CHIRAL AND METABOLOMIC STRATEGIES USING MICRO-SEPARATIVE TECHNIQUES COUPLED TO MASS SPECTROMETRY FOR THE DETERMINATION OF COMPOUNDS OF PHARMACOLOGICAL AND BIOANALYTICAL INTEREST AND THE SEARCH OF POTENTIAL BIOMARKERS

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Two important topics in Analytical Chemistry field are chiral separations and metabolomic analyses. The relevance of chiral separations relies on the fact that two enantiomers of a molecule may possess different biological activities. The separation of enantiomers is considered one of the most challenging tasks in Analytical Chemistry, given their equal physicochemical properties. Therefore, it is clear that continuous research on this topic is needed to further develop methodologies able to separate chiral molecules. This is particularly interesting in the pharmaceutical analysis in which the pharmacologic activity of drugs might be severely influenced if they are chiral, i.e. pharmacological properties of enantiomers might be very different. Also, several endogenous molecules are chiral such as amino acids, that have been proposed to be biomarkers of pathologies. Although their importance has been reported in the previous years there is a continuous interest in improving the existing knowledge and thus, advanced chiral strategies are also needed.

On the other hand, metabolomics covers the comprehensive analysis of the metabolome, i.e. the set of low molecular weight metabolites from a given organism. Particularly, so-called untargeted metabolomics is focused on the unbiased analysis of these molecules which are intermediates of metabolic pathways without a prior knowledge on which ones to focus on. This is particularly useful to find what metabolites are characteristic of a specific pathology or disorder, also considered as potential biomarkers of disease.

*On this sense, the current PhD Thesis entitled "*Chiral and metabolomic strategies using micro-separative techniques coupled to mass spectrometry for the determination of compounds of pharmacological and bioanalytical interest and the search of potential biomarkers*" is focused on: 1) the development of novel advanced analytical methodologies based on Capillary Electrophoresis for the enantiomeric determination of compounds of pharmacological and bioanalytical interest, and 2) the development of metabolomic strategies for the study of biological systems. Specific details on these topics will be further discussed below:*

1. Development of advanced analytical methodologies based on Capillary Electrophoresis for the enantiomeric determination of compounds of pharmacological and bioanalytical interest

The nature of living organisms is chiral. Most of the molecules we are made of present one or several chiral centers what infers them a chiral nature. This is highly relevant in several fields, such as in the pharmaceutical and bioanalytical fields. In the pharmaceutical industry, oftentimes only one enantiomer (eutomer) is responsible for the pharmacological activity whereas that the other enantiomer, named distomer, might be inactive, or might even present unwanted side effects. This is the main reason why several agencies such as the Food and Drug Administration (FDA) demands that the properties of each individual enantiomer must be perfectly studied and defined before deciding whether a drug can be commercialized as a racemic mixture or as an enantiomerically pure formulation. On the other hand, endogenous molecules are also chiral and this influences the different biological mechanisms occurring in living organisms. It was long believed that only L-amino acids were the natural occurring form. However, recent literature demonstrated that D-forms of amino acids were also existing and have been profusely investigated in last years. So far, the physiological function of numerous D-amino acids and/or their role as putative biomarkers has been demonstrated in literature.

Either way, the role of Analytical Chemistry in this field is critical. This demands to develop sensitive chiral separations able to enantioresolve different species in different matrices. Capillary Electrophoresis (CE) is considered as a strong technique in chiral separations due to its numerous advantages derived from the high peak efficiency and high flexibility for incorporating different chiral selectors at different concentrations in the background electrolyte. Thanks to its inherent nature, CE offers also reduced consumption of reagents, solvents and samples, this being a very special feature in analysis of biological samples as in most cases they are available only in small quantities. Advantages of CE can be further expanded if this technique is combined with mass spectrometry (MS) detection. CE-MS offers higher sensitivity than UV detection and better specificity as the molecular mass of the different species can be measured. In

addition, structural information can be obtained if tandem MS experiments (MS^2) are used.

This PhD Thesis includes the development of a chiral method for enantiomeric purity check of the drug duloxetine and the development of a methodology enabling the enantioseparation of all the constituents from the phenyl-alanine metabolic pathway, all of them employing CE-MS^2 strategies.

1.1. Development of a CE-MS^2 method for the enantioseparation of duloxetine. Application to the analysis of pharmaceutical formulations

Duloxetine is a drug used to treat major depressive disorder and anxiety disorders. S-duloxetine has been proven to have more activity than the R-enantiomer. As a consequence, duloxetine is commercialized as a single enantiomer in the S-form, thus, a methodology enabling its proper enantiopure quality control is needed. A screening using different cyclodextrins in an acidic media was conducted to find which one enabled baseline separation for both enantiomers. An interesting result was found. For two of the cyclodextrins that offered enantiorecognition, methyl-γ-cyclodextrin (M-γ-CD) and (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), the enantiomer migration order was opposite [1]. The former enabled the enantiomeric impurity to migrate last whereas that with the latter R-duloxetine migrated first (**Figure 1**).

