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Summary of Ph.D. thesis

Tandem mass spectrometry of sphingolipids with application for metabolic studies and diagnosis of sphingolipidoses

Mgr. Ladislav Kuchař

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Board chairperson: Prof. MUDr. Stanislav Štípek, DrSc.

Supervising department: Institute of Inherited Metabolic Disorders

Supervisor: RNDr. Jana Ledvinová, CSc.

Consultant: RNDr. Befekadu Asfaw, CSc.

The thesis will be available at the Department of Science and Research and International Relations of the Dean's office of the First Faculty of Medicine at least five days before the date of defense.

Abstrakt

V posledních letech se hmotnostní spektrometrie (MS) stala dominantní technologií používanou při lipidomické analýze a silně ovlivnila výzkum a diagnostiku onemocnění lipidního metabolismu jako např. lysosomální střádavá onemocnění (LSD) charakterisovaná poruchami funkcí lysosomů. Soubor poruch lysosomální degradace sfingolipidů (SFL) přísluší ke skupině sfingolipidos. Tento stav má vážné až fatální klinické důsledky.

Prvotním cílem práce bylo zavedení kvantitativní a kvalitativní analýzy SFL pro výzkum a diagnostiku LSD. Na jejím počátku bylo třeba připravit semisynthesou lipidní hmotnostně značené standardy pomocí imobilizované sfingolipid ceramid N-deacylasy. Zavedené metody kvantitativní analysy byly posléze použity k průkazu zvýšené exkrece močových SFL u LSD s charakteristickým střádáním v ledvinách. Vyhodnocení SFL vylučovaných močí prokázalo svůj význam při diferenciální diagnostice deficitu prosaposinu a saposinu B kdy rutinní enzymologie selhává. MS navíc umožňuje sledování jednotlivých molekulárních druhů SFL (isoforem), jenž vedlo ke zjištění, že u některých LSD se jejich profil v moči mění. To následně vedlo k vývoji nové screeningové metody v suchém vzorku moče založeném na vyhodnocování profilu isoforem. Další aplikací MS byla analýza pitevních vzorků tkání nebo buněk u nevyjasněných případů. Fabryho choroba a prosaposinový deficit byly prokázány také analýzou pitevních vzorků ledvin a myokardu, což potvrdilo praktický význam takového postupu. V myokardu pacienta s Fabryho chorobou bylo také prokázáno zvýšení toxického lyso-SFL. MS vyhodnocení SFL v placentě podpořilo nálezy imunohistochemické analýzy a společně ukázalo na specifickou roli apikálního pólu placentálních endothelií. MS analýza se ukázala užitečná nejen při analýze metabolitů, ale též při měření aktivit lysosomálních enzymů, neboť umožňuje používání přirozených substrátů na rozdíl od fluorimetrických metod. Použitím MS se nám podařilo prokázat nulovou aktivitu β-glukocerebrosidasy v kožních fibroblastech pacienta s Gaucherovou chorobou typu II se závažným "collodion baby" fenotypem. Možnost používat hmotnostně značené substráty při dynamických metabolických experimentech namísto běžně užívaných radioaktivních lipidů byla testována v kulturách kožních fibroblastů pacientů s GM1 gangliosidosou. Bylo prokázáno, že hmotnostně značené substráty jsou vhodnou náhradou radioaktivních analogů, což přispívá k eliminaci rizik při práci s radioaktivními sloučeninami.

V rámci této disertační práce bylo zavedeno široké spektrum metod, které byly otestovány při určování metabolických profilů SFL za normálních a patologických stavů. Naše zjištění potvrdila, že MS lipidomika přináší novou, vyšší úroveň citlivosti analýzy i množství dalších detailních informací. Sledování metabolického osudu jednotlivých molekul může přispět k lepšímu pochopení molekulárních mechanismů onemocnění.

Abstract

In recent years, mass spectrometry (MS) become the dominant technology in lipidomic analysis and widely influenced research and diagnosis of diseases of lipid metabolism, e.g. lysosomal storage disorders (LSD) characterized by impairment of the lysosomal functions. Defects in lysosomal processing of sphingolipids SFL belong to the category of sphingolipidoses. This condition has severe and even fatal clinical outcome.

The primary aim of this work was to establish quantitative and qualitative methods of SFL analysis useful for research and diagnosis of LSD. At first, semisynthesis of mass labeled lipid standards utilizing immobilized sphingolipid ceramide N-deacylase was performed. Established methods of quantitative analysis were then used to prove the increased excretion of urinary SFL in LSD with characteristic storage in the kidney. Determination of excreted urinary SFL was found useful for differential diagnosis of prosaposin and saposin B deficiences for which routine enzymology is failing. MS also enabled monitoring of individual molecular species (isoforms) of SFL, which led to the finding that their urinary pattern is changing in some LSD. This resulted in the development of new screening method in dry urinary samples based on isoform profile evaluation. Another MS application referred to analysis of autoptic tissues or cell samples in unresolved cases. Fabry disease and prosaposin deficiency were proved in the autoptic kidney and myocardium which showed the usefulness of this procedure. In the myocardium of Fabry patient, the increase of toxic compound, lyso-SFL was also demonstrated. MS determination of placental SFL supported immunohistochemical analysis and thus pointed to the specific features of placental endothelial apical pole. In addition to metabolites, MS was found very useful for determination of activities of lysosomal enzymes because of use of natural substrates in contrast to fluorimetric methods. Using MS, we were able to demonstrate zero β-glucocerebrosidase activity in skin fibroblasts of Gaucher type II patient with severe collodion baby phenotype. The possibility to use the mass labeled substrates in dynamic metabolic experiments instead of conventional radioactive ones was tested in cultured skin fibroblasts from patients with GM1 gangliosidosis. Mass labeled substrates were found suitable substitutes eliminating the working risk with radioactive compounds.

