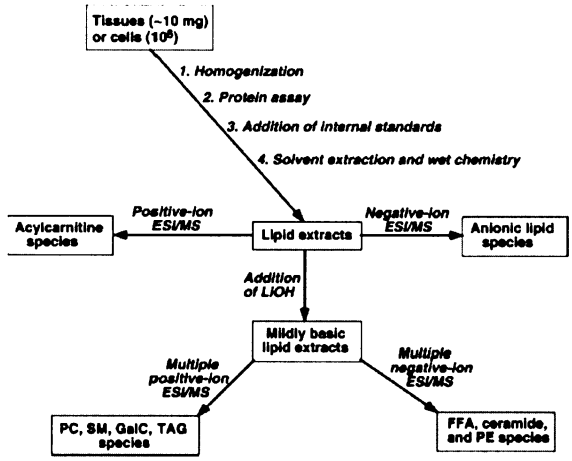


Fig. 8: Experimental strategy in sample preparation from crude cellular extract for lipidomics [52]

2. higher signal-to-noise ratio in comparison to other mass spectrometric approaches
3. nearly linear relationship between the relative intensities of molecular ions and the mass of individual lipids over a 10,000 fold dynamic range
4. independence of ion intensity (within experimental error <5%) on the nature of the polar lipid subclass or the individual molecular species
5. excellent reproducibility of sample measurements <5% of experimental error

Neutral sphingolipids (ceramide, GlcCer, LacCer, Gb_3Cer) produce specific ion after CID, which allows their qualitative analysis by precursor ion scan or quantification by MRM. This ion is formed by cleavage of ceramide molecule to sphingoid base and by additional loss of water. Final product is ion of m/z 264 (Fig. 9) [53, 81-83]. The advantage is in simultaneous analysis of all neutral sphingolipids at once by one product ion. NaCOOH is used as ionization source and mass analyzer works in positive ion mode.

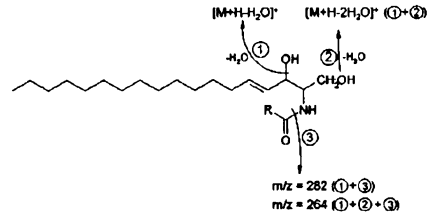


Fig. 9: Fragmentation of ceramide molecule by CID [82]

During tandem MS analysis of **sulphatides** CID produces fragment of m/z 97 [53, 81, 84]. This fragment is a decomposed sulphate group from sulphatide molecule and is used for precursor ion scan or MRM quantification. Sulphatides are negatively charged sphingolipids so they are analysed in negative ion mode without ionization source in sample.

Sphingomyelin produces ion of m/z 184 after CID [53, 81, 83]. This product ion is used for precursor ion scan or MRM quantification. This m/z corresponds also to phospholcholine. Therefore, precursor ion scan of sphingomyelin also contains peaks of phosphatidylcholine. NaCOOH is used as ionization source (see neutral glycosphingolipids above) and mass analyzer works in positive ion mode.

Formation of ion adducts is one of the other approaches in tandem MS analysis of sphingolipids [52, 53, 81]. Na^+ is used for creating positively charged adducts (e.g. in Gb_3Cer tandem MS analysis).

Recently, tandem MS has been designed for determination of catalytic activity of some sphingolipid hydrolases. Special substrates are used for this type of measurements [51].

Immobilisation of enzymes

Principles of enzyme immobilisation

Enzyme immobilisation is a technique, which can provide us with an enzyme system of multiple use. There are many ways of enzyme immobilisation, e.g. adsorption on carrier surface, inclusion into the gel, encapsulation and covalent immobilisation on carrier.

The system with immobilised enzyme is also called "enzyme reactor". This term is derived from terminology in technical chemistry concerning big chemical reactors, but for its conciseness is useful in laboratory conditions, too. There are many kinds of carriers for enzyme immobilisation. The carriers are of different types and forms e.g. tubes, capillary and particles (porous and nonporous). All these carriers are based on different materials like glass, silica, cellulose and many others. Special kind are carriers for covalent immobilisation, which are covered by specific reactive groups. Some carriers have metal core for magnetic separation.

The principles of immobilisation are similar for all carriers. First comes a decision about the kind of carrier and type of immobilisation. The simplest method is an adsorption on surface. This method is based on incubation of a carrier with an enzyme in specific medium with specific ion strength and pH. Protein is held on the surface of the carrier by different interactions like ionic and dipole interactions and also by hydrogen bonds. Another method is an inclusion into the gel when the enzyme is incubated with a mixture of gel polymerisation reagents. However, gels have not good mechanical properties and the enzyme is slowly washed out of the gel matrix. Encapsulation is a method which creates system similar to cell. Microdroplets of the enzyme are covered by thin semipermeable membrane permeable for both substrates and products.

Covalent immobilisation uses a specific chemical reaction and a formation of a covalent bond between an enzyme and a carrier. In this case the type of side groups on a primary polypeptide chain of an enzyme becomes the most important parameter. Function groups of an enzyme should not be used for immobilisation.

Oriented immobilisation is a method of choice where saccharide chains of a glycosylated enzyme protein are covalently bound to the carrier. Side chain groups of a protein molecule are not affected [85-87].

Methods of covalent immobilisation exploit different chemical reactions and reagents. Two of them are good examples of different approaches [88].

First example refers to the reaction between a hydroxyl group and a primary amino group [88]. The initial step is the oxidation of hydroxyl groups of a carrier to aldehyde. Subsequent reactions are based on the formation of Schiff base and its reduction for stabilisation of created bond (Fig. 10).

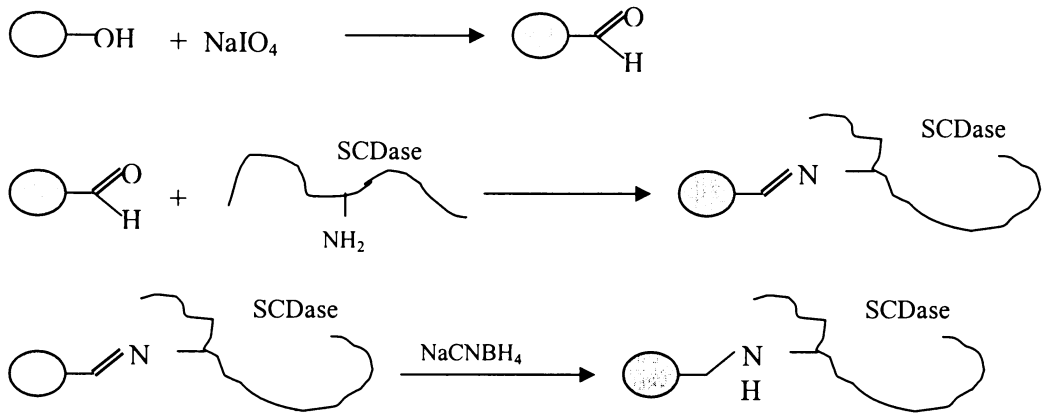


Fig. 10: Immobilisation with oxidation by $NaIO_4$

Second example uses carboxyl groups and primary amino groups to form an amide bond. This reaction is not spontaneous like the first one described above. In this procedure heterobifunctional reagent EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide) is used for amide bond formation. Enhancement of the reaction is made by sulpho-NHS (sulphosuccinimide). In absence of sulpho-NHS the amide bond is formed randomly in one step reaction, whereas its presence allows controlled two step reaction.

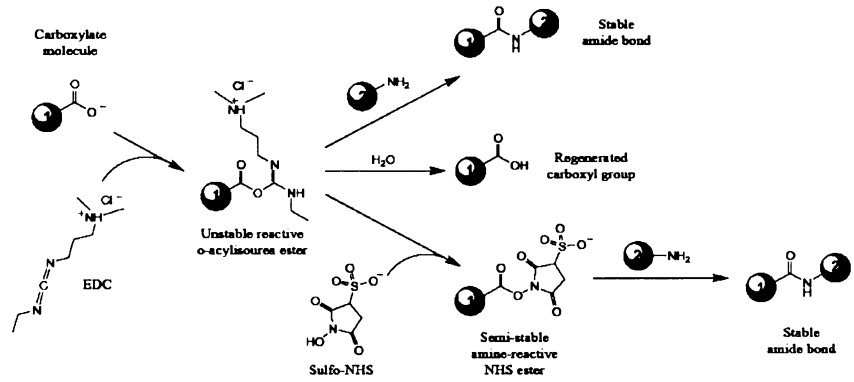


Fig. 11: Immobilisation of proteins with EDC and sulpho-NHS [88]

In two step reaction, first a semi-stable amine-reactive NHS ester is formed by the addition of EDC and sulpho-NHS to the buffer (pH 5, with no carboxyl or amino groups)

containing protein or carrier with carboxyl groups. In the second step the buffer is exchanged for buffer of pH 7 containing protein with primary amino groups. Semi-stable amine-reactive NHS ester is labilized at pH 7 and amide bond is created (see Fig. 11, p. 28) [88-91].

Sphingolipid ceramide N-deacylase

Sphingolipid ceramide N-deacylase (SCDase, EC 3.5.1.69) is a hydrolytic enzyme (acidic hydrolase) isolated by ammonium sulphate precipitation from bacterium Pseudomonas sp. TK4. Its specificity is different from acid ceramidase (EC 3.5.1.23, substrate is ceramide) [92]. SCDase substrates are GSLs and products their lyso-derivatives (N-deacylated GSL). Catalytic activity of SCDase differs using different GSL (Table 3).

Table 3: Substrate specificity of SCDase by TaKaRa BIO, Inc.

Name	Structure	Hydrolysis (%)[1)]	Condensation (%)[2)]
Ceramide	Cer	20	62
Glucosyl ceramide	Glc β1-1'Cer	29	63
Galactosyl ceramide	Gal β1-1'Cer	29	73
Sulphatide	HSO_3-3Gal β1-1'Cer	49	77
Lactosylceramide	Gal β1-4Glc β1-1'Cer	32	-
Asialo GM1	Gal β1-3GalNAc β1-4Gal β1-4Glc β1-1'Cer	63	-
GM1a	Gal β1-3GalNAc β1-4(NeuAc α2-3)Gal β1-4Glc β1-1'Cer	58	75
Sphingomyelin	Choline phosphate- Cer	27	65

1) 2 mU of the enzyme incubated with the 10 nmol substrate at 37°C for 16 hrs in 50 mM acetate buffer pH 6 containing 0.8% Triton X-100.

2) 0.02 mU of the enzyme incubated with the 10 nmol substrate at 37°C for 16 hrs in 50 mM phosphate buffer pH 7 containing 0.1% Triton X-100.

Some properties of SCDase were reported [92]:

- optimal activity at pH 5-6 (hydrolysis)
- stable between pH 4-9
- potently inhibited by: Hg^{2+}, Cu^{2+} and Zn^{2+}
- not inhibited by: Ca^{2+}, Mn^{2+}, Mg^{2+} and EDTA

- retains 80% of its activity after 30 min at 60°C
- can be kept at -85°C for two month without any loss of activity
- addition of Triton X-100 at concentration of 0.4-0.8% increases activity about 10-fold in comparison with reaction in absence of detergent
- molecular mass of an enzyme protein is 52 kDa

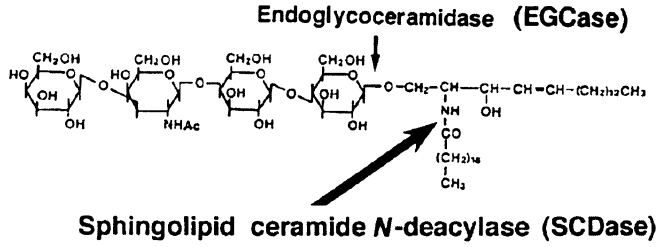


Fig. 12: Function specificity of SCDase (deacylation of AGM1) [92]

The hydrolysis reaction is specific only for amide bond between sphingoid base and fatty acid in ceramide [92] (Fig. 12).

Hydrolysis of GSL has been allosterically inhibited at higher concentrations of released free fatty acids [93]. Therefore biphasic reaction system combining organic and water phases is more advantageous. Organic solvent (heptadecane) contains free fatty acids and thus reaction equilibrium moves to reaction products [93, 94].

SCDase is not only able to hydrolyze amide bond in GSL, but can catalyze condensation reaction at specific conditions, too [93, 95]. The optimal conditions for the condensation are pH 7 and the detergent concentration up to 0.1% [95]. The degree of condensation also depends on types of substrates: lyso-GSL and fatty acid (Table 4).

Table 4: Fatty acids influence on SCDase catalysed condensation reaction [95]

		GM1a		GalCer
Fatty acid	Structure	Hydrolysis	Reverse	Reverse
Acetic acid	C2:0	0	0	0
Caproic acid	C6:0	-	0	8.9
Lauric acid	C12:0	-	50.4	78.3
Myristic acid	C14:0	57.8	62.7	82.9
Palmitic acid	C16:0	100	88.5	78.9
Stearic acid	C18:0	91.2	100	100
Behenic acid	C22:0	47.7	35.4	58.5
Lignoceric acid	C24:0	25.3	28.8	51.6
Cerotic acid	C26:0	-	16.7	26.7
Oleic acid	C18:1 (ω9)	-	29.4	41.4

The quantitative measurement of glycosphingolipids by tandem MS requires appropriate internal standards. Exploitation of the reverse reaction of sphingolipid ceramide N-deacylase to reacylate lyso-GSLs gave very good yields. Furthermore, the hydrolase activity of ceramide N-deacylase can be used to deacylate GSLs to prepare lyso-GSLs, which can then be reacylated enzymically to produce an internal standard [96].

Aims of the diploma thesis:

This work deals with two main tasks: preparation of specific sphingolipid internal standards for tandem MS analysis and establishment of tandem MS procedure for qualitative and quantitative analysis of these lipids. Finally, optimized procedure was developed for studies on sulphatidosis as an example of lysosomal storage disease.

To fulfill these objectives the following steps were planned:

1. **Synthesis of sphingolipid internal standards – enzymic semisynthesis with SCDase**

    - Immobilisation of SCDase - preparation of enzyme reactors

    - Testing and comparison of reactors parameters (catalytic activity and product purity)

    - Enzymic semisynthesis of tandem MS internal standards pointed to sulphatides

2. **Setting up tandem MS method for sphingolipid analysis**

    - Qualitative analysis of isotype composition of sphingolipids

    - Quantitative analysis of sulphatides and its application in diagnosis of sulphatidoses

    - Comparative study of isotype pattern of sulphatides in healthy individuals and patients with sulphatidoses

Materials and methods

Materials

Bovine serum albumin – BSA (Sigma)

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloricum – EDC·HCl or EDAC (Sigma)

Sulphosuccinimide – sulpho-NHS (Pierce)

Millex-LH Filter, 0.45 µm, hydrophilic, PTFE, 4 mm, non-sterile – PTFE syringe pump filters (Millipore)

Sodium periodate (Fluka)

Orcinol (Merck)

HPTLC-Fertigplatten, Kieselgel 60 (Merck)

Triton X-100 (Sigma)

Taurodeoxycholate (Sigma)

Sodium cholate (Sigma)

Sodium cyanoborohydride coupling buffer (Sigma)

GM1 ganglioside (Matreya LLC)

AGM1 ganglioside (Matreya LLC)

lyso-Sulphatide (Calbiochem)

lyso-Gb_3Cer (Matreya, LLC)

C18:0 fatty acid – Stearic acid (Matreya, LLC)

C17:0 fatty acid (Matreya, LLC)

SiMAG-carboxyl (Chemicell GmbH)

Magnetic bead cellulose – op. L 1680; 125-250 μm (Dr M. J. Beneš, Institute of Macromolecular Chemistry, ASCR)

Sphingolipid ceramide N-deacylase – SCDase (TAKARA Bio, Inc)

Sodium chloride (Sigma)

Ceramides (Avanti polar Lipids, Inc)

Glucosylceramides: Gaucher spleen – (Sigma)

Lactosylceramides (Matreya, LLC)

Gb_3Cer (Matreya, LLC)

Sphingomyelin (Avanti polar Lipids, Inc)

Ammonium formiate (Fluka)

All chemicals were of p.a. grade quality.
Organic solvents were redistilled before use.

Biological material for analysis:

Urine samples from sulphatidoses and controls and tissue samples from confirmed case of prosaposin deficiency were from collections of the Institute of Inherited Metabolic Disorders.

Urine samples from SAP-B deficiency and from suspected prosaposin deficiency were from Professor Klaus Harzer, University of Tubingen Germany.

Methods

Immobilisation of sphingolipid ceramide N-deacylase (SCDase)

Basic parameter used in our experiments is defined as mU of immobilised enzyme per amount of carrier. Amount of carrier is expressed in μg for SiMAG-carboxyl (CH) and in μl for magnetic bead cellulose (P).

Magnetic bead cellulose – $NaIO_4$ method

SCDase was immobilised on magnetic bead cellulose using $NaIO_4$ as an oxidizer. 100 μl of particles were washed five times with distilled water, then 100 μl of freshly prepared 0,2 M $NaIO_4$ was added just before starting reaction. This mixture was incubated for 1,5 hrs at laboratory temperature while mixing on rotator. Activated particles were washed ten times with 0,1 M phosphate buffer pH 7, then 250 mU of SCDase (5 mU/μl) and 200 μl of 0,1 M phosphate buffer pH 7 was added. The mixture was incubated for 10 min at laboratory temperature while mixing on rotator. Next, 200 μl of $NaCNBH_3$ was added to stabilize formed Shiff base. Incubation was performed on rotator at 4°C overnight. The particles with immobilised enzyme were washed ten times with 0,1 M phosphate buffer pH 7, followed by ten washings with 1 M NaCl in 0,1 M phosphate buffer pH 7 and then by ten washings with 0,1 M phosphate buffer pH 7. Last step was the activation of an enzyme reactor with 100 nmol of AGM1 ganglioside in 300 μl of 50 mM acetate buffer with 0,8% Triton X-100. Each change of pH was performed by three washings with the corresponding buffer replacing previous one.

