Invited lecture/Research

Omics Mass Spectrometry Analysis of Canine Plasma

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Abstract:
Blood derived products, such as autologous plasma, have high clinical importance and are applied in numerous therapeutic fields. The preparation of autologous plasma from the patient's own blood is easy to perform by centrifugation, however, the preparation procedure can significantly affect the blood cells, platelets and vesicles in the sample. Therefore, it is of utmost importance to understand the impact of sample processing on the chemical composition of plasma preparations as well as on their biological activity. Here, we present a mass spectrometry-based plasma profiling method in which three compound groups: lipids, proteins and glycoproteins are analysed in a single workflow. Analysis of the chemical composition of plasma samples prepared by different centrifugation protocols revealed differences in the lipid and glycoprotein profiles, demonstrating the importance of standardized protocols for the preparation of plasma products.

Keywords: Plasma; Lipids; Proteins; Glycoproteins; Mass Spectrometry, Ion Mobility

1. Introduction

Analytical characterisation of complex biological samples is still a challenging and time-consuming task, especially if a comprehensive analysis is planned. Mass spectrometry (MS) is a leading analytical method for the chemical analysis of biological samples, as MS-based measurements can be easily combined with other analytical and separation techniques. For this reason, mass spectrometry has a key role in "omics" research, where the characterisation of different sets of biomolecules is performed.

Plasma can be produced from blood collected in tubes treated with an anticoagulant. The blood is then processed with centrifugation to remove cells and the autologous plasma is often used for therapeutic purposes (Troha et al., 2023). For example, the eradication of a wound in the middle ear was demonstrated after the application of autologous plasma (Božič et al., 2020). Nevertheless, the exact composition of human plasma varies from individual to individual and is largely affected by the production and storage process. It is therefore important to develop methods to characterise the molecular composition of plasma-derived products. The first step, the centrifugation procedure by which blood plasma is prepared has not yet been standardised.

In this work, the lipid, glycoprotein and protein content of blood plasma was investigated using a newly developed multi-step sample preparation combined with mass spectrometry. Dog (Canis lupus familiaris) plasma samples, subjected to two different centrifugation steps, were compared to detect possible chemical differences due to differences in the preparation.

2. Methods

2.1 Blood sampling

Blood was from expired transfusion bag (**Figure 1**). The donor was a 2 years old female dog without record of the disease. Before pouring the blood the content of the bag was homogenized by gently squeezing the bag. Blood was aliquoted into 3 ml tubes by gently pushing it through attached plastic tubes.

Figure 1. A bag of expired canine transfusion blood.

2.1 Preparation of plasma samples

Two different canine plasma sample sets were prepared by centrifugation at 2000 g for 5 min (at 30 °C), and by centrifugation at 4000 g for 30 mins (at 30 °C). 4-4 technical replicates were prepared for both samples.

2.2 Sample preparation for mass spectrometry

In the first step, lipids were extracted with the following protocol: 150 µL methanol was added to 20-20 µL plasma samples. After mixing, 500 µL methyl-*t*-butyl ether (MTBE) was added and the samples were incubated at room temperature for 1 hour under shaking at 350 rpm. 125 µL MilliQ water was added, and the incubation was repeated for 10 mins. Samples were centrifuged for 10 mins at 14800 rpm. The upper organic phases were collected, and the extraction of lipids was repeated by 500 µL MTBE. The combined organic

phases were vacuum dried. The lower aqueous phases, containing the proteins were further processed in the next steps. 6-6 µL of 0.2%-os RapiGest SF detergent solution were added to 100-100 µL aqueous phases, as well as 2 µL 10 mM dithiotreiol (DTT) solution. Samples were reduced at 60 °C for 30 mins. After cooling to room temperature, 15 µL 200 mM ammonium hydrogen carbonate solution and 2 µL 20 mM iodoacetamide solution were added and the proteins were alkylated for 30 mins in the darkness. Then, 1-1 µL of 0.1 mg/ml trypsin was added and the samples were incubated overnight 37 °C under gentle shaking. 3 µL of formic acid was added to quench the enzymatic reaction and the samples were vacuum dried. In the last step, glycopeptide enrichment was performed. Briefly, the samples were reconstituted in 50 µL of 1% formic acid and 500 µL ice-cold acetone was added to the samples and the samples were then kept overnight at -20 °C. Samples were centrifuged for 10 mins at 12000 rpm to isolate to pellet enriched in glycopeptides. The pellet and the supernatant were then separated and vacuum dried. Peptide solutions were further purified using Pierce™ C-18 Spin columns (the glycopeptide fractions were analysed without additional clean-up)