While working on this Ph.D. thesis, the wide range of methods has been introduced and tested to determine metabolomic profiles of SFL under normal and pathological conditions. Our findings have confirmed that lipidomic MS brings a new, high level of sensitivity and more detailed information. Monitoring of metabolic fate of individual molecules can contribute to better understanding of the molecular mechanisms of the disease.

Klíčová slova:

tandemová hmotnostní spektrometrie, hmotnostní spektrometrie, sfingolipid, lipidomika, lysosom, lysosomální střádává onemocnění, sfingolipidosy, enzym, immobilizace

Key words:

tandem mass spectrometry, mass spectrometry, sphingolipid, lipidomics, lysosome, lysosomal storage disorders, sphingolipidoses, enzyme, immobilization

Abbreviations:

SFL – sphingolipids, LSD – lysosomal storage disorders, ESI – electrospray ionization, FIA – flow injection analysis, MS – mass spectrometry, MS/MS – tandem mass spectrometry, SCDase – sphingolipid ceramide N-deacylase, MMB – magnetic macroporous bead, IPN – isoform profile number, MLD – metachromatic leukodystrophy, Psap-d/pSap-d – prosaposin deficiency, SapB-d – Saposin B deficiency

Content:

1. Introduction

In recent years, increased interest in investigation of metabolism and biological functions of sphingolipid (SFL) biomolecules helped to develop sensitive and accurate methods for their analysis. One of the leading methods, tandem mass spectrometry (MS/MS), fulfils the requirements and provides high selectivity and sensitivity, even using crude lipid extracts. Identification of various molecular species (isoforms) of individual SFL classes in different biological material is an additional, but important, advantage (Grosch S. et al., 2012; Han X. and Gross R. W., 2005; Mills K. et al., 2005; Wennekes T. et al., 2009).

Lysosomal storage disorders (LSD) are caused by defects in genes encoding lysosomal acid hydrolases, their protein activators or other lysosomal proteins, e.g. transporters. Nowadays around 50 diseases are known including a number of sphingolipidoses caused by defects in the catabolic pathway of sphingolipids. These defects lead to the accumulation of non-degraded substrates in the lysosomal compartment resulting in the manifestation of severe, often fatal disease (Kolter T. and Sandhoff K., 2010)

Mass spectrometry (MS) is an analytical method based on measurement of mass to charge ratio of molecular ions in gaseous state (Cole R. B., 2010; de Hoffman E. and Stroobant V., 2002).

MS/MS combines two mass analyses and fragmentation of ions. Therefore, this two dimensional analytical technique has a higher selectivity and sensitivity than MS (de Hoffman E. and Stroobant V., 2002; Sleno L. and Volmer D. A., 2004). MS/MS of sphingolipids uses some of the specific fragments derived from characteristic sphingolipid structures (Han X. and Gross R. W., 2005; Murphy R. C. et al., 2001).

This study focuses on the contribution of MS/MS to sphingolipidomics and its applications in research and diagnostics of inherited lysosomal storage disorders.

2. AIMS

Main objective of this work was to introduce MS/MS as a modern analytical method in sphingolipidomics. This study focuses on the latest techniques in this field and on the development of advanced methods of sphingolipid analysis which would be useful for research and diagnostics of LSD.

Outlines of the study include:

A. Biosynthesis of SFL isoforms labeled by specific fatty acid to be used in MS analysis

B. Analysis of urinary SFL with applications for diagnosis of LSD

C. Sphingolipid analysis in cells and tissues

D. *In situ* and *in vitro* enzymology in LSD utilizing MS/MS

3. Material and Methods

The material analyzed in this study originated from various biological sources e.g. urine, cultured cells and tissue samples from patients with various LSD and from controls. The methods used were mostly based on the extraction of lipids according to Folch (Folch J. et al., 1957) followed by quantitative or qualitative MS analysis via electrospray ionisation tripple quadrupole tandem mass spectrometry (ESI-MS/MS).

The whole range of methods used in this study is described in detail in published articles related to the topic of this Ph.D. thesis. Therefore, only a brief overview follows:

a. ***For preparation of specific SFL,*** Sphingolipid ceramide N-deacylase (SCDase) was immobilized on magnetic macroporous bead (MMB) cellulose via covalent bond using standard protocol (Bilkova Z. et al., 2005). Method utilized formation of the Shiff base between aldehyde on cellulous particles and amino groups of enzyme protein stabilized by reduction of Schiff base using $NaCNBH_3$ (Hermanson G. T., 1996). Specific SFLs with atypical (C17:0) fatty acids were semisynthetised from their deacylated precursors (lyso-SFL) by reverse reaction of immobilized SCDase (Kita K. et al., 2001).

b. ***Methods of SFL extraction from biological material*** were selected according to the source of material.

For direct extraction of urinary SFL, the Folch extraction method utilizing mixtures of chlorofom and methanol was used (Folch J. et al., 1957).