SiMAG-carboxyl – EDC method with sulpho-NHS enhancer

SCDase was immobilised on SiMAG-carboxyl particles using EDC·HCl crosslinker to create amide bond among particles and an enzyme. 0,25 mg of particles was washed five times with 0,1 M phosphate buffer pH 7,3. Washed particles were mixed with 125 mU of SCDase in 50 μl of 0,1 M phosphate buffer pH 7,3 and incubated at laboratory temperature for 20 min, then 1,8 mg of EDC·HCl and 0,3 mg sulpho-NHS were added and buffer volume was filled up to 250 μl. This mixture was incubated overnight at 4°C while mixing

on rotator. After overnight incubation the particles with immobilised enzyme were washed ten times with 0,1 M phosphate buffer pH 7,3. Washed particles were incubated with 0,5% BSA in 0,1 M phosphate buffer, while gently mixed on rotator. Immobilisation was finished by ten washings with 0,1 M phosphate buffer pH 7,3. Last step was the activation of an enzyme reactor with 50 nmol of AGM1 ganglioside in 250 µl of 50 mM sodium acetate buffer with 0,8% Triton X-100. Each change of pH was performed by three washings with the corresponding buffer.

Test of hydrolytic activity of soluble SCDase under different conditions

GM1 ganglioside was the only possible substrate for testing of hydrolytic activity of SCDase. It was chosen because it is close to recommended AGM1 ganglioside. AGM1 ganglioside was used in following experiments concerning immobilisation of SCDase.

20 nmol of GM1 ganglioside was incubated with 0,5 mU of SCDase in 20 µl of sodium acetate buffer pH 5 containing 0,8% solution of different detergents (Triton X-100, taurodeoxycholate and cholate) or 1% BSA (fatty acid free). Experiments were carried out in glass vessels. Reaction was performed for 20 hrs at 37°C. Hydrolysis was tested in both monophasis and biphasic systems. Biphasic system consisted of aqueous and organic phase formed by heptane or hexane [93, 94]. Reaction products were evaporated under nitrogen and dissolved in 20 µl of chloroform:methanol (2:1, v/v) mixture. Semiquantitative analysis was performed using HPTLC/densitometry with orcinol detection.

Performing enzymic reaction using immobilised SCDase

Reaction was performed using standard conditions similar to those with soluble enzyme [93]. Conditions are listed in the table below (Table 5).

Table 5: Reaction conditions for enzymic reactor

Type of reaction	Detergents	Buffer	Temperature
Hydrolysis	0,8% Triton X-100	50 mM acetate pH 5	37°C
Synthesis	0,1% Triton X-100	50 mM phosphate pH 7	37°C

Molar ratio of substrates (i.e. lysosphingolipid and fatty acid) before synthesis was 1:1. Difference from the reaction with a soluble enzyme was in the separation of particles with an immobilised enzyme in the magnetic separator. During reaction reactants were mixed using rotator. Synthetic reaction was performed for 20 hrs.

Evaluation of enzymic reaction products by HPTLC

The products of an enzymic reaction were separated by HPTLC. Two types of solvent systems corresponding to the types of products were used (Table 6). Reaction mixture was evaporated under gentle stream of nitrogen and the residue resolved in the chloroform: methanol (2:1, v/v) mixture.

After separation products and substrates were visualized by orcinol and evaluated by CAMAG TLC Scanner II (Cats3, Camag Scientific, Switzerland).

Detection of glycosphingolipids was performed with orcinol reagent (0,5% orcinol (3,5-dihydroxytoluen) in 2 M H_2SO_4 in ethanol). Plates were sprayed and heated in a vent oven at 105°C for about 5 min until the violet color given by hexose was at a maximum.

Table 6: Mobile phase for HPTLC of different shingolipids

Analysed sphingolipid	Mobile phase
AGM1 ganglioside	chloroform:methanol:0,2%$CaCl_2$ – 5:4:1 (v/v/v)
Gb_3Cer	chloroform:methanol:10% acetic acid – 5:4:1(v/v/v)

Effect (influence) of magnetic carriers on product purity

Magnetic carriers with immobilised enzyme were washed with reaction buffer. Each third washing was evaporated under nitrogen. Samples were reconstituted using the mixture chloroform:methanol (2:1, v/v) and analyzed by HPTLC as described above.

SCDase catalyzed hydrolysis: Products formation in relation to reaction time

80 nmol of AGM1 ganglioside was incubated with enzymic reactor (CH-125mU/250μg or P-250 mU/100 μl) or 0,5 mU of soluble SCDase in 800 μl of 50 mM sodium acetate buffer pH 5 with 0,8% Triton X-100. 100 μl of reaction mixture containing 10 nmol of AGM1 ganglioside were collected in appropriate times. Times for collection of samples aliquots were 1, 3, 5, 8 and 24 hrs. Samples were evaporated under a stream of nitrogen. Products of enzymic reaction were reconstituted in the chloroform:methanol (2:1) mixture. Five nmoles of products were applied on HPTLC plate and analyzed as described above.

SCDase catalyzed synthesis: Determination of appropriate molar ratio of substrates

Both CH (125 mU/250 μg) and P (250 mU/100 μl) reactors were partitioned to four aliquots. Each aliquot was incubated with exact molar ratio of substrates (lyso-Gb_3Cer and C18:0 fatty acid) for enzymic synthesis of C18:0 Gb_3Cer. Molar ratios of lyso-Gb_3Cer/C18:0 fatty acid were 1:1, 1:2, 1:3 and 1:4. Reaction mixture contained the aliquot of enzymic reactor, 20 nmol of lyso-Gb_3Cer and appropriate amount of C18:0 fatty acid in 200 μl phosphate buffer pH7 with 0,1% Triton X-100. This mixture was incubated for 20 hrs at 37°C while mixing on rotator. Products of enzymic synthesis were analyzed using HPTLC and orcinol detection as above.

Preparation of internal standard (ISTD) C17:0 sulphatide by enzymic semisynthesis with immobilised SCDase

30 nmol of lyso-sulphatide and 30 nmol of C17:0 fatty acid were incubated with P reactor (50 mU/20 μl) in 300 μl phosphate buffer pH 7 with 0,7% Triton X-100. Reaction mixture was incubated for 20 hrs at 37°C while mixing on rotator. Medium with reaction product was evaporated under a stream of nitrogen and reconstituted in 300 μl of chloroform:methanol (2:1, v/v). 5 μl aliquot was evaluated on tandem mass spectrometer. Evaluation was done by 1 min precursor ion scan of m/z 97 in negative ion mode.

Tandem mass spectrometry

All tandem mass spectrometry techniques were performed on Perkin Elmer SCIEX API 2000 instrument (MDS Sciex, Concord, Canada) with electrospray ionisation device. Mass spectrometer was connected to Perkin Elmer 200 series Autosampler with direct input interface.

Adjustment of ionization device

Adjustment of ionization source was made to maximize detected signal during mass analysis. This was done by using manual tuning feature of mass analyzer control software. For this purpose solutions of standard samples of tested metabolites (ceramide, GlcCer, LacCer, Gb_3Cer, SM, sulphatides) were prepared. Samples contained 10 μg of sphingolipid (SFL) standard in 1 ml of appropriate solvent. MeOH with 10 mM NH_4COOH was used to dissolve neutral GSL and ceramide [83]. For sulphatides MeOH was used [84]. Samples were introduced to mass spectrometer by syringe pump with flow rate set to 10 μl/min. Optimized conditions of ionisaton are listed in Table 7 below.

Table 7: Optimized ionization parameters for tandem MS of sphingolipids

	Sulphatides	Ceramides	GlcCer	LacCer	Gb_3Cer	SM
Curtain gas (psi)	15	20	20	20	20	20
Collision gas (psi)	2	3	3	3	3	3
IonSpray voltage (V)	-4500	5500	5500	5500	5500	5500
Temperature (°C)	100	200	200	200	200	200
Ion source gas 1 (psi)	20	25	25	25	25	25
Ion source gas 2 (psi)	40	25	25	25	25	25
Interface heater	ON	ON	ON	ON	ON	ON
Declustering pot. (V)	-163	20	25	20	20	70
Focusing potential (V)	-400	400	400	400	400	400
Entrance potential (V)	12	-5	-5	-11	-12	-5
Collision energy (V)	-130	36	48	65	70	38
Collision cell exit potential (V)	-3	11	11	11	11	7

Establishment of tandem MS method for SFL analysis

Introduction of a new method started with choosing an appropriate solvent. MeOH with 10 mM NH_4COOH was used for neutral sphingolipids [83] and MeOH was used for sulphatides [84]. Standard samples (see adjustment of ionization source above) were introduced to mass spectrometer by syringe pump with flow rate set to 10 µl/min.

First scanning of standard samples through m/z range with expected abundance of sphingolipid mass was performed. Mass with the highest intensity was selected for the next measurement of fragmentation. Fragmentation was determined by a product ion scan of previously selected mass. The product ion with the highest intensity was used for precursor ion scan or for MRM quantification.

Extraction of SFLs from cells and tissues

Sphingolipids were extracted using a Folch method [6] which were partially prepared in this laboratory before this work started. Appropriate amount of homogenised tissue (250 mg of tissue wet weight) with 1 ml of H_2O was placed into the 15 ml extraction tube. The sample was extracted by addition of 4 ml of chloroform:methanol mixture (2:1, v/v). Water phase was removed and lower organic phase was collected. Water phase was washed with a mixture of chloroform:methanol:water (86:14:1,v/v/v, theoretical lower layer phase). Lower phases were combined and evaporated under a gentle stream of nitrogen. Upper water phase was discarded. Extracted SFLs were resuspended in 500 µl of chlorofom:methanol (2:1, v/v).

Extraction of SFLs from urine

Sample preparation started with an extraction of SFLs including sulphatides by a method described above. 1 ml of urine (with known creatinine concentration) and 7,5 ml of a chloroform:methanol (2:1, v/v) mixture were placed in 15 ml extraction tube. Sulphatides were extracted by three 1 min vortex mixing in 10 min periods. Tube was left standing at room temperature for 30 min. The mixture was then centrifuged for 5 min at 1500 rpm to separate chloroform and water phase. Water phase was washed by the solvent mixture chloroform:methanol:water (86:14:1, v/v/v). Lower phases were evaporated under gentle stream of nitrogen and reconstituted in 500 µl of chloroform:methanol (2:1, v/v).

1-2,5 mmol/l creatinine aliquots were prepared from the extracts. 200 ng of C17:0 sulphatide ISTD was added to standard urine aliquot (creatinine 1-2,5 mmol/l). This mixture was filtered using a syringe pump with PTFE syringe filters to remove proteins and microelements from the extract. The extract filtrate was evaporated under a stream of nitrogen and reconstituted in 200 µl of MeOH to create final sample. Samples were then vortex mixed and 1 min sonicated in sonic bath.

Qualitative analysis of isotype composition in different tissue samples

Evaporated extracts of 25 mg wet tissue or 100 µg of fibroblast protein aliquots dissolved in MeOH with 10 mM NH_4COOH were used for analysis of neutral sphingolipids. The product ion with the highest mass for neutral GSL was m/z 264,4 (for ceramide, GlcCer, LacCer and Gb_3Cer) and m/z 184,1 for SM. These ions were selected and used for precursor ion scan performed in positive ion mode for 1 min.

26 nmol of brain sulphatides or 50 mg of sulphatides wet tissue extracts from kidney in 500 µl of MeOH were analyzed.

Samples were introduced to mass spectrometer by a syringe pump with a flow rate set to 10 µl/min. Precursor ion scan for m/z 97 was performed in negative ion mode for 1 min.Measured spectra revealed the composition of measured sphingolipid in specific tissue. Sphingolipid composition also showed which isotypes prevail. Prevailing isotypes were used for MRM quantification.

Linearity measurement during MRM quantification of sulphatides

Samples used for linearity measurement contained appropriate amount of sulphatides and 200 ng of C17:0 sulphatide ISTD. Concentrations of sulphatides selected for linearity measurements were 100, 200, 500, 1000, 2000, 5000 and 10000 ng/ml. Measurement was carried out on tandem mass spectrometer in MRM mode. Scanned isotypes were m/z 778, 806, 822, 834, 850, 862, 876, 878, 888, 890, 892, 904, 906 and 792 for ISTD. Injected sample volume was 10 µl and a flow rate set to 10 µl/min. The ratio of sulphatides (except ISTD) cps to ISTD cps (counts per second) was compared to sulphatides concentration to obtain linearity curve.

MRM quantification of sulphatides

Quantification of sulphatides was done by MRM spectra measurement in negative ion mode. 10 µl of standard urine sample and external calibration point (600 ng/ml of sulphatides) were introduced to mass spectrometer with flow rate set to 10 µl/min. Sulphatides species monitored by MRM quantification were of the following m/z: 778, 806, 822, 834, 850, 862, 876, 878, 888, 890, 892, 904, 906 and 792 for ISTD. Data obtained from MRM spectra and used for quantification were the total ion signal of sulphatides (except ISTD) and ISTD signal. Signals were measured in cps.

Quantification was achieved by relating the peak height of urinary sulphatides to the peak height of ISTD and calculation from external calibration point ratio by equation:

$$\frac{\frac{S_{sulphatides}}{S_{ISTD}}}{\frac{S_{EXTR\,CAL}}{S_{ISTD}}} = \frac{c_{sulphatides}}{c_{EXTR\,CAL}}$$

$\frac{S_{sulphatides}}{S_{ISTD}}$ ..... *ratio of signal intensity of measured sulphatides to ISTD signal intensity*

$\frac{S_{EXTR\,CAL}}{S_{ISTD}}$ ..... *ratio of signal intesity of sulphatides in external calibration point to ISTD signal intensity*

$c_{EXTR\,CAL}$ ..... *concentration of sulphatides in external calibration point*

$c_{sulphatides}$ ..... *unknown concentration of sulphatides in measured sample*

Isotype composition of sulphatides in analyzed samples was calculated dividing total sulphatides concentration by the isotype signal ratio from measured MRM spectra.

Results

Immobilisation of SCDase for preparation of ISTD for tandem MS

Test of hydrolytic activity of soluble SCDase in the mono- or biphasic systems

To evaluate the reaction conditions the hydrolytic activity of the enzyme was tested in presence of different detergents (Triton X-100, taurodeoxycholate and cholate) and BSA. BSA stabilizes the enzyme and thus may improve its catalytic effect. BSA can also bind fatty acids from a solution.

The reaction was carried out either in monophasic (aqueous) or in two different biphasic systems using heptane or heptadecane as organic phase.

Reaction products were separated by HPTLC, detected by orcinol-H_2SO_4 reagent (Fig. 13, Fig. 14) and evaluated on CAMAG II densitometer at 545nm (Table 8, Table 9).

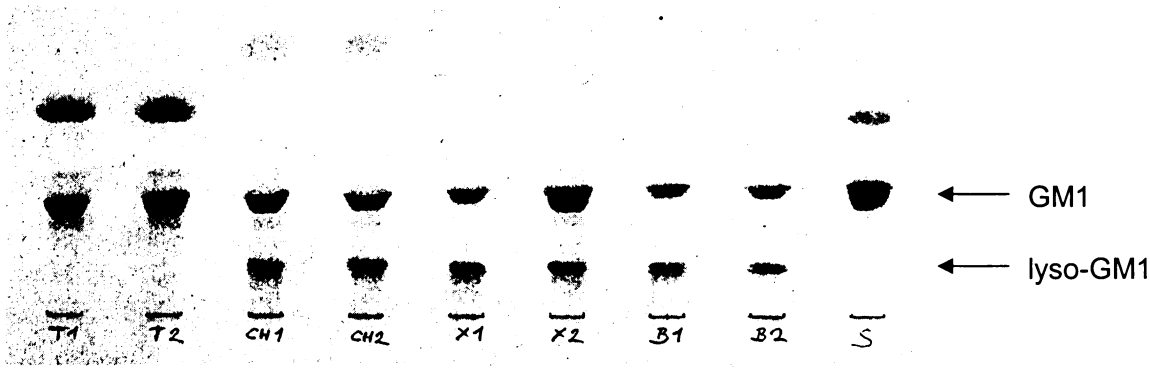


Fig.13: HPTLC of products of soluble enzyme reaction in presence of different detergents either in monophasic (MPS) or biphasic system (BPS).

Reaction conditions: 4 mU of SCDase + 10 nmol GM1 ganglioside in 20 µl of 50 mM sodium acetate buffer (pH 5) with different detergents (0,8% in final sample volume) or 1% BSA in final sample volume, carried out in monophasic or biphasic (aqueous/heptane) systems; 20 hrs incubation at 37°C

HPTLC: mobile phase - chloroform:methanol:0,02%$CaCl_2$ (5:4:1); detection - orcinol

Substrate: GM1 ganglioside; Product: lyso-GM1 ganglioside

Samples: T1-Taurodeoxycholate + MPS; T2-Taurodeoxycholate + BPS; CH1-Cholate + MPS; CH2-Cholate + BPS; X1-Triton X-100 + MPS; X2-Triton X-100 + BPS; B1-BSA + MPS; B2-BSA + BPS; S - GM1 standard

Table 8: Hydrolytic activity of soluble SCDase in presence of different detergents in mono- or biphasic systems – HPTLC/Densitometry evaluation of Fig. 13

	CH1	CH2	X1	X2	B1	B2
GM1 (1)	2757	1978,8	1617,6	2851,7	1790,3	1979
lyso-GM1 (1)	1897,7	1850,7	1741,9	1096,6	1828,1	1488,9
hydrolysis (%)	**40,77**	**48,33**	**51,85**	**27,77**	**50,52**	**42,93**

(1) in arbitrary units

Abbreviations (also see Fig. 13, p. 42): CH1 - Cholate + MPS; CH2 - Cholate + BPS; X1 - Triton X-100 + MPS; X2 - Triton X-100 + BPS; B1 - BSA + MPS; B2 - BSA + BPS

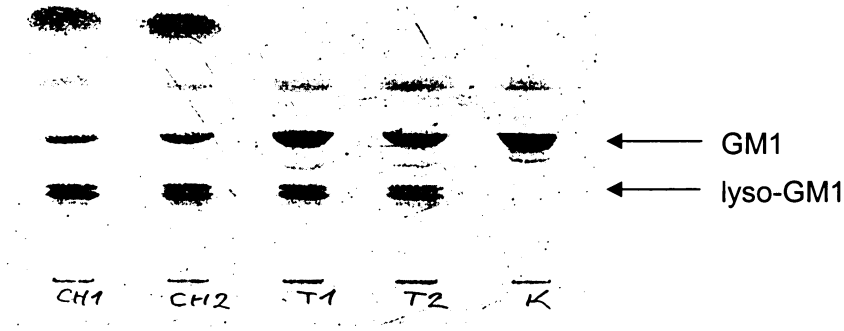


Fig. 14: Effect of detergents on SCDase catalysed hydrolysis of GM1 substrate in the heptadecane/aqueous biphasic system.

