Materials and methods: Mass spectrometry for the determination of phospholipids in cardiac tissue

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) combining HPLC separation on normal phase column with multiple parent-ion or neutral loss scans on a triple quadrupole mass spectrometer was used to analyze the following phospholipid (PL) classes: sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA) and ceramides. Tissue extracts were spiked with 10 µg/ml solution of internal standards (IS) and extracted.[1] The mixture of ISs contained non-naturally occurring lipids: PG(17:0/17:0), PG(17:1/0:0), PA(14:0/14:0), PA(17:0/0:0), PE 14:0/14:0, PE 17:1/0:0, PC14:0/14:0, PC24:0/24:0, PC 17:0/0:0, SM (d18:1/12:0) and Cer (d18:1/17:0) (Avanti Polar Lipids, Alabaster, AL, USA). Samples were mixed with methanol/chloroform (2:1, v/v) and vortexed. All lower phases were combined and evaporated to dryness under nitrogen. The remnant was reconstituted in 200 µl of a mixture of mobile phases 80% A and 20% B, and 10 µl was injected into the LC-MS system. LC-MS system contained a Rheos 2200 HPLC pump (Flux Instruments, Reinach, Switzerland), an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a TSQ Quantum Access triple quadrupole mass analyzer (Thermo Fisher Scientific, Waltham, MA, USA). All spectra and tandem spectra were automatically acquired by a customized sequence operated under Xcalibur software (Thermo Fisher Scientific). Separation of PLs was achieved by using a diol silica-based column (QS Uptisphere 6 OH, 150 x 2.1 mm, 5 µm). The composition of the mobile phase A

was a mixture of hexane/isopropanol/water (70:30:2, v/v) containing 15 mM ammonium formate and the mobile phase B was isopropanol/water (50:2, v/v) containing 15 mM ammonium formate. Gradient elution started with 80% mobile phase A and 20% of B and lasted for the first 7.0 min. Mobile phase B was increased to 40% within 1 min and kept constant for 2 min then mobile phase was increased to 60% within 1 min and kept constant for 12 min. After that the column was re-equilibrated with 80% mobile phase A and 20% of B for 5.0 min. The solvent flow was 0.35 ml/min. The MS parameters were set as follows: 4500 V spray voltage, skimmer voltage was constantly changing in range from 2 to 14 V depending on scan mode, 250°C capillary temperature, and 10 and 6 (arbitrary units) sheath and auxiliary gas, respectively. Tube lens voltages were tuned using a standard mixture of PC(17:0/0:0), PC(14:0/14:0), PC(20:0/20:0) and PC (24:0/24:0), covering a wide molecular mass range. Both Q1 and Q3 resolution were set at 0.7 peak-width (at full-width half-maximum). Multiple parent-ion and neutral loos scan events were performed in positive ionization mode by using 0.7 mTorr collision gas. A neutral loss of masses m/z 115 and 189 from $[M^{+}NH4]^{+}$ ions were used for analysis of PA and PG lipids, respectively. A precursor ion scan of m/z 184 specific for phosphocholine containing lipids was used for PC, SM and LPC. A neutral loss scan of m/z 141 was used for PE and a precursor scanning of m/z 264 was applied for screening of ceramides. Acquired data was analyzed using the Xcalibur software (version 2.0.6) from Thermo Scientific. The identification of the molecular species was based on molecular mass provided by MS, the knowledge about the structure of head group provided from the type of

MS/MS scanning and partly from LC retention time. From each scan segment, a mass-peak list containing parent-ion masses (m/z) and their intensities were generated by using Xcalibur. The mass-peak list from every scan segment was entered to the software LIMSA (The Somerharju Lipid Group, Helsinki, Finland) using a mass tolerance of ±0.7. The output of search gave information about the species, while lipid class was per-defined by type of MS/MS scan. Moreover, isotopic correction for species, which are different in one double bound that correspond to mass difference Δ m/z of two Da was also performed by LIMSA. As result all lipids were assigned to the lipid class and species with defined sum of carbon atoms and double bounds in O-linked fatty acids. Concerning PC, molecular species with odd number of carbon bounds can be attributed to PC species with odd number of carbon atoms in one of acyl chains or represent plasmalogens with vinyl ether linkage at sn1 position of the glycerol backbone. Identification of SM resulted in sum of carbon atoms in sphingoid base and N-linked fatty acid. Quantification was achieved by using ratio intensity to the IS of the lipid species with the same parent mass (m/z). Sensitivity of the method was defined as limit of quantification (LOQ), which was the lowest concentration at which target compounds could be analysed with sufficient accuracy and precision. LOQ was established for each PL class using commercially available standards of lipid species one per class. The established values of LOQs were 0.165 µmol/L for PC, 0.178 µmol/L for SM, 0.5 µmol/L for Cer and LPC, 0.8 µmol/L for PA, 0.3 µmol/L and 0.4 µmol/L for PG and PE, respectively. All CV% for standard measurements of LOQ were below 15%. Extraction recovery of ISs

from plasma varied from highest of 103% for PC to the lowest 68% for PA. Precision of the method was determined by six independent analyses of aliquots of one plasma sample. Precision of measurements of the total concentration of lipid classes was below 15%.

Materials and methods: Mass spectrometry for the determination of sphingoid bases in cardiac tissue

Tissue samples were homogenised in PBS before adding 0.2% Triton X-100 to the homogenates. The homogenates were slowly shaken on a rotary mixer in the cold room for 1 hour and then centrifuged at 16,000 g for 5 min at 4C. LC/MS analysis was performed as described earlier. Lipids were separated on a C18 column (Uptispere 120 Å, 5 µm, 125×2 mm; Interchim, Montluçon, France) and analysed by an MS detector (LCQ, Thermo, Wohlen, Switzerland). The samples were measured as singletons for each participant. Inter- and intra-assay coefficients-of-variation were between 5% and 20% for each sphingoid base.

References

1. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37: 911-7