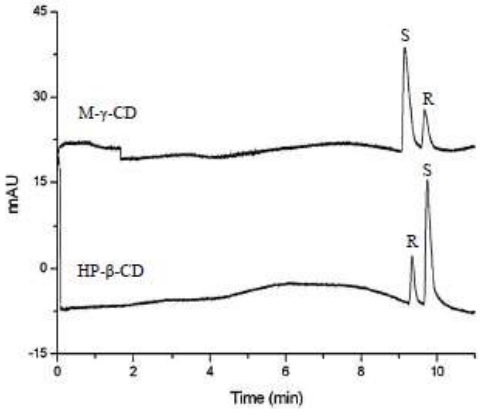

***Figure 1**. Electropherogram of a racemic mixture of duloxetine spiked with S-enantiomer in the presence of 1 % (w/v) of M-γ-CD or HP-β-CD. Reproduced with permission from [1].*

This highlights one of the advantages of CE for chiral separations as its flexibility enables to assay different chiral selectors. In order to evaluate this behavior, nuclear magnetic resonance and MS studied were conducted. It was possible to conclude that the difference in enantiomer migration order was originated by the different electrophoretic mobility of the complexes formed between the enantiomer and the chiral selector and not by their stability. The fact that the enantiomeric impurity migrates first favors its detection at low concentrations in presence of large amounts of the active principle (S-duloxetine). Thus, HP-β-CD was selected as chiral selector for further method development. Using an ion trap analyzer in the MS^2 mode enabled an improvement of 10 times in the method sensitivity when compared to the one obtained in a CE-UV system, obtaining a relative limit of detection of 0.02 % for the enantiomeric impurity [2]. This is the lowest value ever reported for duloxetine and the only one in line with the guidelines of the International Conference on Harmonization (< 0.1 %). This method was applied to the analysis of pharmaceutical formulations where the content of S-duloxetine was in agreement with the established label content and the enantiomeric impurity in all cases was below the limit of detection.

1.2. Development of a CE-MS^2 method for the enantioseparation of the constituents of the phenyl-alanine metabolic pathway. Application to the analysis of plasma samples

The phenyl-alanine (Phe-Tyr) metabolic pathway is based on the conversion of L-phenylalanine (L-Phe) into L-tyrosine (L-Tyr), L-3,4-dihydroxyphenylalanine (L-DOPA), and dopamine (DA), norepinephrine (NE), and epinephrine (EP). Several disorders are associated with this metabolic pathway such as phenylketonuria, an inborn error that causes accumulation of large amounts of Phe in blood and urine and therefore lower amount of its metabolites, what might lead to mental retardation. All these compounds, except DA, are chiral, thus, it is of relevance to study their enantiomeric composition in biological samples. There are several methods reported in the literature to enantioseparate these compounds individually but their simultaneous separation was not yet achieved. This PhD Thesis includes the development of the first chiral methodology enabling the simultaneous separation of all the constituents of the Phe-Tyr metabolic pathway [3]. A dual chiral selector system was needed (180 mM of HP-β-CD and 40 mM of M-β-CD) to achieve the proper enantioresolution. An in-capillary preconcentration method (large volume sample stacking) combined with the use of a MS with ion trap analyzer in the MS^2 mode, allowed to obtain limits of detection between 40 and 150 nM for the studied metabolites (**Figure 2**).

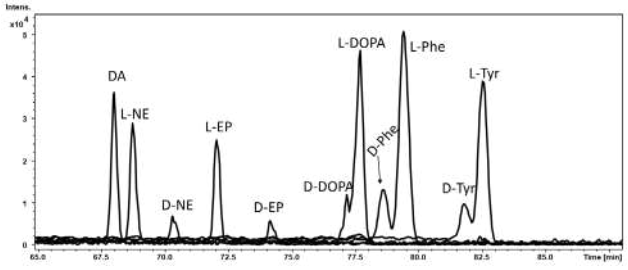

***Figure 2.** Extracted ion electropherogram of the CE-MS^2 enantioseparation of the constituents of the Phe-Tyr metabolic pathway. Reproduced with permission from [3].*

The method was applied to the analysis of plasma samples of rats. However, only L-Phe and L-Tyr were quantified as the rest of compounds were below the limit of detection of the method. This suggests that the future tendency should be redirected towards use of more sensitive approaches such as sheathless MS interfaces or more sensitive preconcentration strategies.