Reaction conditions: 4 mU of SCDase + 10 nmol GM1 ganglioside in 20 µl of 50 mM sodium acetate buffer (pH 5) with 0,8% Triton X-100/Cholate and biphasic heptadecane set; 20 hrs incubation at 37°C

HPTLC: mobile phase - chloroform:methanol:0,02%$CaCl_2$ (5:4:1); detection - orcinol;

Substrate: GM1 ganglioside; Product: lyso-GM1 ganglioside

Samples: CH1, CH2 – Cholate; T1, T2- Triton X-100; K - GM1 standard

Table 9: Effect of detergents on SCDase hydrolytic activity in the heptadecane/ aqueous biphasic system – HPTLC/Densitometry evaluation of Fig. 14

	CH1	CH2	T1	T2
GM1 (1)	1453,3	1752,9	2300,6	1923,7
lyso-GM1 (1)	2676,1	2574,8	1061,1	971,2
hydrolysis (%)	**64,81**	**59,50**	**31,56**	**33,55**

(1) in arbitrary units

Abbreviations (also see Fig. 14): CH1, CH2 – Cholate; T1, T2 - Triton X-100; K - GM1 standard

Hydrolytic activity of enzyme reactor (immobilised SCDase)

Time course of hydrolytic activity of the enzyme reactors (P reactor and CH reactor) were tested. Analysis of HPTLC plates (Fig. 15) was carried out on CAMAG II denzitometer. Results in graphic form are shown in Fig. 16.

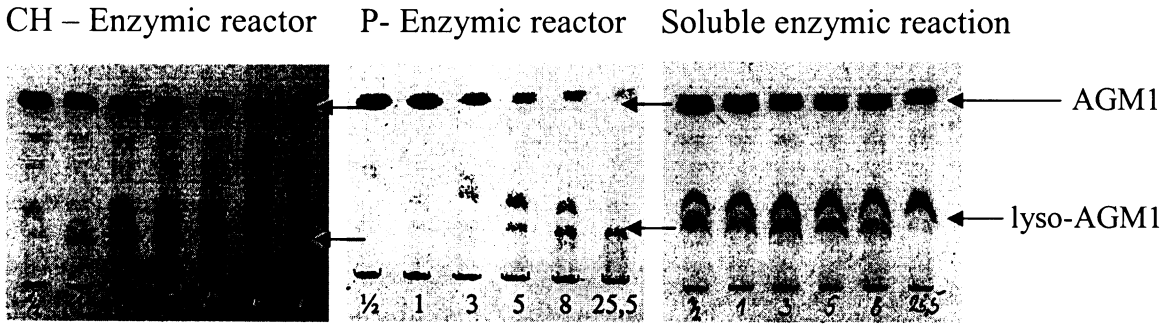


Fig. 15: HPTLC of products of reaction catalysed by immobilised SCDase (CH reactor, P reactor) in comparison with soluble enzyme.

Reaction conditions: 80 nmol AGM1 ganglioside + 0,5 mU SCDase/CH (125 mU/250 μg) or P (250 mU/100 μl) reactor; buffer: 800μl of 50 mM sodium acetate (pH 5) with 0,8% Triton X-100; incubation at 37°C at different times

HPTLC: mobile phase - chloroform:methanol:0,02%$CaCl_2$ (5:4:1); detection - orcinol

Substrate: AGM1 ganglioside; Product: lyso-AGM1 ganglioside

Samples: ½, 1, 3, 5, 8, 25½ - different incubation times in hours; KR – control soluble reaction product and substrate

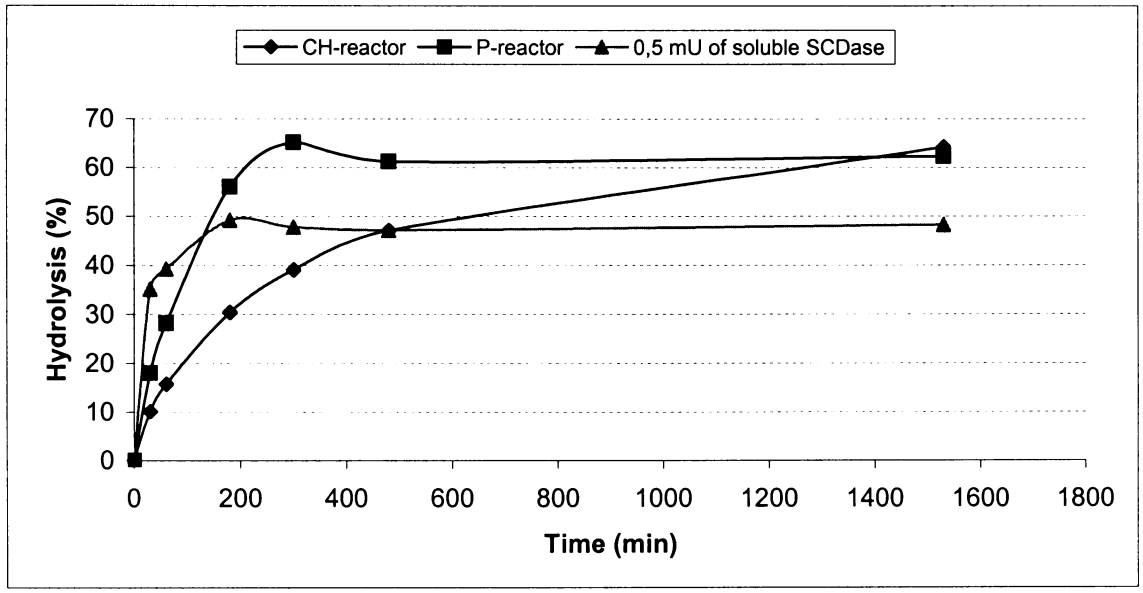


Fig.16: Time course of AGM1 hydrolysis by SCDase in soluble and immobilised form (CH and P reactor)

Comparison of the quality of the products from CH and P reactors

Products releasing into medium after washing steps of CH and P reactors were tested (Fig. 17). In the next experiment particles were mixed during long time reaction and their mechanical stability (fragmentation of bead cellulose), was tested. Polysaccharide fragments of bead cellulose were detected on the chromatogram by orcinol (Fig. 18).

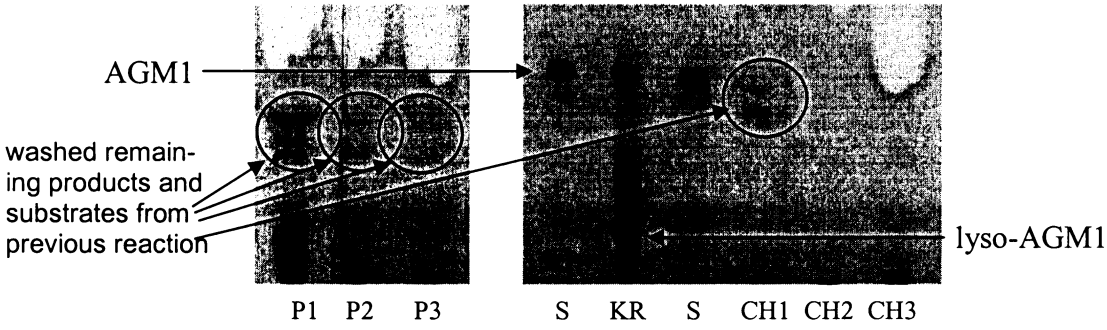


Fig. 17: HPTLC control of purity of washing fractions from CH and P reactors after elution of reaction products

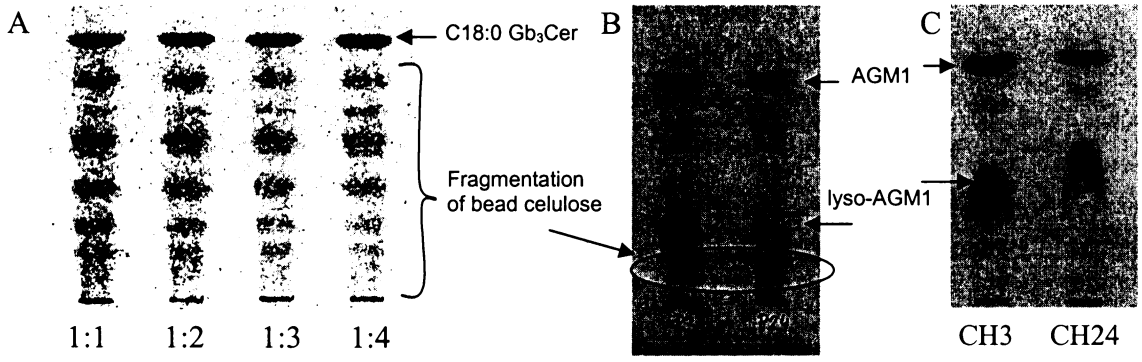
Samples:

P reactor: P1-first wash fraction; P2-fourth wash fraction; P3-seventh wash fraction

CH reactor: CH1-first wash fraction; CH2-fourth wash fraction; CH3-seventh wash fraction

S-AGM1 standard substrate; KR-control reaction product (lyso-AGM1) and AMG1

Mobile phase: chloroform:methanol:0,02%CaCl2 (5:4:1); Detection: orcinol



Synthetic reaction – P reactor P reactor – hydrolysis CH reactor – hydrolysis

Fig. 18: Fragmentation of bead cellulose from P reactor and contamination of samples during long incubation time in comparison with CH reactor

A, Synthesis of C18:0Gb_3Cer with different substrate ratio - incubation for 20 hrs at 37°C

B, P3 - incubation 3 h; P24 - incubation 24 hrs; 37°C

C, CH3 - incubation 3 h; CH 24 – incubation 24 hrs; 37°C

Mobile phase: chloroform:methanol:0,02%$CaCl_2$ (5:4:1) – AGM1; chloroform:methanol:10% acetic acid (5:4:1) – C18:0 Gb_3Cer; Detection: orcinol

SCDase synthetic reaction: optimisation of stearic acid to lyso-Gb_3Cer ratio

Series of reactions were carried out to find the best ratio of substrates for enzymic semisynthetic preparation of GSL. Different substrate ratios of lyso-Gb_3Cer and stearic acid were tested to produce C18:0 Gb_3Cer. The products of the reaction were analysed on HPTLC (Fig. 19) and scanned by densitometer.

Results are shown in Table 10.

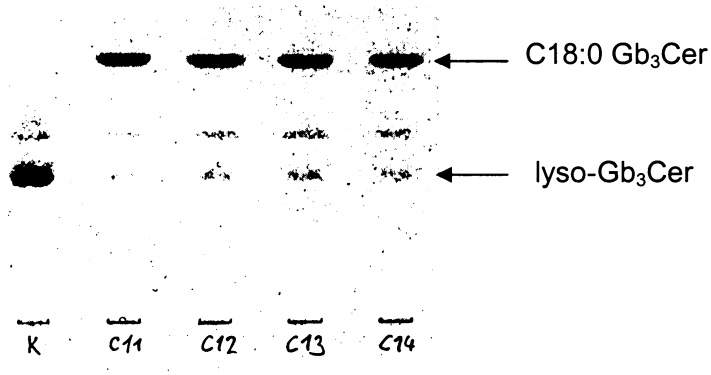


Fig. 19: HPTLC of products of reactions performed with different ratios of substrates

Reaction conditions: 30 nmol lyso-Gb_3Cer + different amount of stearic acid + CH reactor (125 mU/250 μg) in 300 μl of 50 mM phosphate buffer (pH 7) with 0,1% Triton X-100; incubation 20 hrs at 37°C

Mobile phase: chloroform:methanol:10% acetic acid (5:4:1); Detection: orcinol

Substrates: lyso-Gb_3Cer and stearic acid; Product: C18:0 Gb_3Cer

Samples: C11 - lyso-Gb_3Cer:stearic acid=1:1; C12 - lyso-Gb_3Cer:stearic acid=1:2; C13 - lyso-Gb_3Cer:stearic acid=1:3; C14 - lyso-Gb_3Cer:stearic acid=1:4; K - control lyso-Gb_3Cer

Table 10: Formation of acylated products of reverse SCDase reaction with different ratios of substrates: HPTLC/Densitometry evaluation of Fig. 19.

Ratio lyso-Gb_3Cer/stearic acid	**1/1**	**1/2**	**1/3**	**1/4**
lyso-Gb_3Cer (1)	142,6	215,8	218,9	310
C18:0 Gb_3Cer (1)	1401,1	1477,7	1402,5	1307,3
synthesis (%)	**90,76**	**87,26**	**86,50**	**80,83**

Evaluated by densitometry in arbitrary units (1)

Preparation of C17:0 sulphatide internal standard for tandem MS analysis

C17:0 sulphatide internal standard was prepared using both CH and P reactors. Reaction was performed three times with each reactor. Products were analysed by tandem MS on Perkin Elmer SCIEX API 2000 tripple quadrupole tandem mass spectrometer in precursor ion scan mode. Mass spectra (Fig. 20, Fig. 21) clearly demonstrate effectivity of semisynthetic reaction for preparation of internal standards for mass spectrometry.

Multiple uses of reactors also show their effectivity and reliability in comparison to soluble enzyme, especially in case of P reactor with no lose of catalytic activity (Table 11).

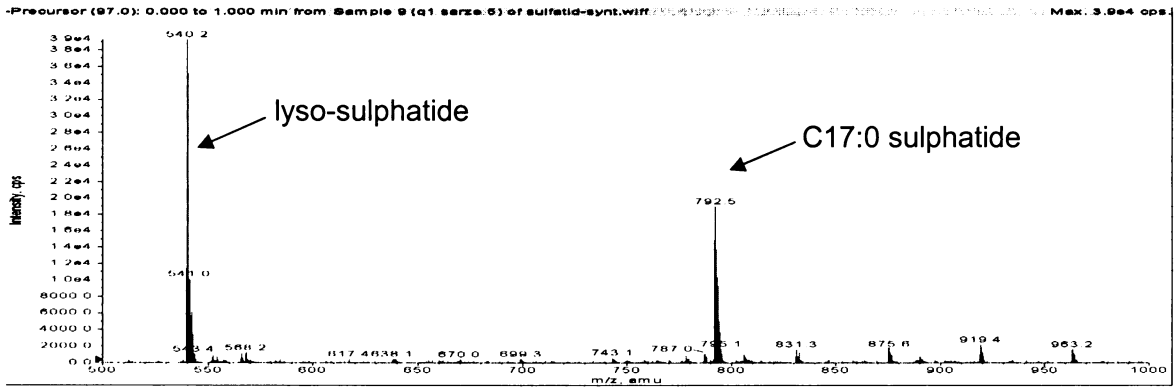


Fig. 20: C17:0 sulphatide ISTD precursor ion scan of m/z 97 (1 min negative ion mode scan); ISTD prepared using CH reactor

Reaction conditions: 30 nmol lyso-sulphatide + 30 nmol C17:0 fatty acid + CH reactor (62mU/125μg) in 300 μl of phosphate buffer pH 7 with 0,1% Triton X-100; 20 hrs incubation at 37°C

Reactor was washed in phosphate buffer (pH 7) with 0,1% Triton X-100 and 1M NaCl before first use

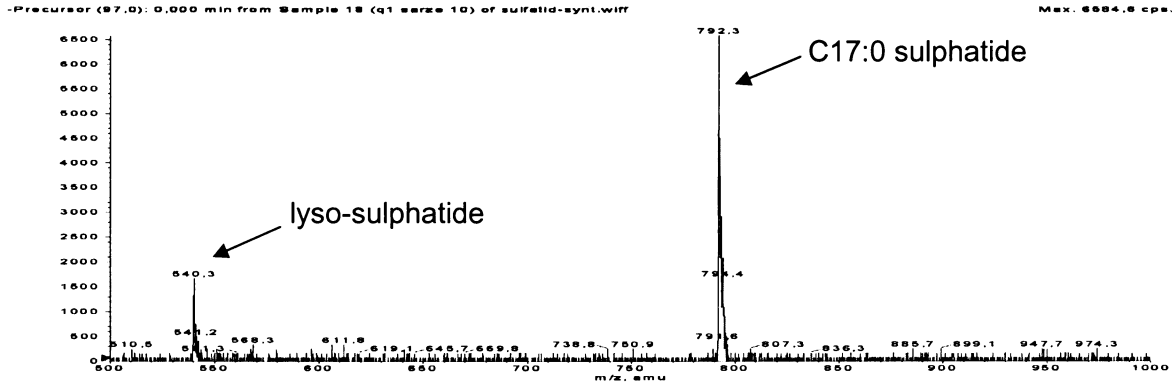


Fig. 21: C17:0 sulphatide ISTD precursor ion scan of m/z 97 (1 min negative ion mode scan); ISTD prepared using P reactor

Reaction conditions: 30 nmol lyso-sulphatide + 30 nmol C17:0 fatty acid + P reactor (50mU/20μl) in 300 μl of phosphate buffer pH 7 with 0,1% Triton X-100; 20 hrs incubation at 37°C

Table 11: Results of synthesis of C17:0 sulphatide ISTD catalysed by P and CH reactors

10th use of CH reactor			
lyso-sulphatide %	**67,42**	*C17:0 sulphatide %*	**32,58**

12th use of P reactor			
lyso-sulphatide %	**20,20**	*C17:0 sulphatide %*	**79,80**

Tandem MS of sphingolipids

All tandem mass spectrometry analyses were performed on Perkin Elmer SCIEX API 2000 instrument equipped with electrospray ionisation device. Mass spectrometer was connected to Perkin Elmer 200 series Autosampler by direct input interface.