2.3 Mass spectrometry

Chromatographic separations were performed using a Waters Acquity I-Class UPLC System, while MS experiments were performed on a high resolution and high mass accuracy hybrid Q-TOF equipped with cyclic ion mobility separation cell (Waters Select Series Cyclic IMS, Waters Corporation) and an electrospray ionization Z-spray source. Single Lock Mass (leucin-enkephalin) was used for mass correction.

Lipid fractions were dissolved in 100 µL 50% A eluent / 50% B eluent mixture (*V*/*V*). Lipidomic LC-MS measurements were performed on an ACQUITY UPLC CSH C18 column (1.7 µm, 2.1x100mm) at 55 °C column temperature and 0.35 mL/min flow rate. Eluent A was 600/390/10 acetonitrile/water/1 M ammonium formate solvent mixture containing 0.1% formic acid (*V*/*V*). B eluent was 900/90/10 isopropanol/ acetonitrile /1 M ammonium formate mixture containing 0.1% formic acid (*V*/*V*). Gradient elution profile was the following: 0 min: 50% B, 0.5 min: 53% B, 4 min: 55% B, 7 min: 65% B, 7.5 min: 80% B, 10 min: 99% B. MS analysis was performed in negative ionization mode in the *m*/*z* 50-1200 range. Collision induced dissociation (CID) was performed in the 25-45 V collision voltage range. Single pass cyclic ion mobility separation was included in the measurements. Data were evaluated and lipids were identified by the Progenesis QI software.

Peptide fractions were dissolved in 25 µL 2% acetonitrile, 0.1% formic acid (*V*/*V*) solvent mixture. Proteomic LC-MS measurements were performed on an ACQUITY UPLC Peptide CSH C18 column (1.7 µm, 1x150mm) at 45 °C column temperature and 20 µL/min flow rate. Eluent A was 0.1% formic acid in water, B eluent 0.1% formic acid in acetonitrile (*V*/*V*). Gradient elution profile was the following: 0 min: 5% B, 1 min: 5% B, 45 min: 35% B, 46 min: 85% B. MS analysis was performed in positive ionization mode in the *m*/*z* 50-2000 range. Collision induced dissociation (CID) was performed in the 19-45 V collision voltage range. Data were evaluated and the proteins were identified by the ProteinLynx Global Server software.

3. Results

The objective of this work was the characterisation and comparison of blood plasma samples in a comprehensive way by a multi-step sample preparation workflow. The sample preparation was divided into three main steps: first, the lipid content of the plasma was isolated using MTBE as organic solvent. Then the lipid-depleted samples containing the plasma proteins were subjected to enzymatic cleavage by trypsin. The resulting peptide mixture was further fractionated. A fraction enriched in glycopeptides was isolated by ice-cold acetone precipitation and the peptide fractions depleted in glycopeptides were also isolated. All three fractions were analysed by UPLC-MS(/MS) and the compounds were identified by database search. Lipids were analysed using cyclic ion mobility separation[3] (cIM) to increase the number of compounds (e.g. isomers) that could be differentiated. **Figure 2**. shows the ion mobility heat map (drift time vs. retention time) of the lipids by LC-

MS under the optimized gradient elution. Using a single pass cyclic ion mobility separation, we were able to resolve several isomeric lipids, which otherwise elute at the same or highly similar retention times.