2. Development of metabolomic strategies for the study of biological systems

Untargeted metabolomics aids in finding differences in the metabolic profile of the samples of study under specific conditions, e.g. healthy vs diseased patients. Untargeted metabolomics is known to be a good approach to generate new hypothesis, given its unbiased nature. This can be applied to the discovery or search of potential biomarkers or as a merely exploratory strategy to further improve knowledge of a particular disease. Main difference between metabolomics and the rest of the *omics* is that it is not possible to measure all metabolites using a single analytical technique. This is due to the fact that metabolites greatly vary not only in concentration but also in physicochemical properties. This limitation could be further minimized by a proper optimization of the sample preparation and sample analysis stages, ideally, combined with the use of multiplatform strategies. In this sense, MS, typically coupled to chromatographic (e.g. liquid chromatography (LC)) or electrophoretic techniques (CE), is the detection system more employed in metabolomics given its high selectivity and sensitivity.

In this PhD Thesis, the development of an analytical methodology to determine polar compounds on reversed phase LC-MS and its further application to the metabolomics analysis of a drug dependence case is carried out. Secondly, the development of a multiplatform strategy based on LC-MS and CE-MS for the analysis of low-volume samples is conducted.

2.1. Design of strategies to study the metabolic profile of highly polar compounds in plasma. Investigation on the combined effect of cocaine and ethanol administration through a metabolomic approach

Amino acids are paramount molecules playing vital roles in many metabolic pathways as they act as building blocks for protein synthesis and are involved in neurotransmitter transport, among others. Unfortunately, the determination of amino acids and other highly polar compounds by the most common strategy in metabolomics, i.e. reversed phase LC-MS, typically has limitations. Polar compounds do not retain in common C18 columns and they elute in the dead volume, suffering ion suppression and hampering their identification as strong peak overlap is occurring.
In this PhD Thesis, three different approaches based on LC-MS were evaluated to find out which one offered better performance: i) a pentafluorophenyl (PFP) column in fully-aqueous conditions, ii) a HILIC column, both without using compound derivatization, and iii) a C18 column using 9-fluorenylmethyloxycarbonyl (FMOC)-derivatization to increase the hydrophobicity of the metabolites and thus enable their retention on C18 columns [4]. The procedure based on FMOC derivatization was the only one enabling to detect the set of compounds assayed (a pool of 35 polar metabolites) with the highest peak efficiency. FMOC derivatization takes place in just 2 min, lower reaction times than other labelling reagents used previously in other metabolomics strategies.

As the objective was to perform metabolomic analysis on plasma samples, the next step consisted on comparing two different sample preparation protocols to eliminate proteins from these samples. Presence of large molecules such as proteins disrupt the chromatographic separation and therefore hampers the metabolomic analysis. The ultrafiltration method was selected over the protein precipitation with organic solvent as the intensity of signals was higher, the number of molecular features was larger and the variability of peak areas for these molecular features was lower. This strategy was applied to investigate the effects of cocaine and/or ethanol on metabolic profiles of rat plasma [5]. 37 *Wistar* rats were randomly assigned to four groups where, during 21 days, they were given any of the following: 15 mg/kg cocaine, 2 g/kg alcohol, 15 mg/kg cocaine + 2 g/kg alcohol, or 0.9 % (w/v) sodium chloride solution (control group). After all samples were analyzed, data processing was performed using the open-source tool XCMS (R package). Data analysis started with unsupervised multivariate analysis. Principal component analysis (PCA) score plot showed a slight grouping of the samples based on the experimental groups. Partial least square discriminant analysis (PLS-DA) on the four groups of samples showed that the main variability happened in cocaine and non-cocaine containing groups.

To highlight what molecular features were affected by cocaine and/or ethanol administration different pair-wise PLS-DA models were plotted and variables with variable importance in the projection (VIP) values higher than 1.5 were selected for further identification. Eight metabolites (carnosine, spermidine, methionine, cystathionine, argininosuccinic acid, 4-hydroxyproline, N-ε-acetyl-L-lysine, and serotonin) could be unequivocally identified through confirmation with the analysis of standards, whereas that three (2-methyl-L-tryptophan, N1-acetyl-spermidine, and β-leucine) were only tentatively annotated by matching the experimental MS/MS fragmentation spectra to the ones in the MS/MS libraries such as HMDB and MassBank. KEGG database was used to get an estimation on where these metabolites could be allocated in the different metabolic pathways. Amino acids-related pathways were mainly affected by the administration of cocaine and/or alcohol. It is important to note that this pilot study can be used as a starting point for further investigations, for example, with a larger number of samples, to fully understand the mechanisms of cocaine and/or alcohol administration.