Spectra of neutral sphingolipids and sulphatides in control tissue samples

First step in our tandem MS study was to identify composition of isotypes of individual sphingolipids in various normal tissue samples and in fibroblasts. The measurement was carried out in precursor ion scan mode. The spectra (Fig. 22, Fig. 23) showed different isotype composition of sphingolipids in different biological sources. Isotype compositions changed in relation to the type of tissue and analysed sphingolipid. Also, different metabolites revealed differences in signal intensities (cps – counts per second).

Neutral GSLs were analysed in following control cells and tissues: cultured skin fibroblasts, grey and white brain matter, myocardium, liver, spleen and kidney. Sulphatides were measured in the brain and kidney.

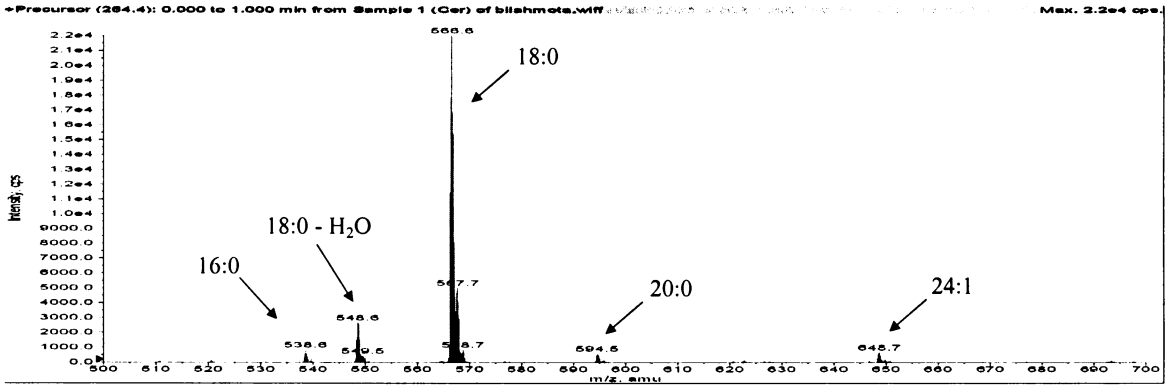


Fig. 22/A: Ceramide composition in the white brain matter - precursor ion scan of m/z 264,4

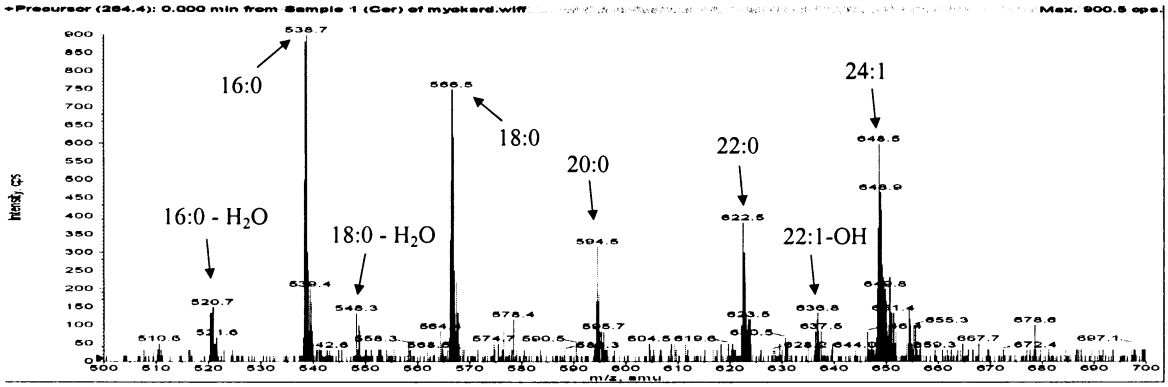


Fig. 22/B: Ceramide composition in the myocardium - precursor ion scan of m/z 264,4

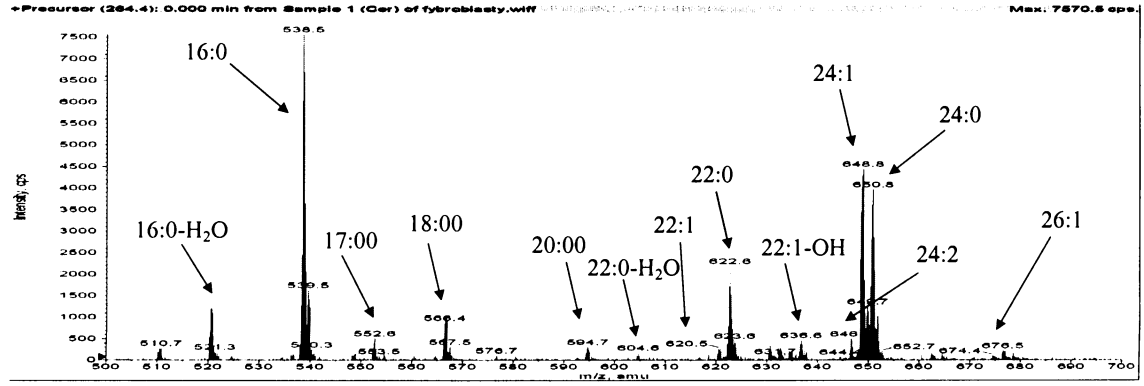


Fig. 22/C: Ceramide composition in cultured skin fibroblasts - precursor ion scan of m/z 264,4

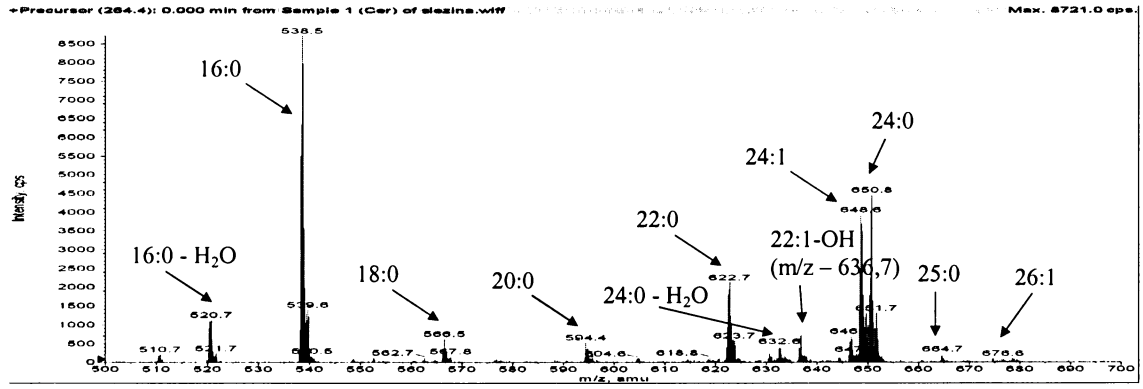


Fig. 22/D: Ceramide composition in the spleen - precursor ion scan of m/z 264,4

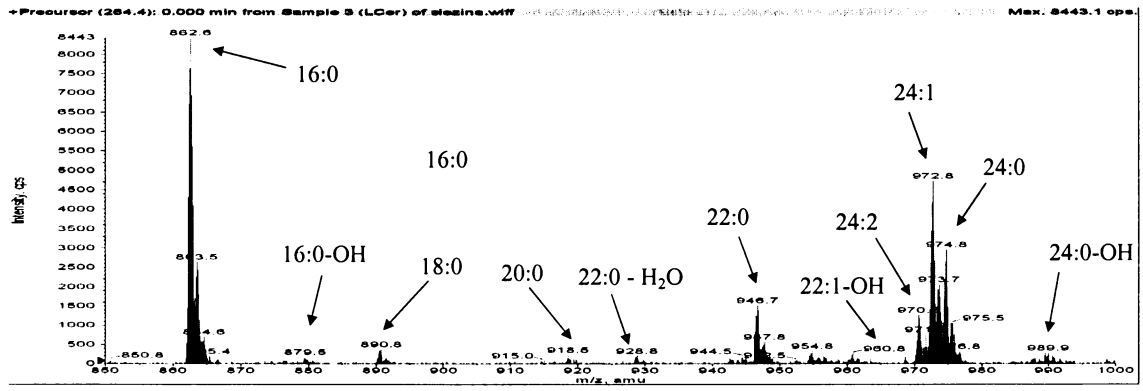


Fig. 22/E: Lactosylceramide composition in the spleen - precursor ion scan of m/z 264,4

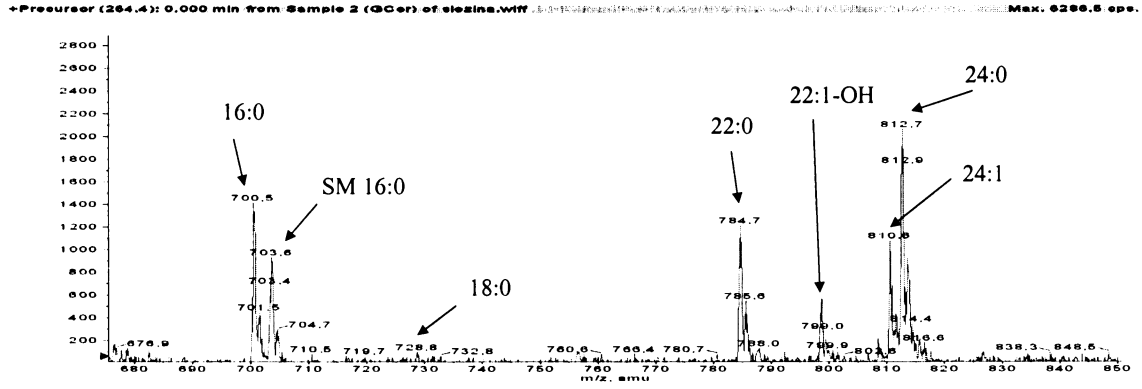


Fig. 22/F: Glucosylceramide composition in the spleen - precursor ion scan of m/z 264,4

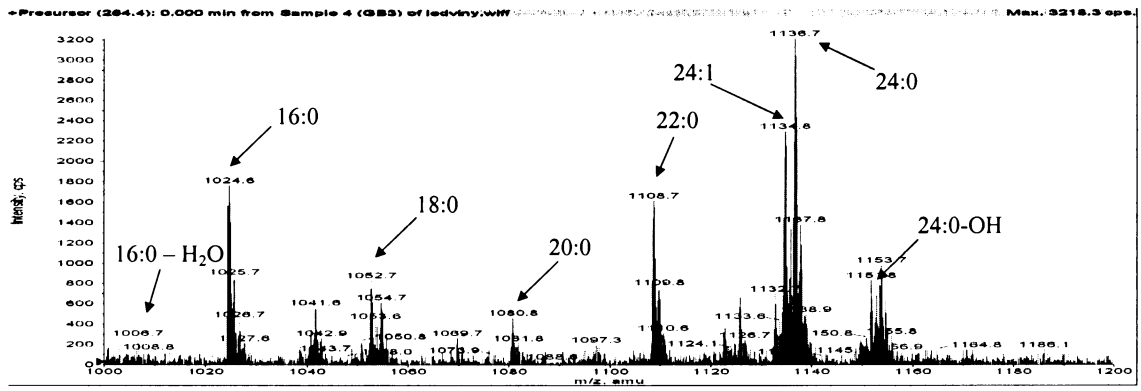


Fig. 22/G: Gb_3Cer composition in the kidney - precursor ion scan of m/z 264,4

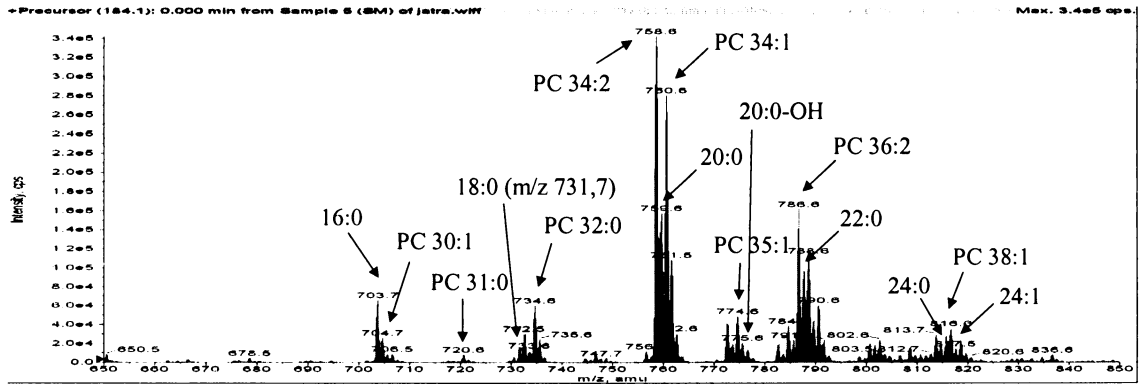


Fig. 22/H: Sphingomyelin composition in the liver - precursor ion scan of m/z 184,1

Comments to Fig. 22/A-H: Precursor ion scan of neutral GSL control samples

Samples: 25 mg wet tissue extract or 100 μg protein aliquot of fibroblasts extract was dissolved in 500 μl MeOH with 10 mM NH_4COOH

Measurement: 1 min scan in positive ion mode

Spectra show different isotype composition and specific loss of H_2O molecule.

Comment to Fig.22/H: Spectra contain also masses of phosphatidylcholine, because it fragmentates to product ion of m/z 184,1 (phosphocholine moiety) by the same way as sphingomyelin

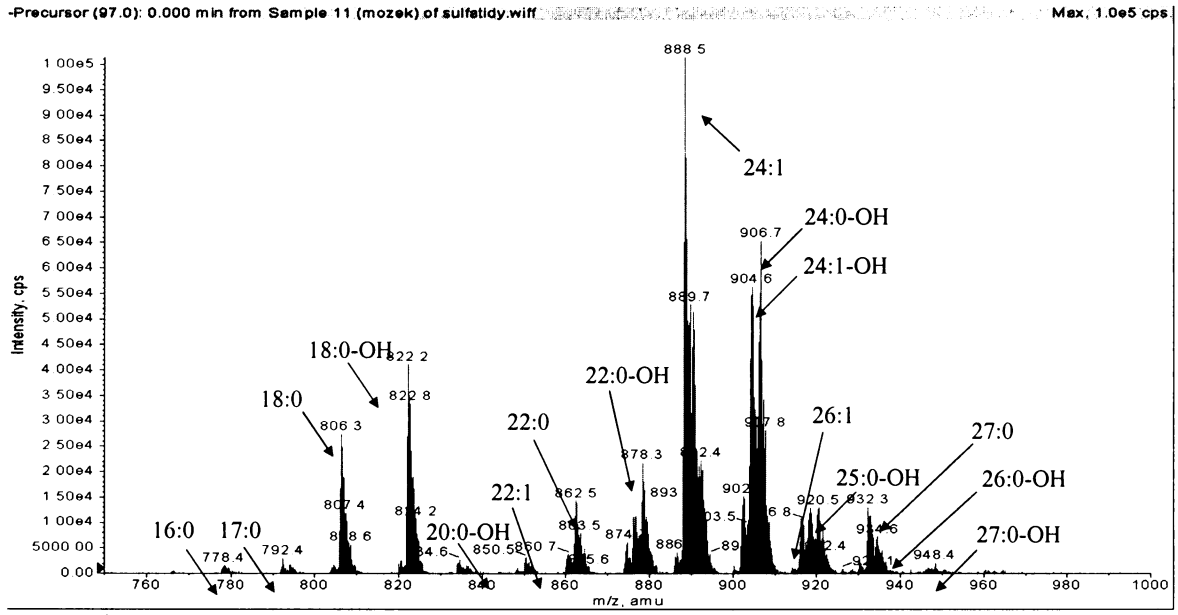


Fig. 23/A: Sulphatides composition in the brain - precursor ion scan of m/z 97

Comment: Precursor ion scan of sulphatides in control sample – 26 nmol of brain sulphatides dissolved in 500 μl MeOH

Measurement: 1min scan in negative ion mode

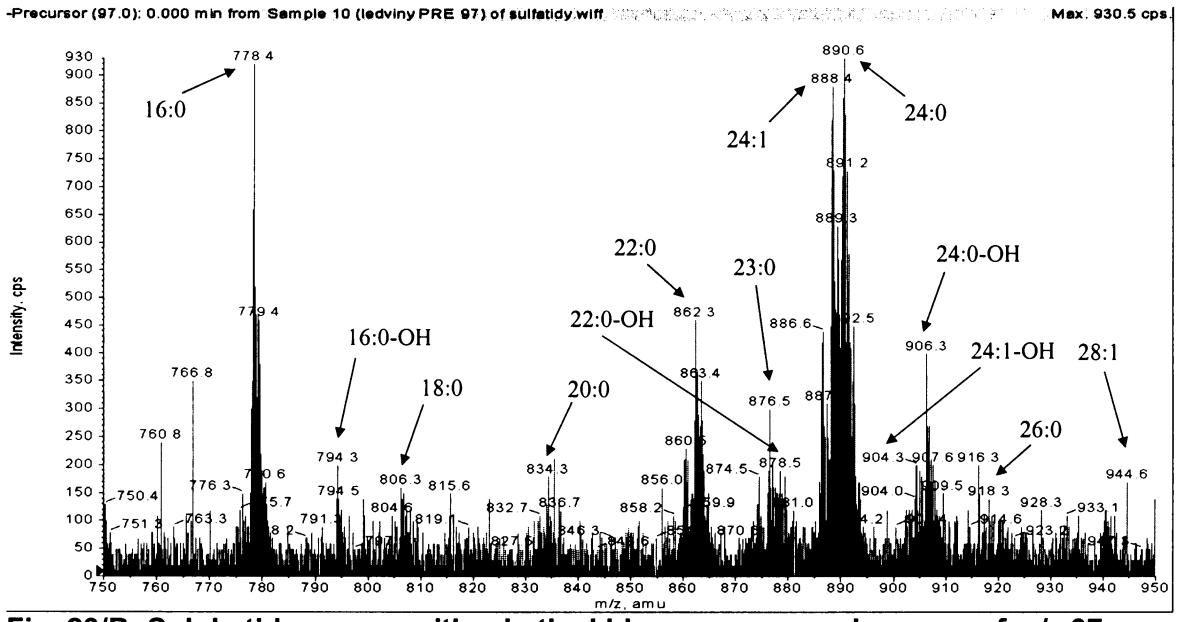


Fig. 23/B: Sulphatides composition in the kidney - precursor ion scan of m/z 97

Comment: Precursor ion scan of sulphatides in control sample – 50 mg of wet tissue extracts dissolved in 500 μl MeOH

Measurement: 1 min scan in positive ion mode

Test MRM spectra of neutral sphingolipids and sulphatides

Tandem mass spectrometer worked in MRM mode. This measurement of MRM spectra was carried out to show reliability of prepared analytical methods. Neutral sphingolipids were evaluated in control and prosaposin deficiency spleens. Sulphatides were analysed in control and MLD urine samples to obtain MRM spectra. Sulphatide urine samples were analysed to obtain control and MLD MRM spectra (Fig. 25).