For preparation and processing of dry samples of urinary sulfatides, DEAE membrane was immersed in a thoroughly mixed urine. Selectively captured sulfatides were then eluted by ammonium acetate in methanol and processed as reported (Kuchar L. et al., 2013).

Cultivated cells were extracted with chloroform-methanol mixture as previously described (Asfaw B. et al., 1998). The organic and water layers were then separated and collected for further analysis depending on the class of analyzed sphingolipids.

Homogenates of tissue samples were sequentially extracted by mixtures of chloroform, methanol and water in various proportions (Natomi H. et al., 1988). The methanol-water upper phase with more hydrophilic sphingolipids (e.g. gangliosides) and organic lower phase containing hydrophobic lipids (ceramides, sulfatides, Gb3Cer etc.) were filtered and used for analysis.

c. ***In vitro measurement of β-glucocerebrosidase activity*** was performed according to a slightly modified Turecek´s method (Turecek F. et al., 2007). 0.05% inactivated bovine serum albumin (BSA) was added to the reaction mixture containing 0.5 μg of the sample protein (cellular homogenate) to stabilize the enzyme.

d. ***In situ metabolic experiments*** were performed on living cells. Mass labeled (or tritium labeled) GM1 ganglioside dissolved in DMSO and mixed with DMEM medium was added to cell cultures of human skin fibroblasts. Cells were then cultivated for a defined number of days with the addition of conduritol-B-epoxide (inhibitor of acid β-glucocerebrosidase) in the cultivation medium as previously described (Asfaw B. et al., 1998)

e. ***For MS/MS analysis of sphingolipids,*** appropriate aliquots of lipid extracts were mixed with internal standards and dissolved in ammonium formiate to produce $[M+H]^+$ ions in the positive ion mode or in methanol to generate $[M-H]^-$ ions in the negative ion mode. Analysis was done on triple quadrupole tandem mass spectrometer (AB/MDS SCIEX 3200 or 4000) under previously

optimized conditions. Lipids were measured using a flow injection analysis (FIA) in the methanol mobile phase. Internal standard method using one concentration point was used for quantification (Kuchar L. et al., 2012).

4. Results

A. Biosynthesis of sphingolipid isoforms labeled by specific fatty acids

The sphingolipids containing C17:0 fatty acid were prepared by reverse enzymatic reaction of SCDase bound to MMB cellulose. Reaction gave a high yield of enzymatically converted lyso-derivatives: 80% for sulfatide >90% for glucosylceramide and GM1 ganglioside. Also isoform purity of products was high (97%) in comparison with previously used soluble non-immobilized enzyme (36%).

B. Analysis of urinary sphingolipids for diagnostic purposes

Pitfalls of the quantification of urinary sphingolipids

So far, the quantity of urinary sphingolipids has been measured and normalized using creatinine or, less often using urinary volume. Comparison of both normalizing parameters is shown on Fig. 1.