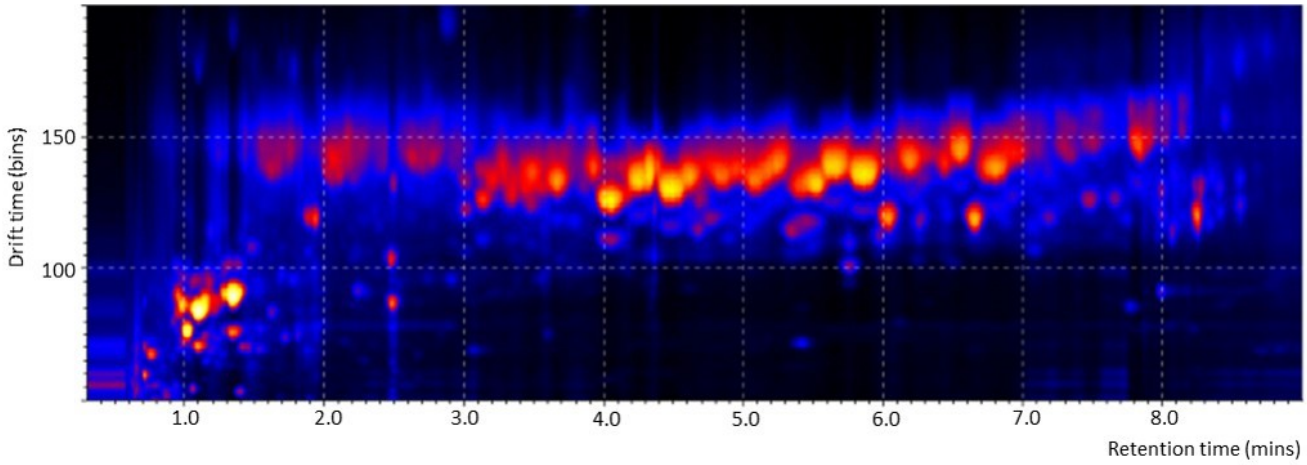

Figure 2. 2D LC-cIM-MS heat map (mobilogram) of the extracted lipids from canine plasma centrifuged at 2000 g for 5 min.

Main lipids were identified by database search. Analytical data and possible structures of the most intensive lipid species are summarized in Table 1. Note that in many cases, the exact structure of the lipids is ambiguous, due to identical masses and similar fragmenta tion patterns. Here, the collision cross sections (the drift times) are different. The most intensive species correspond to phosphatidylcholines (PC), sphingomyelins (SM) and ether-linked phosphatidyl-ethanolamines (PE). Under these conditions, formate adducts ($[M+FA-H]^-$) were dominant in the mass spectra.

Table 1. The 15 most intensive plasma lipids identified by LC-cIM-MS from canine plasma centrifuged at 2000 g for 5 min.

m/z	Retention time (min)	CCS (Å)	Formula	Possible lipids	Adduct type
747.5681	4.03	286.1	C39H79N2O6P	SM(34:1)	$[M+FA-H]^-$
750.5459	6.64	278.3	C43H78NO7P	PE(O-38:5)	$[M-H]^-$
802.5631	4.48	291.2	C42H80NO8P	PC(34:2)	$[M+FA-H]^-$
804.5766	5.48	291.2	C42H82NO8P	PC(34:1)	$[M+FA-H]^-$
826.5615	3.66	294.3	C44H80NO8P	PC(36:4)	$[M+FA-H]^-$
826.5624	4.23	295.4	C44H80NO8P	PC(36:4)	$[M+FA-H]^-$
828.5776	4.58	296.4	C44H82NO8P	PC(36:3)	$[M+FA-H]^-$
830.5932	5.86	297.5	C44H84NO8P	PC(36:2)	$[M+FA-H]^-$
832.6086	6.79	299.6	C44H86NO89	PC(36:1)	$[M+FA-H]^-$
854.5922	5.24	299.5	C46H84NO8P	PC(38:4)	$[M+FA-H]^-$
854.5932	5.6	300.5	C46H84NO8P	PC(38:4)	$[M+FA-H]^-$
856.6079	6.16	301.6	C46H86NO8P	PC(38:3)	$[M+FA-H]^-$
857.6759	7.81	307.8	C47H93N2O6P	SM(42:2)	$[M+FA-H]^-$
882.6243	6.53	308.7	C48H88NO8P	PC(40:4)	$[M+FA-H]^-$
885.5517	4.32	300.3	C47H83O13P	PI(38:4)	$[M-H]^-$

CCS: Collision Cross Section

The type of the major lipids was identical in the samples, however, comparison of the samples prepared by different centrifugation revealed differences in the intensity of specific lipid species. **Figure 3** shows the intensities of two lipid ions in the LC-MS chromatograms. While the intensity of PC(O-36:4) did not change, there is a remarkable decrease in the intensity of PE(O-38:5) due to centrifugation with elevated spin and longer time.