MRM spectra included m/z of internal standard to find possible interferences with other isotype masses, but internal standards were not included in the samples. The spectra revealed interference of SM and GlcCer (Fig. 24), since C16:0 SM had the same fragmentation patern as C16:0-^{3}D GlcCer (ISTD). Another interference was observed in MRM spectra of sulphatide internal standard, because C17:0 sulphatide (ISTD) showed isotopic splitting and interfered with C16:0-OH sulphatide (Fig. 26).

Measured isotypes proposed for quantification of neutral sphingolipids and those used for quantification of urinary sulphatides are listed in Table 12 and Table 13.

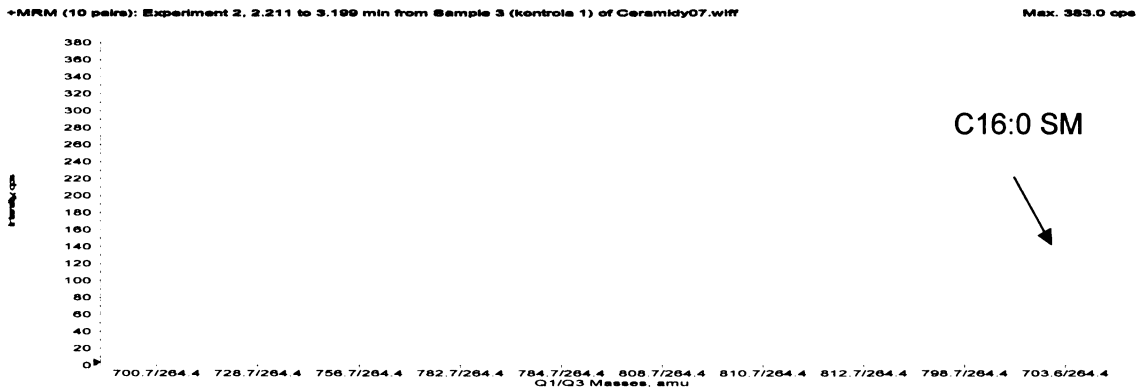


Fig. 24: GlcCer in the control spleen – MRM spectra

Sample: 250 μg of wet tissue extract dissolved in 500 μl of MeOH with 10 mM NH_4COOH

Measurement: MRM spectra in positive ion mode, 10 μl of sample for 10 min scan

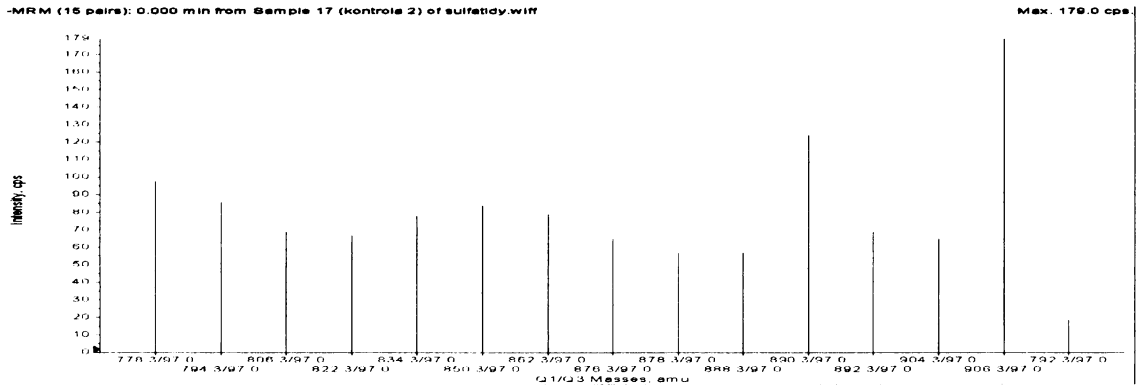


Fig. 25/A: MRM spectra of sulphatides in control urine sample

Sample: 2ml of urine extracted by Folch method and dissolved in 200 μl of MeOH

Measurement: negative ion mode; 10 μl of sample for 10 min

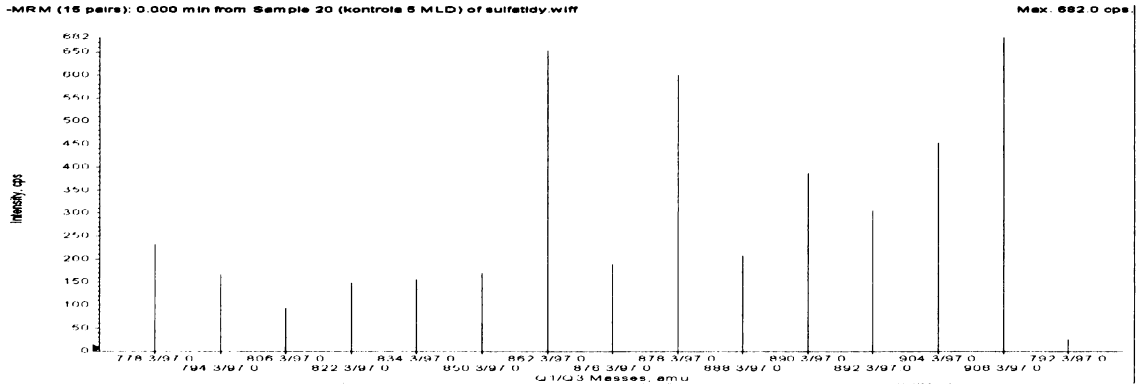


Fig. 25/B – MRM spectra of sulphatides in MLD urine sample

Sample: 1ml of urine extracted by Folch method and dissolved in 200 μl of MeOH

Measurement: negative ion mode; 10 μl of sample for 10 min

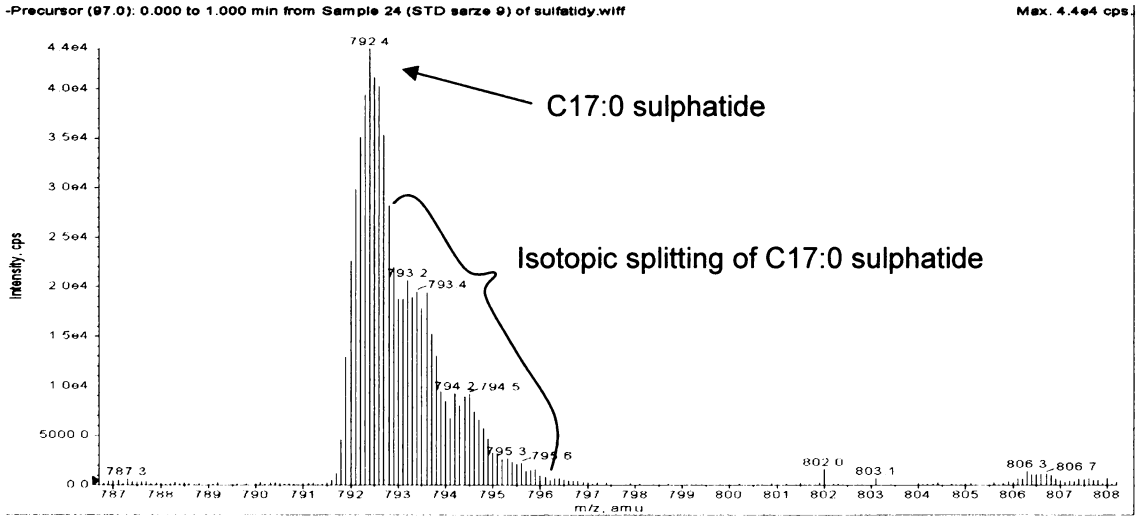


Fig. 26: Isotopic splitting of C17:0 sulphatide - negative ion mode scan

Comment: Sample of C17:0 sulphatide ISTD - 500 μg dissolved in 500 μl of MeOH

Measurement: negative ion mode; 1 min precursor ion scan of m/z 97

Table 12: MRM spectra showing major isotypes of neutral sphingolipids in the spleen used for further quantification

Isotype	Cer (m/z)	GlcCer (m/z)	LacCer (m/z)	Gb_3Cer (m/z)	SM (m/z)
14:0	510,6				
16:0	538,7	700,7	862,4	1024,7	703,6
16:1					701,6
18:0	566,7	728,7	890,7	1052,7	731,6
18:1					729,6
18:0-OH	581,4				
20:0	594,7	756,7	918,7	1080,7	759,6
20:1-OH					773,6
22:0	622,7	784,7	946,7	1108,7	787,6
22:1		782,7	944,7	1106,7	785,6
22:1-OH	636,8	798,7	960,7		
24:0	650,7	812,7	974,7	1136,7	815,6
24:1	648,7	810,7	972,7	1134,7	813,6
24:2	646,7	808,7	970,7	1132,7	811,6
24:0-OH			990,7	1152,7	
24:1-OH				1150,7	
24:2-OH				1148,7	
26:0	678,7				
26:1	676,7				
26:2	674,7				
ISTD	552,5	*672,7 (C12:0)*	865,6	1038,8	717,6

Comment: m/z 703,6 was former C16:0-^{3}D GlcCer ISTD for GlcCer, but it interfered with C16:0 SM (Fig. 24, p. 53); new ISTD – C12:0 GlcCer was proposed (shown in italics)

Table 13: MRM spectra representing major sulphatide isotypes in urine used for quantification

Isotype	Sulphatide (m/z)
16:0	778
18:0	806
18:0-OH	822
20:0	834
20:0-OH	850
22:0	862
23:0	876
22:0-OH	878
24:1	888
24:0	890
23:0-OH	892
24:1-OH	904
24:0-OH	906
ISTD	792

Determination of concentration linearity of sulphatides during tandem MS analysis

Tandem mass spectrometer worked in MRM mode. The analysis was made to determine in which scale the response of measurement is linear. Linearity showed efficient range in which the measurement proceeded without distortion (Fig. 27).

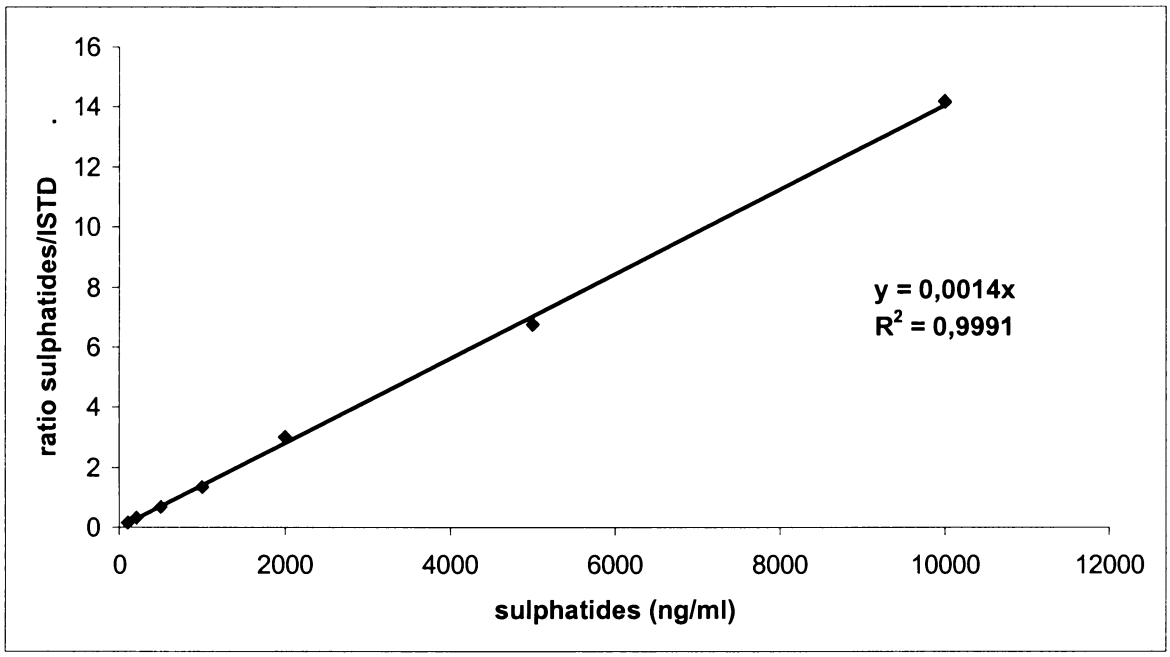


Fig. 27: Linearity of sulphatides concentration

Comment: Samples contained described amount of sulphatides with 200 ng of C17:0 sulphatide ISTD in 500 µl of MeOH

Quantitative measurement of sulphatides in urine for diagnosis of sulphatidoses

Measurements were carried out in MRM mode on tandem mass spectrometer. C17:0 sulphatide was used as an internal standard. We analysed urine samples of 15 control individuals, 13 samples of MLD patients and single samples of an MLD heterozygote, a patient with SAP-B deficiency and of a suspected prosaposin deficiency infant. The cohort of MLD patients consisted of 6 late infantile, 1 juvenile and 6 adult clinical forms.

Repeated measurements of one MLD sample were performed to determine, which quantification approach (either peak height or peak area) was better (Table 14).

All samples were quantified using external calibration point with known amount of sulphatides. Results of these measurements were sorted in tables and graphs (Table 15, p. 58, Fig. 28, p. 58).

Table 14: Accuracy of sulphatides quantification using peak area vs. peak height calculation

no. of measurement	*sulphatides/ISTD (peak area)*	*sulphatides/ISTD (peak height)*
1	1,376923	1,370079
2	1,532258	1,416667
3	1,446154	1,373984
4	1,451128	1,357724
5	1,460938	1,358974
6	1,492188	1,389831
7	1,453846	1,381356
SD	**0,043854**	**0,018888**

Table 15: Quantity of sulphatides in urine of patients with different types of MLD and SAP deficiencies

	Sulphatides ng/ml STD urine*			
	median	mean	SD	range
Controls n=15	**279,59**	**287,54**	**67,32**	**170-380**
MLD n=13	**2537,62**	**2572,84**	**1106,52**	**712-4391**
MLD-late infantile n=6	**3543,58**	**3271,03**	**1034,92**	**1512-4391**
MLD-adult n=6	**2147,33**	**2184,62**	**503,62**	**1454-3212**
Individual cases of MLD:	ng/ml STD urine*		MLD type	
1	4390,64		*late infantile*	
2	2668,03		*late infantile*	
3	3610,77		*late infantile*	
4	1511,97		*late infantile*	
5	3476,40		*late infantile*	
6	3968,39		*late infantile*	
7	712,99		*juvenile*	
8	2181,79		*adult*	
9	2537,62		*adult*	
10	1610,10		*adult*	
11	1453,59		*adult*	
12	3211,72		*adult*	
13	2112,87		*adult*	
SAP–B deficiency	**3021,71**		*four-month infant*	
Susp. prosaposin defficiency	**14265,73**		*two-month infant*	

*STD urine is urine adjusted to standard creatinine concentration of 2,5 mmol/l

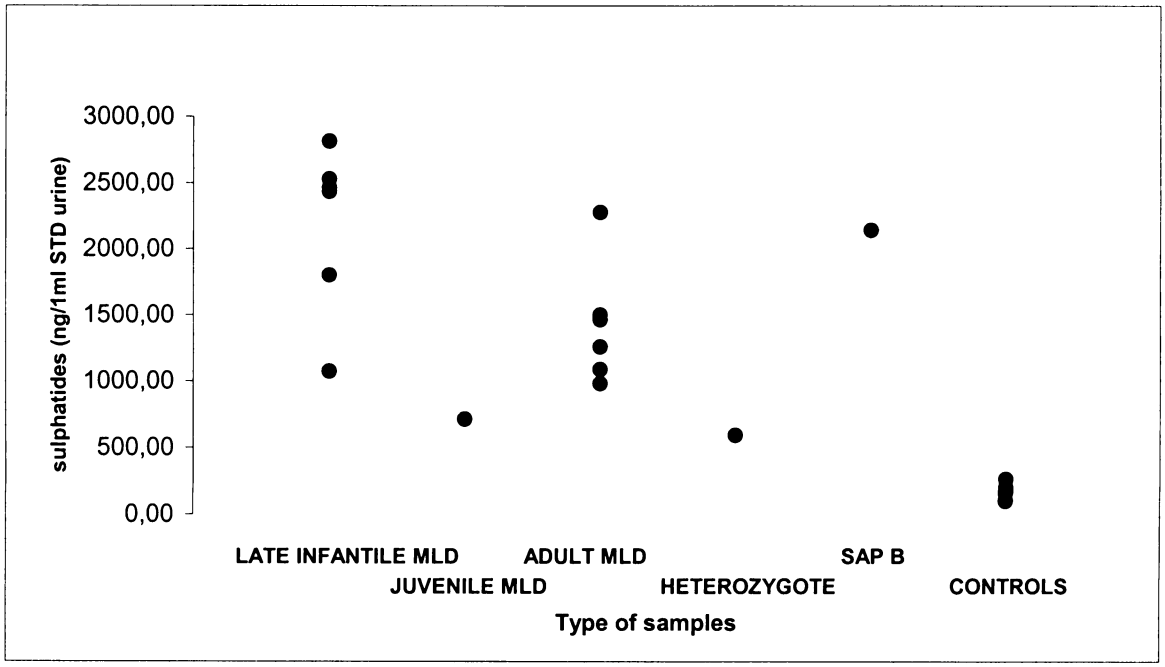


Fig. 28: The level of stored sulphatides in urine samples of patients and control individuals

Isotype composition of sulphatides in control and sulphatidosis urine samples

Tandem MS data measured in control and sulphatidosis urine samples were processed to obtain isotype composition (Fig. 29).