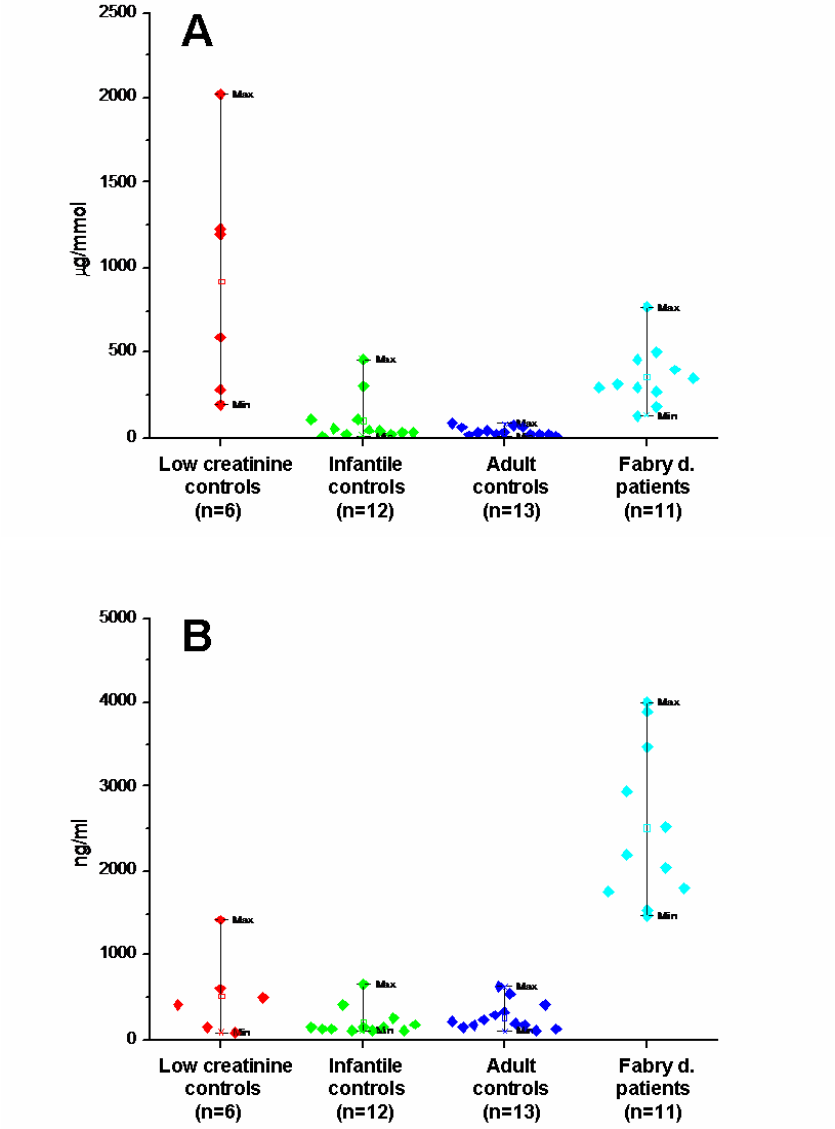

Fig. 1 Urinary Gb3Cer normalized by urinary (A) creatinine (B) volume
controls with low creatinine: creatinine ≤1 mM; infantile and adult controls with creatinine within a normal range: creatinine >1 mM-15 mM; Fabry patients: creatinine was within a normal range

Data presented on Fig. 1A showed overlap of control and patient groups. Therefore, this method is not suitable for diagnostic purposes and either urine volume (Fig. 1B) or sphingomyelin ought to be used as more reliable normalizers of SFL concentration in urine.

Quantity of urinary sphingolipids

The measurement of the quantity of three diagnostically most important urinary SFL is sumarized in Tab. 1. Increased excretion of relevant SFL in urines of LSD patients normalized by urinary sphingomyelin is clearly evident.

Tab 1. Quantity of selected urinary sphingolipids

	Lipid values[a] expressed as μg/100 μg sphingomyelin		
	Sulfatide	**Globotriaosylceramide**	**Ceramide**
Patient 1 (pSap-d) 44-day-old	67[c]	208[c]	17[c]
Patient 2 (SapB-d) 50-month-old	145[c]	51[c]	6.3
Metachromatic leukodystrophy 1- to 5-year-old (n = 6)	120[c] 38[d]	8.8 3.3	3.8 1.1
Fabry disease males 24- to 54-year-old (n = 10)	6.8 2.5[d]	201[c] 102	4.8 1.7
Infantile/late-infantile controls 0.5- to 12-year-old (n = 16)	14 5.2[d]	15 8.2	4.3 1.8
Adult controls males and females[b] 17- to 60-year-old (n = 12)	9.7 2.5[d]	21 14	5.8 2.8

[a]Mean of three determinations for patients 1 and 2. For the analytical reproducibility, see Patients and Methods Section.
[b]Fabry carrier status was excluded in control females molecularly.
[c]Statistical significance $P < 0.001$.
[d]Standard deviation.
[e]Statistical significance $P < 0.01$.

prosaposin deficiency (pSap-d), saposin B deficiency (SapB-d)

Also, the percentage distribution of urinary SFL is diagnostically informative and shows a characteristic decrease in sphingomyelin and conversely, an increase of relevant SFL in all examined LSD (Kuchar L. et al., 2009)

Search for novel urinary sphingolipid biomarkers: the development of a new method using DEAE membrane

Examination of detailed composition of molecular species (isoforms) of urinary SFL (Fig. 2) revealed a shift of the sulfatide isoform pattern to species with longer chain fatty acids (carbon length from 22 to 24) in urines of patients with metachromatic leukodystrophy (MLD), prosaposin deficiency (pSap-d, Psap-d) and Sap-B deficiency (SapB-d), and similarly of the Gb3Cer species in Fabry disease (Kuchar L. et al., 2012).

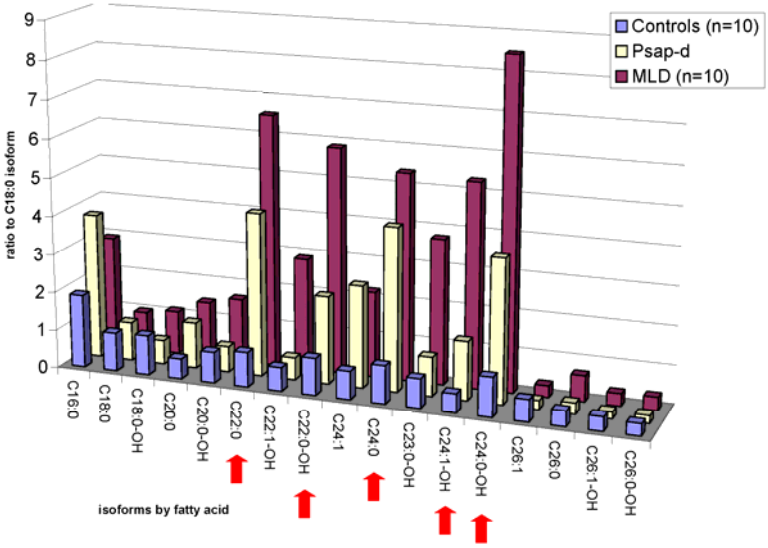

Fig. 2 Comparison of urinary sulfatide isoform profiles from MLD and prosaposin deficiency (Psap-d) patients and controls. Arrows indicate components undergoing major changes.

Urinary sulfatides bound on DEAE membrane were eluted and MS/MS analyzed. Ratio of major elevated sulfatide isoforms toward the C18:0 isoform was defined as an isoform profile number (IPN) and used as a new MLD biomarker (Fig. 3).