2.2 Development of a multiplatform metabolic workflow for biomass-restricted tissue samples. Application to the metabolomic analysis of polycystic kidney disease samples

Given the differences in metabolite concentrations (ranging from pmol to mmol) and physicochemical properties (different polarity and molecular weights, among others) the whole spectrum of the metabolome cannot be covered with one solely technique. Multiplatform strategies can be

useful on this aspect. However, this might be a challenging task if samples are only available in low amounts such as needle-biopsies, histological material, or body fluids from experimental animals e.g. mice cerebrospinal fluid or microdialysates.

In this Thesis, a multiplatform setup based on LC with two orthogonal columns (C18 and HILIC) and two ionization modes (positive and negative, respectively) and on CE in positive ionization mode was developed, for metabolomic analysis of low volume samples, i.e. 20 µm-thick sections of mice kidney [6, 7]. Under normal conditions, mouse kidney is about 0.5 – 0.8 % of total weight, therefore, a 20 µm-thick kidney section is a good example of biomass-restricted sample. To test the feasibility of the multiplatform setup, an animal model of polycystic kidney disease (PKD) was used. PKD combines numerous cystic diseases that involve development of fluid-filled cysts and fibrosis in the kidneys. The sample preparation was optimized in such a way that, first, samples were analyzed by reversed phase LC and after subsequent evaporation of the remaining sample in the vial and further reconstitution in compatible media, it was analyzed in HILIC. Then, the remaining sample was again evaporated and reconstituted, now in a much lower volume, for CE-MS analysis. Only 2.5 µL of sample volume in the vial was enough to guarantee repeatable injections in CE-MS (**Figure 3**).

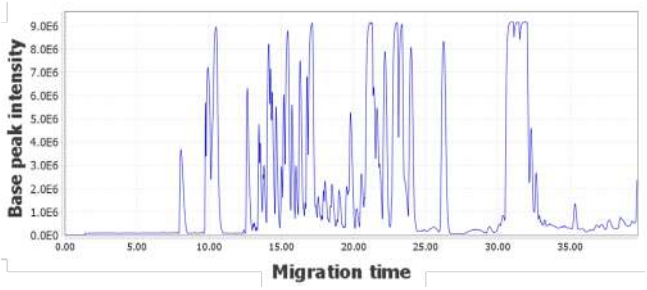

Figure 3. *Base peak electropherogram of a quality control sample of the kidney section extracts analyzed by the CE-MS sheathless platform. Reproduced from [7] through a Creative Commons CC BY licence.*

The metabolomics analysis of the PKD study was conducted. 20 mice were equally divided in four experimental groups: non-PKD young mice (Wt0 group), adult mice with mild-stage PKD (MCK group), adult mice with end-stage PKD (ESCK group), and a control group adult mice (Wt14 group). Three tissue sections were acquired per mouse (total number of 60 samples). Again, XCMS tool (R package) was used for data processing. In the case of CE-MS metabolomic analysis signals had to be aligned using the *Msalign2* algorithm. The total number of features found were: 125, 119, and 112 for RPLC-MS, HILIC-MS, and CE-MS, respectively. This means that the number of signal features was very similar although different platforms were used. This shows the potential of the sheathless CE-MS as, although it has not been used often in metabolomics, it offers many advantages as high sensitivity and low consumption of samples. Metabolomic sequences showed good analytical consistency as the quality control samples clustered together in PCA. The next step was to focus on the variables that were significant from the Wt0 vs MCK and/or MCK vs ESCK comparisons but not in Wt0 vs Wt14 (age-related metabolites). Most of them showed differences from MCK to ESCK groups, i.e. they changed from mild to end-stage PKD. Only a few were different from Wt0 to MCK, as well as from MCK to ESCK. Concerning the latter scenario, only methylhippuric and citric acids in RPLC-MS, methylhippuric acid and 3,4-dihydroxyphenylglycol-O-sulfate in HILIC-MS, and 4-guanidinobutanoic acid in CE-MS met such requirements, i.e. they could be used to study the PKD progression from the beginning of the disease. Herein, it could be concluded that the multiplatform setup described properly the progression of PKD. However, this needs to be carefully considered as it is just a pilot study. Further research should include a larger number of observations and preferably include a targeted approach to accurately measure the absolute concentrations of the metabolites of interest in these samples.

This last part of the PhD thesis was conducted as a result of two internships of a total of 6.5 months in the Center for Proteomics and Metabolomics from the Leiden University Medical Center in Leiden, the Netherlands, under supervision of Dr. Oleg A. Mayboroda.

Overall, this thesis has contributed to the Analytical Chemistry field in terms of developing methodologies which help understanding biological processes by chiral or metabolomic strategies as well as improving the evaluation of pharmaceuticals by chiral analysis.

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