Isotype composition showed different dominant isotype for patients and for controls. There were also differencies in composition among clinical forms of sulphatidosis. Isotype patterns of individual cases are shown in Fig. 30, p. 60, Fig. 31 and Fig. 32, p. 31.

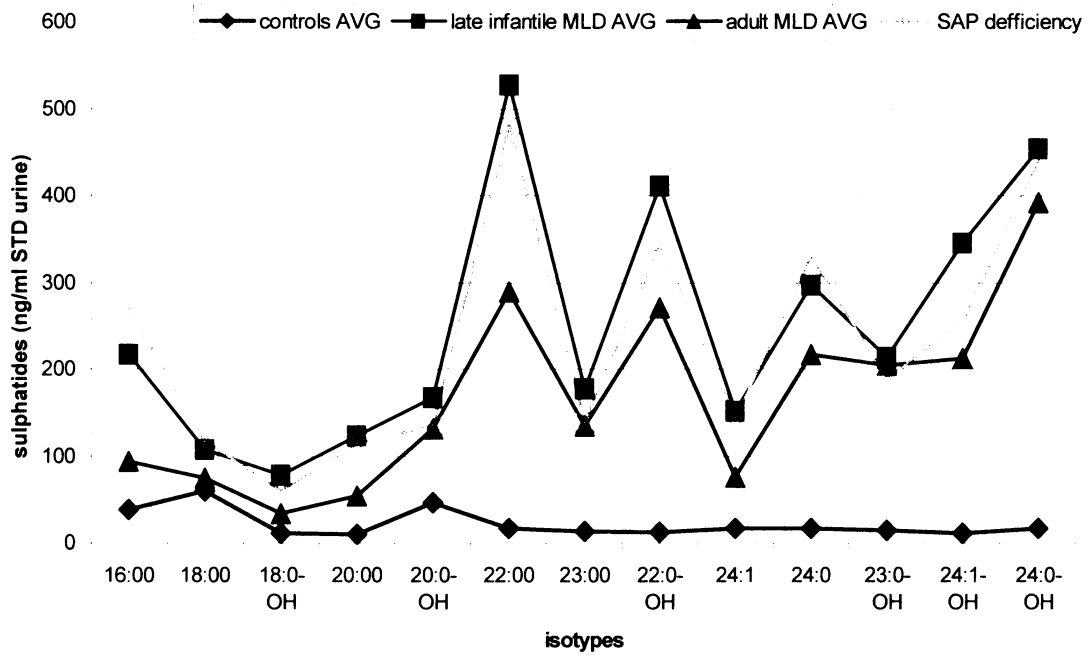


Fig. 29: Average isotype composition of sulphatides in MLD compared to SAP-B deficiency and controls

Comment: controls n=15, late infantile n=6, adult n=6; AVG=average

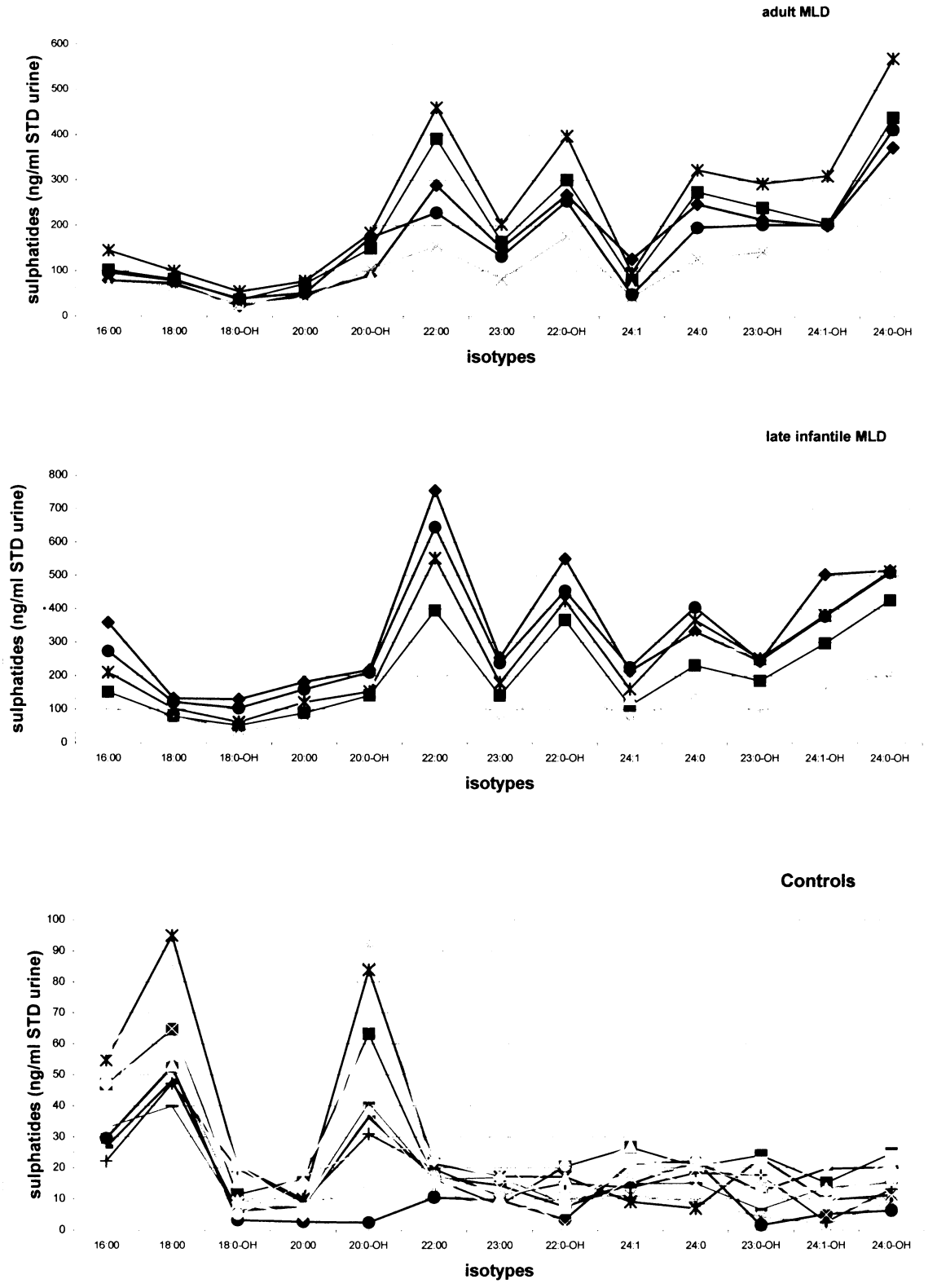


Fig. 30: Isotype composition of sulphatides in adult MLD, late infantile MLD and in controls

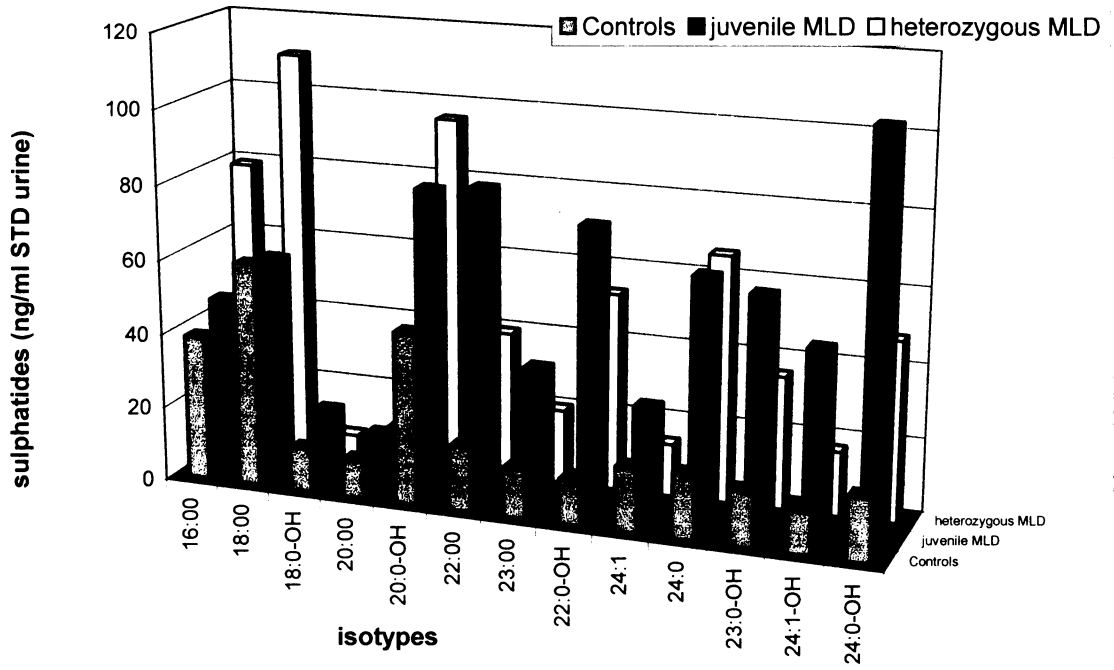


Fig. 31: Isotype composition of sulphatides in heterozygous MLD, juvenile MLD and in controls

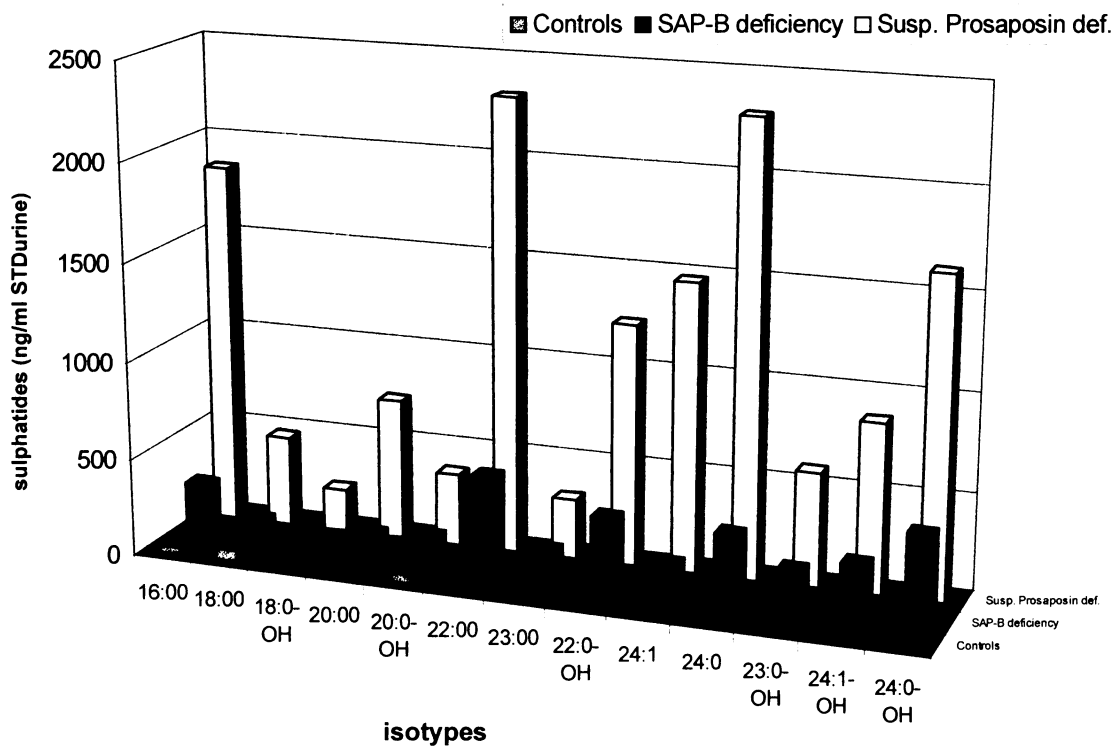


Fig. 32: Isotype composition of sulphatides in SAP-B deficiency, suspected prosaposin deficiency and in controls

Discussion

Synthesis of sphingolipid internal standards

Sphingolipid ISTD for tandem MS analysis should have some necessary attributes, e.g.:

- it must be of the same class of compounds as the analyte to undergo the same ionization and fragmentation
- it does not occur naturally in human tissues
- its mass must differ from naturally occurring metabolite for at least 4 m/z, but it should be as close to the mass of the main natural isotype as possible.

Sulphatides suitable as ISTD for tandem MS analysis are not commercially available. One possibility was to prepare ISTD by chemical synthesis. Among several complicated procedures with low yields only one recently reported method had the rate of conversion about 90% [97]. This result was excellent, but salts with Cl^- ions were formed as reaction byproducts and had to be removed by desalting. Cl^- ions create adducts with sphingolipids [52, 53] dividing thus the signal between adduct and ionized form which results in problematic quantification. Remaining salts decrease signal intensity during tandem mass analysis since they interfere with ionized analyte. Therefore we decided to prepare the ISTD for tandem mass spectrometry by enzymic semisynthesis described recently by Ito *et al* [93, 95].

Hydrolysis and semisynthesis of sphingolipids with the use of SCDase

Sphingolipid ceramide N-deacylase is an enzyme able to cleave amide bond in the sphingolipid molecule and under changed conditions to catalyze a reverse reaction, too [95]. The procedure was found highly specific without byproduct formation and seemed to be the best choice for ISTDs preparation. This work began with testing of reaction conditions according to the literature recommendations [93-95].

Three types of detergents (taurodeoxycholate, cholate, Triton X-100) with recommended optimal concentrations [94, 95] for hydrolysis and synthesis were tested first. In the hydrolytic step their role was found essential, because without detergents the reaction did not proceed or with minimal rate of conversion [94]. It can be explained by hydrophobic nature of lipid compounds which must be solubilized to reach the active center of a hydrophilic enzyme. Their function is similar to that of saposins in the lysosomal

degradation of simple hydrophobic sphingolipids [24, 75]. Recommended concentration of 0,8% detergent solutions was used in hydrolytic reaction [94]. Different situation was in case of reverse reaction, i.e. acylation of lyso-sphingolipids with appropriate fatty acids. Lyso-sphingolipids reveal lower hydrophobicity because of the loss of one fatty acid. Therefore synthesis requires lower detergent concentration (up to 0,1%). Formation of micelles from both substrates (lyso-sulphatides and fatty acids in the optimal ratio) is crucial for the progress of synthetic reaction [95] because in this form substrates can reach the enzyme active center.

Triton X-100 was chosen as the best detergent in the monophasic system, which was consequently used in semisynthetic reaction with SCDase.

pH is another important factor for reverse reactions catalyzed by SCDase. It has been reported that hydrolysis prevails at pH 5-6 [92-94]. Synthetic reaction starts from pH 7 to higher values [95].

pI of SCDase used in our experiments (commercial product originated from Pseudomonas sp. TK4) has not been determined yet. According to our preliminary analysis on immobilline stripes in both reductive or non-reductive environments, two main bands of commercial SCDase corresponded to pI 6±1 (data not shown) which is in accordance with pI of similar enzyme of different origin [98]. Literature data showed the break point between hydrolysis and synthesis at pH 6±1 [95] suggesting the importance of ionized side groups on enzyme protein and their role in sphingolipid hydrolysis or synthesis. Exact mechanisms of SCDase catalytic activity has not been investigated yet. However, characterization of SCDase was not the subject of this work but has been found as an interesting and important topic for future investigation.

Enzymic semisynthesis showed good potential for preparation of ISTD of sphingolipids. Reactions with lyso-Gb_3Cer and stearic acid revealed ≥90% conversion of substrates to the product (C18:0 Gb_3Cer) [96]. This result was very satisfactory, but enzyme preparation was used up in one reaction which made enzymic semisynthesis expensive. This problem had simple solution in immobilisation of an enzyme on a carrier allowing multiple use of the enzyme.

Preparation of enzyme reactors – immobilisation of SCDase

The choice of the carrier and type of immobilisation was the first task. We employed experience and excellent collaboration with Assoc. Prof. RNDr. Z. Bílková, Ph.D. from the

University of Pardubice and tested for our purposes two methods used in her laboratory [85-87, 89-91]. Methods utilizing magnetic particles with covalently immobilised enzyme were chosen for simple separation of the product from a reaction mixture using strong magnet.

Two different approaches to immobilisation of an enzyme protein were tested. Principle of the first method was creating an amide bond between the primary amino group on an enzyme protein and the aldehyde group on porous bead cellulose through the Schiff base formation. The advantage is in the formation of reactive aldehyde groups on carrier particles whereas enzyme molecule is not affected by oxidation. Porous particles with huge surface are favourable for this reaction. Created Schiff base was mildly reduced by cyanoborohydride to form amide bond [88]. This approach had no influence on enzyme protein.

Second method is more aggressive since it utilizes EDC (EDAC) and sulpho-NHS to form amide bond between the enzyme primary amino groups and the carboxyl groups of the carrier, concentration of which is an important basic parameter. Tested carrier was SiMAG-Carboxyl coated by carboxyl groups on silica surface. The disadvantage of this approach is a vigorous EDC attack on all carboxyl groups on both carrier and enzyme molecule. In presence of EDC three situations can occur. The first possible situation is that during the reaction enzyme carboxyl groups are not affected and primary amino group reacts with modified carboxyl group of the carrier. This is the desired reaction resulting in immobilisation of an enzyme on carrier surface. In second case enzyme carboxyl groups are modified and react with primary amino groups of another enzyme molecule. Enzyme molecules create enzyme complex, which may concentrate enzymes into one place. It could be an advantageous situation in the case of immobilising the complex on carrier surface. The third possible variation is that enzyme carboxyl groups are modified and react with primary amino groups on the same enzyme molecule. This is the worst situation resulting in irreversible conformational change of the enzyme protein. Thus, EDC attack of carboxyl groups might irreversibly destroy enzyme itself. Concentration of EDC and sulpho-NHS reagent is crucial for the immobilising reaction. High concentration of EDC is dangerous because of enzyme damage. Low concentration results in low binding effect. Creation of enzyme sites must be controlled by reagent concentrations. EDC method of immobilisation needs optimizations of all reaction steps [88, 89].