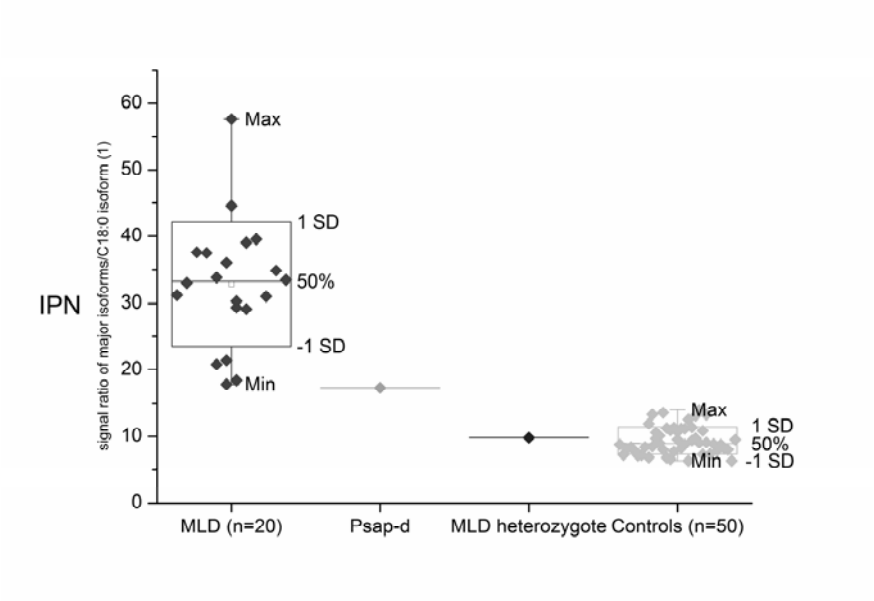

Fig. 3 IPN for MLD and Psap-d patients, MLD heterozygote and controls

Sulfatides bound to DEAE membrane were long time stable at laboratory temperature even after 100 days of storage.

C. Sphingolipid analysis in cells and tissues

Sphingolipids in cultured skin fibroblasts

Quantity of selected SFL in cultured skin fibroblasts is presented in Tab. 2. showing the increase in lipids relevant to the specific LSD.

Tab. 2 Sphingolipids in cultured skin fibroblasts

	Cer	**CMH**	**CDH**	**Gb3Cer**	**SM**
Prosaposin def.	**34,48**	**14,27**	**25,75**	**27,06**	45,70
saposin B def.	6,87	1,44	1,86	**21,36**	107,33
Fabry disease	3,76	1,89	2,10	**35,68**	42,62
Nieman-Pick A	4,15	1,67	5,63	0,51	**195,17**
Control 1	6,21	3,25	5,17	0,52	68,28
Control 2	1,18	2,67	1,37	5,17	21,04

concentrations are in ng/µg protein

Cer - ceramide; CMH - ceramide monohexoside; CDH - ceramide dihexoside; Gb3Cer - globotriaosylceramide; SM - sphingomyelin

Sphingolipids in human kidney and myocardium

Post-mortem analysis of kidney tissue samples revealed accumulation of nondegraded SFL in Fabry disease (Gb3Cer) and in pSap-d (all simple sphingolipids; Tab 3).

Tab. 3 Sphingolipids in the human kidney (autoptic samples)

	Cer	**CMH**	**CDH**	**Gb3Cer**	**sulfatide**
Fabry disease	0,5	1,0	3,7	**115,2**	0,5
Prosaposin def.	**39,1**	**23,9**	**49,8**	**57,6**	**39,6**
Control (n=3)	11,2	0,7	3,2	10,2	1,1

concentrations are in ng/µg protein

Cer - ceramide; CMH - ceramide monohexoside; CDH - ceramide dihexoside; Gb3Cer - globotriaosylceramide

In the Fabry myocardium, an increase in the toxic deacylated derivative lyso-Gb3Cer was found (0,7 vs 0,2 ng/mg protein) in addition to the massive storage of major compound, Gb3Cer (286,6 vs 1,4 ng/mg protein).

Sphingolipids in human placenta

Mass spectrometry identified Gb3Cer and GM3 ganglioside as the major neutral and acidic SFLs in the placental tissue. Another sialylated glycolipids, sialylparagloboside (SPG) and GM1 ganglioside were identified in one SPG+GM1 fraction. Concentrations of measured sphingolipids are reported in Tab. 4.

Tab 4 - Placental tissue sphingolipids,

	Gb4Cer	Gb3Cer	CDH	CMH	SPG + GM1	GM3
C1	12.8	27.1	27.8	22.7	7.0	51.9
C2	11.5	32.1	15.2	16.7	5.4	37.6
C3	19.1	20.4	15.1	20.2	4.3	50.1
C4	15.9	35.1	12.5	23.2	4.5	51.6
C5	13.5	35.3	16.0	19.2	N.Q.	N.Q.

SPG - sialylparagloboside ($IV^3NeuAc\text{-}nLc_4Cer$)
concentrations are in pmol/nmol of sphingomyelin
C1-C5 - five different control placentas

D. *In vitro* and *in situ* enzymology in LSD utilizing tandem mass spectrometry

Lysosomal enzyme activities in cell homogenates demonstrated on Gaucher disease

Determination of lysosomal β-glucocerebrosidase activity using natural substrate (glucosylceramide with C12:0 fatty acid) in fibroblasts homogenates revealed zero activity in Gaucher patient with severe "collodion baby

phenotype" and small, but measurable residual activities in fibroblasts from the other patients with type I and type II Gaucher disease (Fig. 4).