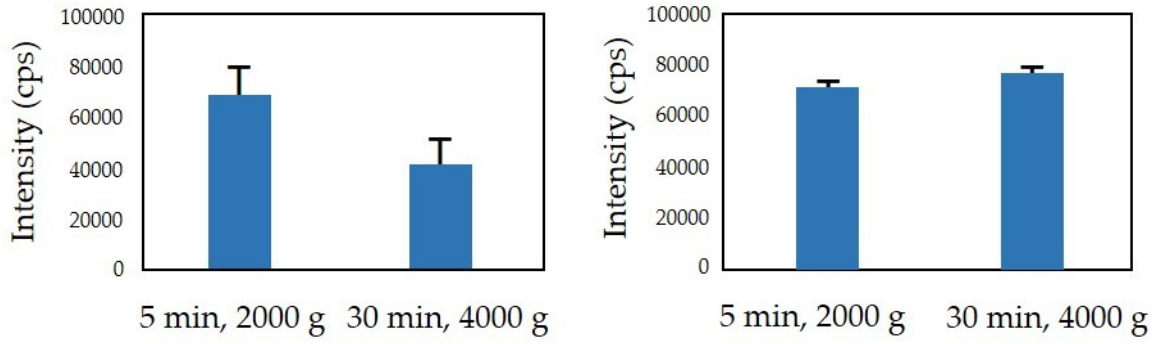

Figure 3. Effect of the centrifugation on plasma lipids. A.) Intensity of *m*/*z* 750.5459, PE(O-38:5); B.) Intensity of *m*/*z* 812.5822, PC(O-36:4).

Similarly, the protein and glycoprotein content of the samples were also compared. Protein contents of the samples were analysed after tryptic digestion. Fractions enriched and depleted in glycopeptides were prepared and proteins were identified by MS/MS peptide sequencing and database search. Database search revealed the presence of the most typical and abundant plasma proteins, including for example Apolipoproteins, Hemoglobin, Serum albumin, Haptoglobin and Fibronectin. While the peptide fraction did not show significant differences between the two sample groups, the intensity of the glycopeptides was increased in the plasma sample set which was centrifuged at higher spin and longer time (**Figure 4**). Based on glycopeptide marker ion intensities, larger spin and longer centrifugation time increased the intensity of glycopeptides, demonstrating that the relative ratio of glycoproteins increases in the prepared plasma. **Figure 4** shows the total intensity of the glycopeptide MS/MS fragment ions at *m*/*z* 366.14 for the samples prepared by different centrifugation protocols.

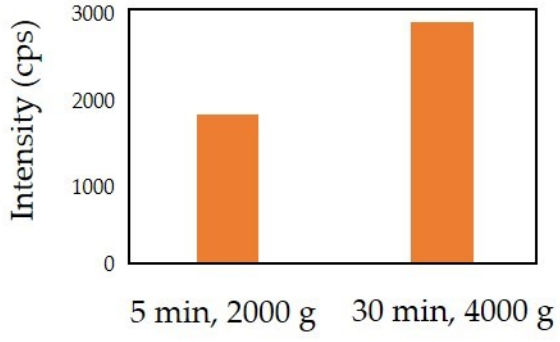

Figure 4. Effect of the centrifugation on plasma glycoproteins. Intensity of *m*/*z* 366.14 glycopeptide marker fragment ion in the MS/MS spectra of the LC-MS/MS chromatograms.

4. Discussion

LC-MS-based comparison of plasma samples produced by different centrifugation methods revealed differences in their chemical compositions. In the case of lipids, certain species showed significant decrease while other species showed increase based on their LC-MS intensities. Differences were also observed in the glycopeptide fraction obtained by tryptic digestion of differently centrifuged samples: longer centrifugation times resulted in a more intense glycopeptide pattern. Our results demonstrate that the centrifugation steps, used for the preparation of plasma products, changes the chemical composition. Therefore, it is essential to obtain more information on the effect of preparation protocols and the composition of plasma products in the future.

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Conflicts of Interest: The authors declare no conflict of interest.

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