Testing and comparison of reactors parameters (catalytic activity and product purity)

Both immobilisation methods were successfully used for SCDase. Differences between both reactors were in catalytic activity during hydrolysis and synthesis. CH reactor (SCDase immobilised on SiMAG-Carboxyl) showed higher total hydrolytic activity with AGM1 ganglioside as a testing compound in comparison with P reactor (SCDase immobilised on magnetic bead cellulose) which revealed higher activity only at the beginning of the hydrolysis. Synthesis of C17:0 sulphatide ISTD showed higher rate of conversion with P than CH reactor.

Purity of the reaction product was better in case of CH reactor. Since bead cellulose is porous and holds the products and substrates inside, P reactor required prolonged and rigorous washings after use. However, long-term rigorous mixing on rotator should be avoided because it caused fragmentation of bead cellulose detectable on TLC plate after orcinol detection.

For the synthetic reaction molar ratio of 1:1 substrates (fatty acid:lyso-sulphatide) was confirmed as the best one (for both P and CH reactors). Other reaction conditions were the same as the ones for soluble SCDase [93-95].

Enzymic semisynthesis of C17:0 sulphatide ISTD

First semisynthetized compound was C17:0 sulphatide ISTD which was required for tandem MS quantification of sulphatides. P reactor was found satisfactory, with the rate of conversion of lyso-sulphatide to C17:0 sulphatide mostly above 80%, although minor disadvantages occured (e.g. saccharide fragmentation). Synthesized compound was tested on tandem mass spectrometer with very good results. Multiple use of reactor was the main advantage in comparison with soluble SCDase reaction. P reactor retained its catalytic activity even after 10^{th} and more use (see Results, p.48) whereas CH reactor has lost it completely at that stage of use.

Beside preparation of ISTDs, this system with immobilised SCDase will be useful for preparation of sphingolipids specifically labelled on fatty acid either isotopically or with fluorescent markers. This opens a way to further promising applications in different fields of sphingolipid biochemistry.

Since a fragmentation of carrier particles could be disadvantageous for some applications, other immobilisation procedures ought to be tested in future experiments. One such

example is the biotinylated SCDase (prepared using biotinylation kits) immobilised on particles coated by streptavidin (e.g. SiMAG-affinity coated with streptavidin).

Sphingolipid analysis by tandem MS

Tandem mass spectrometry is a modern and powerful method for shingolipid analysis with broad spectrum of applications in routine diagnosis and in research programs related to lysosomal storage disorders [53, 83, 84]. Proof of stored sphingolipids by tandem mass analysis in urine samples, cells or tissues provides important information for diagnosis which is confirmed by determination of catalytic activity of corresponding hydrolases in the next step. Tandem mass spectrometry can be successfully exploited for determination of enzyme activity using specific substrates set [51].

Setting up of tandem MS methods

The work on tandem mass spectrometry of sphingolipids started with preparation of standard samples for adjustment of ionization [83, 84]. After that, measurements leading to determination of fragmentation patterns of studied sphingolipids began. Major fragment produced by neutral sphingolipids (Cer, GlcCer, LacCer and Gb_3Cer) had m/z 264 corresponding to sphingoid base (sphingosine) with loss of two water molecules [82]. Sphingomyelin main fragment had its origin in phosphocholine with m/z 184, but fragment with m/z 264 typical for all sphingolipids was also produced.

Fragmentation of the sulphatide standard revealed the only fragment with m/z 97 (sulphate group). This fragment allows direct analysis of sulphatides without their separation from other sphingolipids. This fact also demonstrated the versatility of tandem MS for analysis of complex biological samples [52].

Fragmentation measurements allowed us to measure precursor ion spectra of the sphingolipids in normal tissue samples.

Qualitative analysis of sphingolipids

Evaluation of precursor ion spectra revealed isotype composition of the neutral sphingolipids in different biological samples which gave a set of isotypes necessary for quantification. C16:0 sphingomyelin isotype had the same mass as GlcCer ISTD (C16:0-^{3}D GlcCer) with m/z 703 resulting in an interference with GlcCer ISTD. Therefore, C16:0-

^{3}D GlcCer ISTD could not be used for GlcCer quantification and another ISTD had to be selected (C12:0 GlcCer was proposed).

Differences in signal intensities were observed through different tissue types. Isotype patterns in neuronal tissues showed prevailing isotypes with longer chain fatty acids in comparison with non-neuronal tissues. This can be explained by lower conductivity of longer chains which plays an important role for insulation of neural fibers by myelin sheath [72].

MRM spectra of neutral sphingolipids in the control spleen confirmed that the method is prepared for quantification measurements in patients´ samples. These measurements will follow in further studies.

Precursor ion spectra of sulphatides were measured in the brain and in the kidney. MRM spectra of sulphatides were measured in urine extracts. Isotypes with long chain fatty acids dominated in the brain tissue [72]. Comparison of precursor ion scans of kidney tissue extracts to MRM spectra of urine samples showed that the pattern of kidney sulphatide isotypes is identical to the one in urine [84].

Precursor ion spectra of synthesized C17:0 sulphatide showed isotope splitting which interferes with C16:0-OH isotype and therefore was removed from the quantification measurements.

Quantitative analysis of sulphatides

Total quantity of 13 isotypes of urinary sulphatides (except ISTD) was calculated. Concentration of individual isotypes was also evaluated. We presumed from reported data [84] that d18:1 sphingosine was the predominant base in urinary sulphatides.

First step in MRM quantification was done by evaluation of linearity of sulphatides concentration. Linearity was approved in the concentration range from 100 – 10000 ng/ml but according to published data this would persist up to 1 000 000 ng/ml [52].

Usage of two or more ISTDs corresponding to distribution of sample mass is recommended and they will be prepared for further studies as also suggested by others [84]. Practically it means to have one ISTD for isotypes with C16:0 – C18:0 fatty acids and second one for C24:0 fatty acids. It is also necessary to mention the importance of another ISTD for the correction of extraction step [82].

Different quantification approaches were also tested. Quantification with external calibration point was chosen because of high level of linearity through measured

concentration of sulphatides together with exclusion of interferences caused by ISTD concentration. Role of ISTD is to keep signal during measurement linear. Therefore we compared sulphatides signal in samples to signal of sulphatides in external calibration point representing complex mixture of different isotypes.

Extracts of urine samples were measured in MRM mode. Signal intensity dropped with rising of creatinine concentration and resulted in loss of the peak top. Deformed peaks gave imprecise sulphatide quantification. Therefore urinary samples were adjusted to equal range of creatinine concentrations corresponding to 1-2,5 mmol/l. Measured quantities of sulphatides were related to STD urine sample having creatinine concentration 2.5 mmol/l. This adjustment was found very efficient.

Quantification of sulphatides in control and MLD patient urine samples

Optimized tandem MS procedure in MRM mode was used for analysis of sulphatides in 15 control urine samples, 13 MLD patients with confirmed diagnosis and 1 MLD heterozygote. The cohort of MLD patients comprised of 6 late infantile, 1 juvenile and 6 adult forms of the disease.

Results confirmed massive excretion of stored sulphatides in urine of MLD patients in comparison with controls. Concentration of sulphatides also reflected clinical type of MLD. Infantile cases stored more sulphatides than the others. Only juvenile type of MLD had different trend, but this particular case also showed differences in dynamic metabolic studies probably due to higher residual activity of arylsulphatase A (personal comm. – J. Ledvinová). Lower storage was also proved in urine sample of heterozygous MLD caused by higher residual ASA activity.

Quantification of sulphatides in SAP-B and prosaposin deficiencies

We also had a unique opportunity to analyze urine samples from patients with suspected very rare defects, SAP-B and prosaposin deficiencies, which have not been quantified by tandem mass spectrometry yet. In several published cases only few urine samples were analyzed mostly qualitatively by TLC of lipid extracts [30]. In both cases our results corresponded perfectly with proposed diagnoses. Excretion of sulphatides was in the range of infantile MLD cases, that of prosaposin deficiency even 10 fold higher. This confirmed our previous presumption of effectiveness of urinary lipid analysis in cases suspected of protein activator deficiences [99]. The sample of SAP-B deficiency confirmed the role of

SAP-B in degradation of sulphatides and their massive excretion in urine. The study showed that a tandem mass analysis is a new useful diagnostic method, which is able together with determination of ASA activity and function tests in cell cultures to confirm this type of rare defects [75].

The urine sample from a suspected prosaposin deficiency patient revealed the highest concentration of sulphatides (3 - 10 fold higher than in our MLD cases). However, the diagnosis must be confirmed by further analyses (e.g. proof of normal ASA activity in leukocytes, excretion of Gb_3Cer and shorter sphingolipids in urine, function experiments in fibroblast culture, mutation analysis in prosaposin gene).

Total concentration levels measured in our samples were different from concentrations published by Whitfield *et al.* [84]. This was caused by different quantification method and by usage of C16:0 sulphatide ISTD. In our work we preferred C17:0 sulphatide as ISTD, because it is not present or only in negligible amounts in normal isotype pattern. C16:0 sulphatide, on the other hand, is relatively abundant isotype of natural sulphatides. C16:0 represented 12% in control samples, 4-7% in MLD samples, 13% in suspected prosaposin deficiency and 9% in SAP-B deficiency. These portions cannot be ignored otherwise quantification is not precise. Therefore ratio of ISTD signal to sulphatides signal used for quantification was also inaccurate in the above mentioned study especially in case of MLD samples [84]. Furthermore, only eight isotypes were scanned which made total concentration underestimated. All these differences together with different sulphatides concentration unit resulted in different output concentration values.

Study of sulphatide isotypes in patients with sulphatide storage disorders

Isotype composition of urinary sulphatides in healthy individuals and in patients was newly studied by tandem MS in this work [100]. Our results revealed isotype shift to longer fatty acids in sulphatides composition of MLD samples. This isotype pattern was characteristic for all defects in sulphatides degradation. Predominant stored isotypes were C16:0, C22:0, C22:0-OH, C24:0 and C24:0-OH. There is also a characteristic change in ratio of C16:0 isotype to C18:0 isotype. The level of C16:0 rises above C18:0 level in MLD samples. This pattern was compared to that of Gb_3Cer in same urine samples (personal comm. – L. Berná). However, the pattern of Gb_3Cer isotypes in urines of our

group of MLD patients showed normal isotype composition comparable to control urine samples. This confirms that the composition of other sphigolipid series is not affected.
Shift in isotype pattern should be explained hypothetically by „enzymic theory". The presupposition is that ASA degrades especially isotypes synthetised by sulphotransferase. These isotypes are C16:0 in the first period of life (immaturated tissues) and shifted to C22:0, C22:0-OH, C24:0 and C24:0-OH in the maturated tissues [72]. Since MLD is a defect of ASA catalytic activity, which results in storing of sulphatides in lysosomes during the life span, higher isotypes are increasingly accumulated together with continually synthetized and nondegraded shorter types. Interestingly, C18:0 isotype might be regulated by another mechanism, because its level is almost the same in control and MLD samples.

Future research in this field is desirable. It is not resolved yet why sulphatides with longer chain fatty acid are stored in comparison to those with palmitic or stearic fatty acid. These research works should be done as dynamic metabolic studies with specific labelled sulphatide isotypes (e.g. prepared by enzymic semisynthesis). Catalytic activity of ASA towards individual isotypes should be also interesting and can possibly explain some details of mechanism of sulphatides storage. Quantification using another membrane sphingolipid (e.g. sphingomyelin) as the reference value is also worth of further study, because it better represents the cellular source of stored sphingolipids. Biosythesis could be investigated together with degradation to evaluate turnover of particular sphingolipid. Methods of molecular biology should be included to show e.g. gene expression of enzymes included in these pathways.

Conclusions

This work was focused on tandem MS analysis of sphingolipids, its use in diagnosis of disorders of sphingolipid degradation and in related research studies. This aim was fullfiled as follows:

1. Synthesis of sphingolipid internal standards on immobilized SCDase

Preparation of enzymic reactor with immobilised SCDase

- SCDase was immobilised on two different magnetic carriers - SiMAG-carboxyl (CH reactor) and magnetic porous bead cellulose (P reactor). A special immobilisation method for each carrier was established

Testing and comparison of parameters of two reactors (P and CH)

- CH reactor showed slightly higher total hydrolytic activity with AGM1 after 24 hrs, whereas P reactor revealed higher rate of conversion of lyso-sulphatide to C17:0 sulphatide ISTD. Certain disadvantage of P reactor was in fragmentation of bead cellulose and in unavoidable rigorous washings after use but it had any negative influence on tandem MS analysis

Enzymic semisynthesis of tandem MS internal standards

- C17:0 sulphatide was successfully synthesized using P reactor and its identity confirmed by tandem MS. The yield was up to 80% of C17:0 sulphatide converted from lyso-sulphatide.
- The main advantage of immobilised SCDase was in its multiple use (at least 12 uses without loss of activity).

2. Setting up tandem MS method for sphingolipid analysis

- Two methods for SFL analysis were developed: first for analysis of neutral SFLs (ceramide, GlcCer, LacCer, Gb_3Cer and SM) and second one for analysis of sulphatides.

Qualitative analysis of isotype composition of sphingolipids:

- Using precursor ion scan, isotype composition of neutral SFLs and sulphatides in different tissues was obtained. The composition was specific according to the type of the analysed tissue and SFL.
- Sulphatides in control and patient urine samples were evaluated by MRM. The isotype composition resembled the isotypes found in kidney tissue. Significant differences between control and patients´ urine samples were demonstrated.

Quantitative analysis of sphingolipids directed to the diagnosis of specific storage diseases

- MRM scans were performed to quantitate sulphatides in control and MLD patients urine samples standardised to creatinine concentration of 1-2,5 mmol/l.
- Results showed that patients excreted 2 to 25 times more sulphatides than controls. Distinct differences between clinical forms of MLD were observed.
- Unique defect of SAP-B deficiency was evaluated by MRM scan of urinary sulphatides. Storage was similar to MLD patients with the most severe (i.e. late infantile) clinical form.
- Highest level of sulphatides storage was found in a patient with suspected prosaposin deficiency - 3 fold higher than in the most storing case of MLD. Final confirmation of diagnosis is in progress (tandem MS of other short SFLs, feeding studies, DNA analysis).
- Analysis confirmed reliability of tandem MS to prove increased excretion of sulphatides (and other sphingolipids) in urine as the important diagnostic marker in SAPs deficiences

Comparative study of sulphatide isotypes in patients with sulphatide storage disorders and in healthy individuals

- Results revealed specific isotype patterns for controls and patients with sulphatidosis (MLD and SAPs deficiences) with significant isotype shift to longer fatty acid in all sulphatidosis cases. In MLD, isotypes of another sphingolipids were not changed. The cause of these changes is not clear and needs further investigation.

References

1. Hakomori, S.I., *Glycosphingolipids in Cellular Interaction, Differentiation, and Oncogenesis.* Annual Review of Biochemistry, 1981. **50**: p. 733-764.
2. Stults, C.L., C.C. Sweeley, and B.A. Macher, *Glycosphingolipids: structure, biological source, and properties.* Methods Enzymol, 1989. **179**: p. 167-214.
3. *IUPAC-IUB joint commission on biochemical nomenclature (JCBN) nomenclature of glycolipids recommendations 1997.* J Mol Biol, 1999. **286**(3): p. 963-70.
4. Ulrich-Bott, B. and H. Wiegandt, *Micellar properties of glycosphingolipids in aqueous media.* J Lipid Res, 1984. **25**(11): p. 1233-45.
5. Curatolo, W., D.M. Small, and G.G. Shipley, *Phase behavior and structural characteristics of hydrated bovine brain gangliosides.* Biochim Biophys Acta, 1977. **468**(1): p. 11-20.
6. Folch, J., M. Lees, and G.H. Sloane Stanley, *A simple method for the isolation and purification of total lipides from animal tissues.* J Biol Chem, 1957. **226**(1): p. 497-509.
7. Gillard, B.K., R.G. Clement, and D.M. Marcus, *Variations among cell lines in the synthesis of sphingolipids in de novo and recycling pathways.* Glycobiology, 1998. **8**(9): p. 885-90.
8. Merrill, A.H., Jr., *De novo sphingolipid biosynthesis: a necessary, but dangerous, pathway.* J Biol Chem, 2002. **277**(29): p. 25843-6.
9. Mandon, E.C., G. van Echten, R. Birk, et al., *Sphingolipid biosynthesis in cultured neurons. Down-regulation of serine palmitoyltransferase by sphingoid bases.* Eur J Biochem, 1991. **198**(3): p. 667-74.
10. Stoffel, W., D. LeKim, and G. Sticht, *Biosynthesis of dihydrosphingosine in vitro.* Hoppe Seylers Z Physiol Chem, 1968. **349**(5): p. 664-70.
11. Shimeno, H., S. Soeda, M. Sakamoto, et al., *Partial purification and characterization of sphingosine N-acyltransferase (ceramide synthase) from bovine liver mitochondrion-rich fraction.* Lipids, 1998. **33**(6): p. 601-5.
12. Michel, C. and G. van Echten-Deckert, *Conversion of dihydroceramide to ceramide occurs at the cytosolic face of the endoplasmic reticulum.* FEBS Lett, 1997. **416**(2): p. 153-5.
13. Mandon, E.C., I. Ehses, J. Rother, et al., *Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydrosphinganine reductase, and sphinganine N-acyltransferase in mouse liver.* J Biol Chem, 1992. **267**(16): p. 11144-8.
14. van Meer, G., *What sugar next? Dimerization of sphingolipid glycosyltransferases.* Proc Natl Acad Sci U S A, 2001. **98**(4): p. 1321-3.
15. Lannert, H., K. Gorgas, I. Meissner, et al., *Functional organization of the Golgi apparatus in glycosphingolipid biosynthesis. Lactosylceramide and subsequent glycosphingolipids are formed in the lumen of the late Golgi.* J Biol Chem, 1998. **273**(5): p. 2939-46.