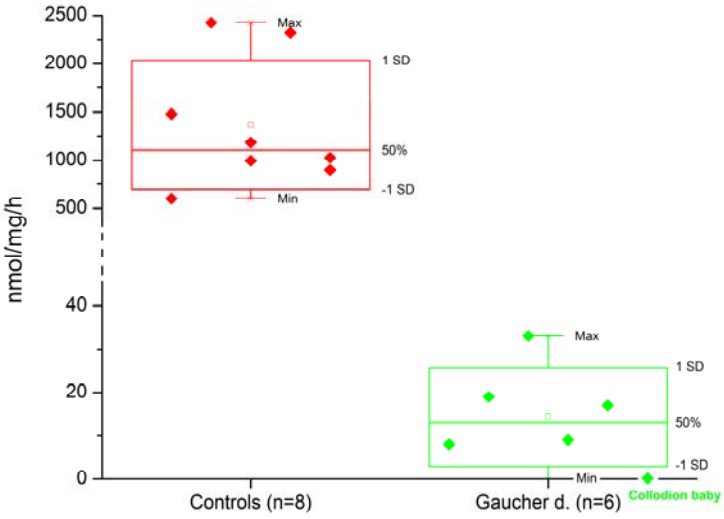

Fig. 4 *In vitro* activity of lysosomal acid β-glucocerebrosidase in Gaucher and control fibroblasts analyzed by FIA-ESI-MS/MS

In situ loading experiments in skin fibroblast cultures: monitoring the sphingolipid degradation pathways

Loading experiments (dynamic metabolic experiments, feeding experiments) utilizing mass labeled and radiolabeled substrates were performed in model system of fibroblast cultures from patients with GM1 gangliosidosis and controls as it is shown on Fig 5.

This experiment clearly identified the defect in degradation of GM1 ganglioside. Comparison of residual activities from late-onset forms (b,c,d,e) and severe infantile forms of the disease (f,g) with normal activity of healthy control (a) is demonstrated on Fig. 5B.

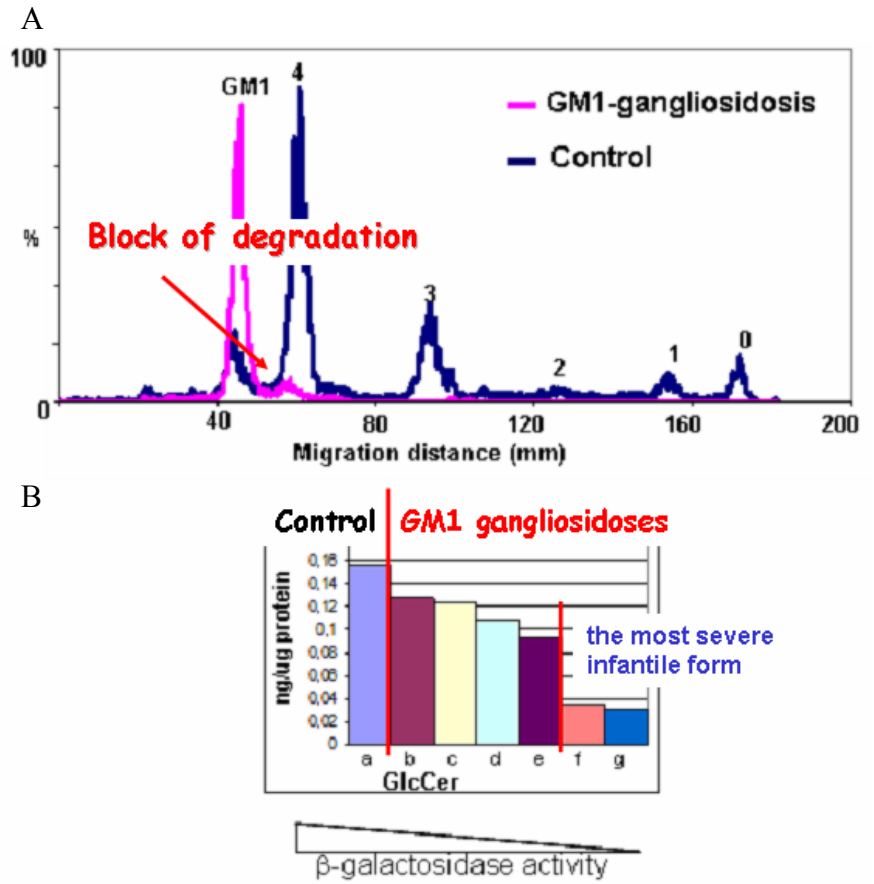

Fig 5. Results from dynamic metabolic experiments utilizing (A) radiolabeled or mass labeled (B) substrate

A - Degradation pattern of [^{3}H]GM1 ganglioside in control and β-galactosidase-deficient cell evaluated by HPTLC and radioscanner . 0 to 4 = degradation products of GM1 ganglioside,
B - MS/MS quantification of GlcCer product formed from loaded stable isotope-labeled GM1 ganglioside (C18:0-D_3). GlcCer degradation was inhibited with CBE.
Clinical phenotypes: a-control, b-adult GM1 gangliosidosis, c-Morquio B, d-adult GM1 gangliosidosis/Morquio B, e-juvenile GM1 gangliosidosis, f-infantile GM1 gangliosidosis, and g-infantile GM1 gangliosidosis

