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Development of innovative LC-MS/MS methods for the quantitation of disease biomarkers in biological fluids

PhD Thesis Summary

PhD Thesis developed and co-supervised in collaboration with the Laboratory for the analysis of Medicines, CIRM, University of Liège, Belgium

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Târgu Mureș, Liège 2019

One of the most important sources of biomarkers in the clinical settings represents the circulating and non-circulating endogenous or xenobiotic molecules. From a chemical point of view, this biomolecules are belonging to different chemical and biochemical classes. Thus for the proper identification and futher quantification of this biomolecules different analytical approaches are used in the clinical routine settings or in the medical or biomedical research. For routine analysis in clinical settings, analytical platforms that are based on Ligand Binding Assays, Immunoassays or ELISA-enzyme linked immunosorbent assays, Flow Cytometry and Immunohistochemistry are among the most encountered analytical methods. Even though many of the routine aforementioned analytical methods can be used for biomarker discovery experiments being capable of multiplex analysis, new complementary or alternative methods are beginning to find their places in the clinical laboratory. In this category, it is worth mentioning liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and capillary electrophoresis coupled to tandem mass spectrometry (CE-MS/MS).

The PhD Thesis entitled „Development of innovative LC-MS/MS methods for the quantitation of disease biomarkers in biological fluids" is structured in two main parts. The first part presents the current state of knowledge concerning the main analytical methods including LC-MS/MS (liquid chromatography-mass spectrometry) methods for the analysis of biomarkers in different pathologies. In addition, the pathological implications of acute renal failure, chronic kidney disease and the pathophysiology of thyroid function are discussed. At the same time, the main biomarkers that will be analyzed in the context of the various pathologies are summarized while underlining the main analytical methods known for the targeted molecules: iohexol, thyroid hormones (TH), human bradykinin and two peptide fragments. (B 1-9, B 1-8 and B 1-7) and the NGAL (Neutrophil Gelatinase Associated Lipocalin) protein.

The personal contribution of the PhD Thesis is structured in four different studies corresponding to different types of biomerkers that are involved in different pathologies. Thus the experimental part can be regarded from two different perspectives. The first perspective is constructed based on different pathologies that are debated while the second more pronounced perspective is constructed based on innovation of different analytical approaches, sample management and sample processing methods for analytes that are belonging to different biochemical classes: iohexol, hydrophilic, low molecular weight xenobiotic molecule, thyroid hormones (TH)-hydrophobic low molecular weight, endogenous molecules, human bradykinin and bradykinin fragments as intermediate molecular weight peptides and finally, NGAL as high molecular weight protein. New state of the art microsampling systems such as VAMS - Volumetric Absorbative Microsampling Systems that accurately collect 10 or 20 µL of capillary blood and the HemaPEN devices that accurately sample 2.74 µL of capillary blood obtained by finger prick, have been used for the analysis of Iohexol and Thyroid hormones in the context of different pathologies. Chromatographic behavior and mass spectral fragmentation patterns of peptides have been studied using human bradykinin and two of its fragments (B 1-9, B 1-8 and B 1-7). This study represented a bridge between the analysis of low molecular weight compounds and high molecular weight compounds-proteins. Finally, a targeted bottom up proteomics approach assisted by LC-MS/MS has been used for the analysis of Neutrophil Gelatinase Associated Lipocalin (NGAL) in human plasma.

The first study was centered on the LC-MS/MS analysis of iohexol from whole human blood in the context of measuring glomerular filtration rate in Chronic Kidney Disease. Iohexol

was analysed using VAMS and HemaPEN microsampling systems. For this, different kind of studies have been realised. A new LC-MS/MS method, sample preparation and extraction protocol for iohexol have been developed. This step followed by short to medium term stability of iohexol in VAMS prelevated samples. Stability studies continued with iohexol stability in dried processed extracts stored at -80 ^{o}C and iohexol stability in the autosample. Matrix effects studies followed by the assessment of process efficiency and extraction recovery rates were performed in order to critically evaluate the impact of the sample processing method over the ionisation process, overall recovery of the analyte and variability at different concentration ranges. HemaPEN studies have been conducted in order to assess the possibility of analysing iohexol from ony 2.74 µL of blood. Indeed iohexol was able to be analysed from HemaPEN samples evaluating and comparing in the same time extraction recovery rates with those obtained using VAMS devices. Futher on, the entire analytical process was validated according to FDA criteria for VAMS microsampling and eventually was tested on real samples in order to assess the pharmacokinetic profile of iohexol based on VAMS. Thus the study underlines the possibility of analysing iohexol from only 10 µL (VAMS) or 2.74 µL (HemaPEN) of capillary blood obtained by finger prick which can be eventually be used for the assessement of the Cronic Kidney disease.