16. Vos, J.P., M. Lopes-Cardozo, and B.M. Gadella, *Metabolic and functional aspects of sulfogalactolipids.* Biochim Biophys Acta, 1994. **1211**(2): p. 125-49.
17. Futerman, A.H., B. Stieger, A.L. Hubbard, et al., *Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus.* J Biol Chem, 1990. **265**(15): p. 8650-7.
18. Coste, H., M.B. Martel, and R. Got, *Topology of glucosylceramide synthesis in Golgi membranes from porcine submaxillary glands.* Biochim Biophys Acta, 1986. **858**(1): p. 6-12.
19. Futerman, A.H. and R.E. Pagano, *Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver.* Biochem J, 1991. **280 (Pt 2)**: p. 295-302.
20. Paul, P., Y. Kamisaka, D.L. Marks, et al., *Purification and characterization of UDP-glucose:ceramide glucosyltransferase from rat liver Golgi membranes.* J Biol Chem, 1996. **271**(4): p. 2287-93.
21. Jeckel, D., A. Karrenbauer, K.N. Burger, et al., *Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions.* J Cell Biol, 1992. **117**(2): p. 259-67.
22. Ichikawa, S. and Y. Hirabayashi, *Glucosylceramide synthase and glycosphingolipid synthesis.* Trends Cell Biol, 1998. **8**(5): p. 198-202.
23. Nomura, T., M. Takizawa, J. Aoki, et al., *Purification, cDNA cloning, and expression of UDP-Gal: glucosylceramide beta-1,4-galactosyltransferase from rat brain.* J Biol Chem, 1998. **273**(22): p. 13570-7.
24. Sandhoff, K. and T. Kolter, *Biosynthesis and degradation of mammalian glycosphingolipids.* Philos Trans R Soc Lond B Biol Sci, 2003. **358**(1433): p. 847-61.
25. Sandhoff, K. and T. Kolter, *Topology of glycosphingolipid degradation.* Trends Cell Biol, 1996. **6**(3): p. 98-103.
26. Jeyakumar, M., T.D. Butters, R.A. Dwek, et al., *Glycosphingolipid lysosomal storage diseases: therapy and pathogenesis.* Neuropathol Appl Neurobiol, 2002. **28**(5): p. 343-57.
27. Furst, W. and K. Sandhoff, *Activator proteins and topology of lysosomal sphingolipid catabolism.* Biochim Biophys Acta, 1992. **1126**(1): p. 1-16.
28. van der Goot, F.G. and J. Gruenberg, *Oiling the wheels of the endocytic pathway.* Trends Cell Biol, 2002. **12**(7): p. 296-9.
29. Peters, C. and K. von Figura, *Biogenesis of lysosomal membranes.* FEBS Lett, 1994. **346**(1): p. 108-14.
30. Harzer, K., B.C. Paton, A. Poulos, et al., *Sphingolipid activator protein deficiency in a 16-week-old atypical Gaucher disease patient and his fetal sibling: biochemical signs of combined sphingolipidoses.* Eur J Pediatr, 1989. **149**(1): p. 31-9.
31. Vogel, A., G. Schwarzmann, and K. Sandhoff, *Glycosphingolipid specificity of the human sulfatide activator protein.* Eur J Biochem, 1991. **200**(2): p. 591-7.
32. Bradova, V., F. Smid, B. Ulrich-Bott, et al., *Prosaposin deficiency: further characterization of the sphingolipid activator protein-deficient sibs. Multiple glycolipid elevations (including lactosylceramidosis), partial enzyme deficiencies and ultrastructure of the skin in this generalized sphingolipid storage disease.* Hum Genet, 1993. **92**(2): p. 143-52.

33. Furst, W., J. Schubert, W. Machleidt, et al., *The complete amino-acid sequences of human ganglioside GM2 activator protein and cerebroside sulfate activator protein.* Eur J Biochem, 1990. **192**(3): p. 709-14.
34. Furst, W., W. Machleidt, and K. Sandhoff, *The precursor of sulfatide activator protein is processed to three different proteins.* Biol Chem Hoppe Seyler, 1988. **369**(5): p. 317-28.
35. Schuette, C.G., B. Pierstorff, S. Huettler, et al., *Sphingolipid activator proteins: proteins with complex functions in lipid degradation and skin biogenesis.* Glycobiology, 2001. **11**(6): p. 81R-90R.
36. Sandhoff, K. and T. Kolter, *Processing of sphingolipid activator proteins and the topology of lysosomal digestion.* Acta Biochim Pol, 1998. **45**(2): p. 373-84.
37. Sandhoff, K., T. Kolter, and G. Van Echten-Deckert, *Sphingolipid metabolism. Sphingoid analogs, sphingolipid activator proteins, and the pathology of the cell.* Ann N Y Acad Sci, 1998. **845**: p. 139-51.
38. Coetzee, T., K. Suzuki, and B. Popko, *New perspectives on the function of myelin galactolipids.* Trends Neurosci, 1998. **21**(3): p. 126-30.
39. Huwiler, A., T. Kolter, J. Pfeilschifter, et al., *Physiology and pathophysiology of sphingolipid metabolism and signaling.* Biochim Biophys Acta, 2000. **1485**(2-3): p. 63-99.
40. Tettamanti, G., M. Masserini, A. Giuliani, et al., *Structure and function of gangliosides.* Ann Ist Super Sanita, 1988. **24**(1): p. 23-31.
41. Koerner, T.A., Jr., J.H. Prestegard, P.C. Demou, et al., *High-resolution proton NMR studies of gangliosides. 1. Use of homonuclear two-dimensional spin-echo J-correlated spectroscopy for determination of residue composition and anomeric configurations.* Biochemistry, 1983. **22**(11): p. 2676-87.
42. Riboni, L., S. Sonnino, D. Acquotti, et al., *Natural occurrence of ganglioside lactones. Isolation and characterization of GD1b inner ester from adult human brain.* J Biol Chem, 1986. **261**(18): p. 8514-9.
43. Myers, M., C. Wortman, and E. Freire, *Modulation of neuraminidase activity by the physical state of phospholipid bilayers containing gangliosides Gd1a and Gt1b.* Biochemistry, 1984. **23**(7): p. 1442-8.
44. van Meer, G. and Q. Lisman, *Sphingolipid transport: rafts and translocators.* J Biol Chem, 2002. **277**(29): p. 25855-8.
45. Wertz, P.W. and B. van den Bergh, *The physical, chemical and functional properties of lipids in the skin and other biological barriers.* Chem Phys Lipids, 1998. **91**(2): p. 85-96.
46. Kaye, E.M., *Lysosomal Storage Diseases.* Curr Treat Options Neurol, 2001. **3**(3): p. 249-256.
47. Neufeld, E.F., *Lysosomal storage diseases.* Annu Rev Biochem, 1991. **60**: p. 257-80.
48. Meikle, P.J., J.J. Hopwood, A.E. Clague, et al., *Prevalence of lysosomal storage disorders.* Jama, 1999. **281**(3): p. 249-54.
49. Conzelmann, E. and K. Sandhoff, *Partial enzyme deficiencies: residual activities and the development of neurological disorders.* Dev Neurosci, 1983. **6**(1): p. 58-71.
50. Rapola, J., *Lysosomal storage diseases in adults.* Pathol Res Pract, 1994. **190**(8): p. 759-66.
51. Li, Y., C.R. Scott, N.A. Chamoles, et al., *Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening.* Clin Chem, 2004. **50**(10): p. 1785-96.

52. Han, X. and R.W. Gross, *Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics.* J Lipid Res, 2003. **44**(6): p. 1071-9.
53. Han, X. and R.W. Gross, *Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples.* Mass Spectrom Rev, 2005. **24**(3): p. 367-412.
54. Ramsay, S.L., I. Maire, C. Bindloss, et al., *Determination of oligosaccharides and glycolipids in amniotic fluid by electrospray ionisation tandem mass spectrometry: in utero indicators of lysosomal storage diseases.* Mol Genet Metab, 2004. **83**(3): p. 231-8.
55. Biffi, A., M. De Palma, A. Quattrini, et al., *Correction of metachromatic leukodystrophy in the mouse model by transplantation of genetically modified hematopoietic stem cells.* J Clin Invest, 2004. **113**(8): p. 1118-29.
56. Krivit, W., J.H. Sung, E.G. Shapiro, et al., *Microglia: the effector cell for reconstitution of the central nervous system following bone marrow transplantation for lysosomal and peroxisomal storage diseases.* Cell Transplant, 1995. **4**(4): p. 385-92.
57. Martino, S., A. Consiglio, C. Cavalieri, et al., *Expression and purification of a human, soluble Arylsulfatase A for Metachromatic Leukodystrophy enzyme replacement therapy.* J Biotechnol, 2005. **117**(3): p. 243-51.
58. Radin, N.S. and R.R. Vunnam, *Inhibitors of cerebroside metabolism.* Methods Enzymol, 1981. **72**: p. 673-84.
59. Ringden, O., C.G. Groth, A. Erikson, et al., *Ten years' experience of bone marrow transplantation for Gaucher disease.* Transplantation, 1995. **59**(6): p. 864-70.
60. Schaison, G., P. Bordigoni, and G. Leverger, *Bone marrow transplantation for genetic and metabolic disorders.* Nouv Rev Fr Hematol, 1989. **31**(2): p. 119-23.
61. Sillence, D.J., V. Puri, D.L. Marks, et al., *Glucosylceramide modulates membrane traffic along the endocytic pathway.* J Lipid Res, 2002. **43**(11): p. 1837-45.
62. Vunnam, R.R. and N.S. Radin, *Analogs of ceramide that inhibit glucocerebroside synthetase in mouse brain.* Chem Phys Lipids, 1980. **26**(3): p. 265-78.
63. Desnick, R.J., *Enzyme replacement and enhancement therapies for lysosomal diseases.* J Inherit Metab Dis, 2004. **27**(3): p. 385-410.
64. Barranger, J.A., E.O. Rice, and W.P. Swaney, *Gene transfer approaches to the lysosomal storage disorders.* Neurochem Res, 1999. **24**(4): p. 601-15.
65. Barranger, J.M. and E.A. Novelli, *Gene therapy for lysosomal storage disorders.* Expert Opin Biol Ther, 2001. **1**(5): p. 857-67.
66. Consiglio, A., A. Quattrini, S. Martino, et al., *In vivo gene therapy of metachromatic leukodystrophy by lentiviral vectors: correction of neuropathology and protection against learning impairments in affected mice.* Nat Med, 2001. **7**(3): p. 310-6.
67. Naffakh, N., D. Bohl, A. Salvetti, et al., *Gene therapy for lysosomal disorders.* Nouv Rev Fr Hematol, 1994. **36 Suppl 1**: p. S11-6.
68. Salvetti, A., J.M. Heard, and O. Danos, *Gene therapy of lysosomal storage disorders.* Br Med Bull, 1995. **51**(1): p. 106-22.
69. Pagano, R.E., V. Puri, M. Dominguez, et al., *Membrane traffic in sphingolipid storage diseases.* Traffic, 2000. **1**(11): p. 807-15.
70. Maria, B.L., K.M. Deidrick, H. Moser, et al., *Leukodystrophies: pathogenesis, diagnosis, strategies, therapies, and future research directions.* J Child Neurol, 2003. **18**(9): p. 578-90.

71. Gieselmann, V., U. Matzner, B. Hess, et al., *Metachromatic leukodystrophy: molecular genetics and an animal model.* J Inherit Metab Dis, 1998. **21**(5): p. 564-74.
72. von Figura, K., V. Gieselmann, and J. Jaeken, *Metachromatic Leukodystrophy*, in *The Metabolic & Molecular Bases of Inherited Disease*, C.R. Scriver, et al., Editors. 2001, The McGraw-Hill Companies, Inc. p. 3695-3724.
73. Lukatela, G., N. Krauss, K. Theis, et al., *Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis.* Biochemistry, 1998. **37**(11): p. 3654-64.
74. von Bulow, R., B. Schmidt, T. Dierks, et al., *Defective oligomerization of arylsulfatase a as a cause of its instability in lysosomes and metachromatic leukodystrophy.* J Biol Chem, 2002. **277**(11): p. 9455-61.
75. Sandhoff, K., T. Kolter, and K. Harzer, *Sphingolipid Activator Proteins*, in *The Metabolic & Molecular Bases of Inherited Disease*, C.R. Scriver, et al., Editors. 2001, The McGraw-Hill Companies, Inc. p. 3371-3388.
76. Mehl, E. and H. Jatzkewitz, *[A cerebrosidesulfatase from swine kidney].* Hoppe Seylers Z Physiol Chem, 1964. **339**(1): p. 260-76.
77. Dulcks, T. and R. Juraschek, *Electrospray as an ionisation method for mass spectrometry.* Journal of Aerosol Science, 1999. **30**(7): p. 927-943.
78. McLuckey, S.A. and J.M. Wells, *Mass analysis at the advent of the 21st century.* Chem Rev, 2001. **101**(2): p. 571-606.
79. Jennings, K.R., *The changing impact of the collision-induced decomposition of ions on mass spectrometry.* International Journal of Mass Spectrometry, 2000. **200**(1-3): p. 479-493.
80. *LC/MS Operator Training CD-ROM.* 1999, PE Biosystems.
81. Murphy, R.C., J. Fiedler, and J. Hevko, *Analysis of nonvolatile lipids by mass spectrometry.* Chemical Reviews, 2001. **101**(2): p. 479-526.
82. Liebisch, G., W. Drobnik, M. Reil, et al., *Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS).* J Lipid Res, 1999. **40**(8): p. 1539-46.
83. Fuller, M., P.C. Sharp, T. Rozaklis, et al., *Urinary lipid profiling for the identification of fabry hemizygotes and heterozygotes.* Clin Chem, 2005. **51**(4): p. 688-94.
84. Whitfield, P.D., P.C. Sharp, D.W. Johnson, et al., *Characterization of urinary sulfatides in metachromatic leukodystrophy using electrospray ionization-tandem mass spectrometry.* Mol Genet Metab, 2001. **73**(1): p. 30-7.
85. Bilkova, Z., J. Mazurova, J. Churacek, et al., *Oriented immobilization of chymotrypsin by use of suitable antibodies coupled to a nonporous solid support.* J Chromatogr A, 1999. **852**(1): p. 141-9.
86. Bilkova, Z., M. Slovakova, D. Horak, et al., *Enzymes immobilized on magnetic carriers: efficient and selective system for protein modification.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **770**(1-2): p. 177-81.
87. Bilkova, Z., M. Slovakova, A. Lycka, et al., *Oriented immobilization of galactose oxidase to bead and magnetic bead cellulose and poly(HEMA-co-EDMA) and magnetic poly(HEMA-co-EDMA) microspheres.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **770**(1-2): p. 25-34.
88. Hermanson, G.T., *Bioconjugate Techniques*. 1st ed. 1996: Academic Press, Inc.

89. Bilkova, Z., M. Slovakova, N. Minc, et al., *Functionalized magnetic micro- and nanoparticles: Optimization and application to mu-chip tryptic digestion.* Electrophoresis, 2006. **27**(9): p. 1811-1824.
90. Bilkova, Z., R. Stefanescu, R. Cecal, et al., *Epitope extraction technique using a proteolytic magnetic reactor combined with Fourier-transform ion cyclotron resonance mass spectrometry as a tool for the screening of potential vaccine lead peptides.* Eur J Mass Spectrom (Chichester, Eng), 2005. **11**(5): p. 489-95.
91. Slovakova, M., N. Minc, Z. Bilkova, et al., *Use of self assembled magnetic beads for on-chip protein digestion.* Lab Chip, 2005. **5**(9): p. 935-42.
92. Ito, M., T. Kurita, and K. Kita, *A novel enzyme that cleaves the N-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms.* J Biol Chem, 1995. **270**(41): p. 24370-4.
93. Ito, M., K. Kita, T. Kurita, et al., *Enzymatic N-deacylation of sphingolipids.* Methods Enzymol, 2000. **311**: p. 297-303.
94. Kurita, T., H. Izu, M. Sano, et al., *Enhancement of hydrolytic activity of sphingolipid ceramide N-deacylase in the aqueous-organic biphasic system.* J Lipid Res, 2000. **41**(5): p. 846-51.
95. Kita, K., T. Kurita, and M. Ito, *Characterization of the reversible nature of the reaction catalyzed by sphingolipid ceramide N-deacylase. A novel form of reverse hydrolysis reaction.* Eur J Biochem, 2001. **268**(3): p. 592-602.
96. Mills, K., A. Johnson, and B. Winchester, *Synthesis of novel internal standards for the quantitative determination of plasma ceramide trihexoside in Fabry disease by tandem mass spectrometry.* FEBS Lett, 2002. **515**(1-3): p. 171-6.
97. Mills, K., S. Eaton, V. Ledger, et al., *The synthesis of internal standards for the quantitative determination of sphingolipids by tandem mass spectrometry.* Rapid Commun Mass Spectrom, 2005. **19**(12): p. 1739-48.
98. Furusato, M., N. Sueyoshi, S. Mitsutake, et al., *Molecular cloning and characterization of sphingolipid ceramide N-deacylase from a marine bacterium, Shewanella alga G8.* J Biol Chem, 2002. **277**(19): p. 17300-7.
99. Hulkova, H., M. Cervenkova, J. Ledvinova, et al., *A novel mutation in the coding region of the prosaposin gene leads to a complete deficiency of prosaposin and saposins, and is associated with a complex sphingolipidosis dominated by lactosylceramide accumulation.* Hum Mol Genet, 2001. **10**(9): p. 927-40.
100. Philippart, M., L. Sarlieve, C. Meurant, et al., *Human urinary sulfatides in patients with sulfatidosis (metachromatic leukodystrophy).* J Lipid Res, 1971. **12**(4): p. 434-41.

Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a příjmení s adresou	Číslo OP	Datum vypůjčení	Poznámka