5. Discussion

A. Biosynthesis of sphingolipid isoforms labeled by specific fatty acids

Reusable system for enzymatic semisynthesis of specific isoforms of SFL based on immobilization of SCDase on MMB cellulose particles was developed (Kuchar L. et al., 2010; Kuchar L. et al., 2012). The main advantage of immobilized enzyme over its soluble form is the high isoform purity of products. Reusability and long-term stability (system remained active after 15 reuses and 1,5 year of storage without loss of activity), high rate of conversion of lyso-SFL to acylated products and ease of operation are further advantages of the procedure (Kuchar L. et al., 2010) in comparison with other reported methods (Fauler G. et al., 2005). This system has a universal application for the preparation of SFL specifically labeled in the fatty acid moiety and is useful for studies in various fields of SFL biochemistry. Using this method we have prepared atypical SFL isoforms suitable as internal standards for MS/MS C17:0 isoforms of sulfatide, GlcCer and GM1 ganglioside (Hulkova H. et al., 2012; Kuchar L. et al., 2010).

B. MS/MS analysis of urinary sphingolipids and its application for diagnosis of LSD

Laboratory diagnosis should be always focused on utilization of non invasive biological material. Therefore, we intended to develop a simple and reliable method for assessing urinary sphingolipid markers typical for LSD.

Pitfalls of urinary sphingolipids quantification

First, it was necessary to find a suitable normalization parameter for lipids in urine. Evaluation of commonly used reference factors for the expression of lipid concentration excluded creatinine for biased results at its low values (see

Fig. 1A) (Kuchar L. et al., 2012). Criticism of this method of evaluation has been already published (Forni S. et al., 2009; Kuchar L. et al., 2012) but so far it has no significant impact. To improve this situation we recommend to replace the creatinine normalizer by urinary volume or by sphingomyelin, the major membrane SFL as more reliable parameters (Berna L. et al., 1999; Kuchar L. et al., 2009; Kuchar L. et al., 2012).

Quantity of sphingolipids in urine

Quantitative analysis of elevated urinary sphingolipids, e.g. Gb3Cer, dihexosyl- and monohexosylceramides, sulfatides and ceramides is important pre-diagnostic step in some sphingolipidoses (Kuchar L. et al., 2009; Kuchar L. et al., 2012). In particular, it concerns those that are caused by defects of activator proteins where standard enzyme assays fails (Sandhoff K. et al., 2001)

Massive excretion of nondegraded lipids reliably indicated MLD, Fabry patients and both saposin deficiences. Ceramide was elevated only in pSap-d and may thus serve as a marker for distinguishing SapB-d and pSap-d (Kuchar L. et al., 2009).

Percentage distribution of urinary sphingolipids also gives diagnostic information with the advantage that normalizing parameter is not necessary. Decrease of sphingomyelin and increase of percentage of critical lipids in urine is characteristic for all studied LSD (Kuchar L. et al., 2009).

Changes in the pattern of sphingolipid urinary isoforms: new markers for diagnosis of MLD, Fabry disease and saposin B deficiences

Shift in the Gb3Cer isoform pattern to species with longer chain fatty acids (C22:0, C24:0 and C24:1) in urines of patients with Fabry disease was reported (Kuchar L. et al., 2012; Paschke E. et al., 2011). We found similar shift in the

molecular pattern of sulfatides in MLD as well as in pSap-d and SapB-d (Kuchar L. et al., 2012). Ratio of major elevated sulfatide isoforms toward the C18:0 isoform was defined as isoform profile number (IPN) and was used as a new MLD biomarker (Kuchar L. et al., 2013).

This may be explained by changes in cellular types in the urinary sediment of LSD patients, because lipid laden kidney epithelial cells are preferentially desquamated. However, in normal urine, urothelial cells are the main cell types (Kuchar L. et al., 2013; Warnock D. G. et al., 2010).

New method based on the selective capture of sulfatides by DEAE membrane and facilitating the transport of liquid urine samples has been developed and tested on 50 controls and 21 patients with no false negative or false positive results. Sulfatides bound to DEAE membrane were long time stable at laboratory temperature. All this indicates that this method has good potential to be used for screening of MLD (Kuchar L. et al., 2013).

C. Analysis of sphingolipids in human tissues and cells - contribution to general knowledge of LSD pathology

Sphingolipid analysis in tissues and cells may have the specific diagnostic applications. In this respect, the MS/MS technique was found to be very effective for the diagnosis of unsolved cases. For example, analysis of SFL in the autoptic kidney samples, skin fibroblasts and some other tissues confirmed the diagnosis of Fabry disease and pSap-d, later verified by DNA analysis (Kuchar L. et al., 2012). Analysis of Fabry myocardium showed - in addition to the massive accumulation of Gb3Cer, small increase in the concentration of toxic lyso-derivative which may contribute to the manifestations of disease (Kuchar L. et al., 2012). These derivatives are supposed to have important regulatory and signaling functions but their role in the lysosomal pathology has not yet been fully elucidated (Aerts J. M. et al., 2008).