The second study was focused on the LC-MS/MS analysis of thyroid hormones (TT3, TT4 and rT3) in the context of thyroid function assessement. Thyroid hormones were analysed using microsampling devices such as VAMS (20 µL) and HemaPENs (2.74 µL). The beginning of the studies was centered on developing a targeted LC-MS/MS analytical method and a new sample processing and extraction protocol for TH using VAMS and HemaPENs. After this step, stability studies for TH were conducted: short to medium term stability of TH in VAMS prelevated samples; stability studies of TH in dried processed extracts stored at -80 ^{o}C and stability of TH in the autosampler in the final injection solvent. For all three thyroid hormones, studies regarding matrix effects, extraction recovery rates and process effciency have been carried on. HemaPEN studies have led to the conclusion that 2.74 µL of capillary blood suffice to analyse TTH from human blood. However, it turned out that the samples obtained after the processing of HemaPEN-derived samples are less impacted by the matrix effects and are prone to higher extraction rates compared to VAMS-derived samples. Finally, using VAMS samples, endogenous levels of TH have been assessed for real samples derived from euthyroid status samples while the results being comprised in the range of normal plasmatic physiological values for total amount of thyroid hormones (TT3 & TT4). The second study confirms the ability of using VAMS and HemaPEN samples for the quantification of total amount of thyroid hormones from whole blood using microsampling devices.

The third study aimed in underlining the impact of some chromatographic parameters over the separation process of peptides. Since the peptides are intermediate molecular weight molecules, somewhere between low molecular weight molecules and high molecular weight molecules (proteins) it is considered that, when employing reversed-phase separation mechanisms, peptides tend to exhibit rather adsorbtion at the level of stationary phase then partion between stationary phase and mobile phase as low molecules do. The impact of the chromatographic temperature and mobile phase gradient slope over the selectivity was studied and it turned out that the influence of this two parameters are in close connection with some of peptide descriptors further on correlated with the peptides physico-chemical properties such as hydrophobicity index, Gravy index and so on. Considering the mass spectral behavior, it turned

out that human bradykinin fragments generating low abundant product ions , the most intense one being the b_1 ion (proline-derived immonium ion or doubly charged arginine fragment). Indeed all three peptides generated *b*-type fragment ions at the level of the collision cell. All the three related peptides were succesfully base-line separated while proving that salts and protein precipitation from urine samples do not impact substantially overall peptide recovery.

The fourth study managed to prove the applicability of analysing NGAL protein using a LC-MS/MS method approach and the principles of targeted bottom-up proteomics employing a surrogate isotope-labeled protein, in the context of acute kidney injury. This study comprised multiple steps. First, computerized *in-silico* digestion simulations have been performed for natural NGAL, synthetic NGAL and surrogate internal standard in order to choose the right proteotypic peptides. Next, a suitable LC-MS/MS was developed for the SYP and SYN proteotypic peptides, using a dedicated column for peptides separation. Following this, a solid phase extraction dedicated protocol based on weak cation exchanger sorbent was developed for the peptide isolation and sample cleanup. Subsequently, a new targeted digestion protocol for NGAL and surrogate QpEST surrogate internal standard was developed while assessing the entire digestion process. After this, the entire analytical workflow protein digestion-SPE-LC-MS/MS analysis was validated according to FDA criteria for concentration ranges covering both physiological and pathological values. Finally, following the FDA validation, the new method was employed for real samples that were divided in two groups-the control group and the study group. In the last group samples derived from sources with different pathologies and comorbidities were included , for which elevated levels of NGAL were expected. Indeed it turned out that applying the new method it was possible to statistically distinguish ($p<0.05$) between the control group and the study group for which elevated concentration levels of NGAL have been found, relative to control group. Thus, the new LC-MS/MS protemics approach was able to analyse NGAL protein from human plasma for low and high levels of NGAL, respectively.

In conclusion, LC-MS/MS is a versatile analytical technique that can be used for different kinds of analytes in different clinical settings. Indeed the automatation of the present day employed analytical platforms assures high throughput while reducing to a minimum the cost per analysis, however LC-MS/MS is beginning to find its place in the clinical laboratory assuring alternativity and complementarity.