In the placenta, GM1 ganglioside was for the first time reported (Hulkova H. et al., 2012). MS identified Gb3Cer and GM3 ganglioside as the most abundant SFL. The presence of Gb3Cer, GM1 and GM3 gangliosides and cholesterol along with caveolin 1 immunohistochemically demonstrated in the placental blood villous capillaries suggests the existence of caveola-associated microdomains (rafts) (Pang H. et al., 2004; Parton R. G., 1994) at the apical pole of endothelial cells.. This represents a unique finding pointing to specific features of endothelial apical pole of placental villous capillaries and is open for further investigation (Hulkova H. et al., 2012).

D. *In vitro* and *in situ* enzymology in LDS utilizing tandem mass spectrometry

Tandem mass spectrometry applications for in vitro enzymology

MS was found suitable for determination of activities of lysosomal enzymes because it can use natural substrates. Using MS/MS and C12:0GlcCer substrate, we were able to evaluate residual β-glucocerebrosidase activities in fibroblasts of both Gaucher types (I and II) and to confirm zero activity in Gaucher Type II-collodion baby phenotype (Kuchar L. et al., 2012). It refines previous observations based on artificial substrate only (Finn L. S. et al., 2000).

In situ monitoring the sphingolipid degradation pathways by MS/MS

Loading experiments in cell cultures (also called feeding experiments) are frequently used to track the metabolic fate of labeled exogenous compounds in living model systems (Asfaw B. et al., 2002; Leinekugel P. et al., 1992; Martin O. C. and Pagano R. E., 1994; Sonderfeld S. et al., 1985). The main advantage of such experiments is that they assess the dynamics of the entire apparatus of

living cells, including nonenzymatic cofactors e.g. like saposins which are not detectable by routine enzymology.

Results from loading experiments utilizing mass- and radiolabeled substrates in GM1 gangliosidosis fibroblast cultures as a model system clearly showed hindered degradation of critical glycosphingolipids. Mass labeled substrates are therefore considered as possible substitutes of radiolabeled analogues. Their application increases the accuracy of quantification and eliminates the risk when working with radioactive substances (Kuchar L. et al., 2012).

6. Conclusions

We have introduced MS/MS methods of complex sphingolipid analysis in various biological material related to research and diagnosis of LSD. In this study, the following objectives have been met:

- development of method of semisynthesis of mass labeled sphingolipid standards using immobilized SCDase and their use for qualitative and quantitative MS analysis (Hulkova H. et al., 2012; Kuchar L. et al., 2009; Kuchar L. et al., 2012)
- development of methods of quantitative SFL analysis in urine for diagnosis of several LSD (MLD, Fabry disease, pSap-d and SapB-d) (Kuchar L. et al., 2009; Kuchar L. et al., 2012)
- development of a new method for screening of MLD and saposin deficiences in dry urine samples using DEAE membrane on the principle of changes in the profile of sulfatide isoforms (Kuchar L. et al., 2013)

Applications of established MS methods were effective for tissue and cell analysis in unsolved LSD cases - (demonstrated on autopsy tissues from Fabry disease and prosaposin deficiency) (Kuchar L. et al., 2012).

MS and immunohistochemical analyses in the human placenta pointed to the specific features of placental endothelial apical pole of endothelial cells where the presence of Gb3Cer, GM1 and GM3 gangliosides and cholesterol along with caveolin 1 suggested the existence of caveola-associated microdomains (rafts). The presence of GM1 ganglioside was for the first time demonstrated in this tissue. (Hulkova H. et al., 2012)

MS/MS applications in enzymology *in vitro* showed an advantage of using natural substrates of lysosomal hydrolases and enables to more accurately assess residual activities (Kuchar L. et al., 2012)

Use of mass labeled substrates in metabolic experiments instead of conventional radioactive lipids was found advantageous due to accuracy of quantification, safety and simplification of analytical work (Kuchar L. et al., 2012)

In conclusion, MS/MS is a robust and sensitive analytical procedure efficient in determining the composition of endogenous sphingolipid classes in various biological materials and is effective in monitoring their metabolic fate. Its ability to establish metabolomic profiles of SFL under normal and pathological conditions in cells and tissues can contribute to a better understanding of the biological significance of sphingolipid molecules.

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Publications:

1A Publications in Impacted Journals related to the topic of this Ph.D. thesis

Kuchar L., Rotkova J., Asfaw B., et al., *Semisynthesis of C17:0 isoforms of sulphatide and glucosylceramide using immobilised sphingolipid ceramide N-deacylase for application in analytical mass spectrometry.* Rapid Commun Mass Spectrom, 2010. **24**(16): p. 2393-2399

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1B Non impacted publications related to topic of this Ph.D. thesis

Chapter in the Book

Kuchar L., Asfaw B., and Ledvinova J., *Tandem Mass Spectrometry of Sphingolipids: Application in Metabolic Studies and Diagnosis of Inherited Disorders of Sphingolipid Metabolism*, in *Tandem Mass Spectrometry - Applications and Principles*, Prasain, Editor 2012, InTech: Rijeka. p. 739-768. ISBN: 978-953-51-0141-3

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Kuchar L., Hlavatá J., Asfaw B., et al., *Changed isoform profiles in sphingolipid storage disorders: a new pre-diagnostic tool.* Int J Clin Pharm Th, 2010. **48**(Suppl. 1):p. S76

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2A Publications in Impacted Journals not related to topic of this Ph.D. thesis

Musalkova D., Lukas J., Majer F., et al., *Rapid isolation of lysosomal membranes from cultured cells.* Folia Biol (Praha), 2013. **59**(1): p. 41-46