Monitoring the reaction of corticoids with lysine by mass spectrometry: towards the development of analytical methodologies for the differential diagnosis of Arterial Hypertension

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Abstract

Arterial hypertension (HTN) affects more than 1 billion people worldwide, and its associated complications are responsible for 9.4 million annual deaths worldwide. Thus, developing a methodology for its differential diagnosis is of extreme importance. One of the key players in resistant HTN and HTN-related chronic kidney disease (CKD) is aldosterone. However, currently available clinical methods focused on determining the free plasmatic/urinary levels of this corticosteroid are not effective as predictive or differential diagnosis tools.

With the ultimate goal of identifying better biomarkers of resistant HTN and CKD than free aldosterone levels, this work is set at exploring mass spectrometry-based methodologies to investigate if: i) aldosterone can covalently modify blood proteins, yielding stable covalent adducts; and ii) hydrazide-based methodologies can be used to enrich aldosterone-protein adducts prior to their identification by mass spectrometry.

In vitro modification of human serum albumin (HSA) with aldosterone, followed by digestion to amino acids and LC-HRMS analysis, allowed the identification of lysine modified residues, consistent with the formation of covalent adducts stemming from Schiff base stabilization upon Heyns rearrangement. Similar results were obtained with other steroids such as prednisolone, dexamethasone, cortisol and corticosterone, thereby suggesting that this constitutes a general ability of acyloin containing corticosteroids. A pre-analysis enrichment methodology, based on the use of hydrazide resins, was tested for this type of adducts. Whereas further optimization is required, the results obtained suggest that this enrichment procedure can be very useful for detecting protein adducts formed with HTN-related steroids in biologic samples.

1. Introduction

Arterial hypertension (HTN) affects more than 1 billion people worldwide and is related to several health problems, such as heart and kidney diseases. Complications associated with HTN are responsible for 9.4 million annual deaths worldwide. Not only that, but HTN also has a substantial impact on both society and the economy. The total cost associated with HTN in the United States of America is $131 billion per year [1, 2].

HTN can cause, among others, headaches, shortness of breath, dizziness, and heart palpitations. When left untreated, it can lead to cardiovascular disease, dementia, chronic kidney disease (CKD), and aneurysms [2, 3].

Effective blood pressure control significantly reduces the occurrence of complications associated with HTN. However, a high proportion of hypertensive patients under treatment cannot achieve long-term blood pressure control and develop resistance to treatment [4]. Resistant hypertension (RHTN) is the leading cause of HTN's mortality and mobility and can be defined as elevated blood pressure despite using three different antihypertensive drugs as treatment. Patients with RHTN have a higher risk of poor outcomes when compared with patients without it. For example, they are more likely to suffer from myocardial infarction, death, heart failure, stroke, and CKD than hypertensive patients whose blood pressure is effectively controlled by antihypertensive drugs. Therefore, the correct

treatment for this type of hypertensive patients is of extreme importance [5].

Current diagnosis tools are not able to stratify hypertensive patients, causing all patients to be prescribed the same treatment and HTN to be used as a general umbrella to classify a multigenic disease that is highly influenced by environmental factors [5-7]. Therefore, to provide better treatment, the development of strategies to improve HTN control is essential, and they need to involve precision medicine strategies supported by efficient differential diagnosis tools [7].

Several studies indicate that excessive aldosterone levels may contribute to treatment resistance and lead to RHTN [5, 8]. Aldosterone is a corticosteroid produced from cholesterol in the zona glomerulosa of the adrenal gland, located in the kidneys, and responsible for blood pressure regulation through electrolyte regulation and fluid homeostasis. Angiotensin II and extracellular potassium concentration regulate aldosterone, and it is recognized as a critical factor in several diseases, such as HTN, heart failure, arrhythmia, and metabolic and kidney diseases [6, 9].

Despite elevated aldosterone levels being associated with HTN, independently of the aldosteronism diagnosis, the measurement of aldosterone serum levels has failed as a discriminating biomarker for HTN. This can be due to inadequate sensitivity of the existing diagnostic techniques used to detect aldosterone levels in tissues and interindividual aldosterone level fluctuations related to its up and downstream metabolism. Also, aldosterone has a short plasma half-life of less than 20 minutes, making it difficult to quantify. Measuring serum aldosterone levels has another associated problem: high plasma levels of aldosterone do not necessarily mean that in the kidney, where aldosterone exerts all of its functions, aldosterone levels are also high [10].

The covalent modification of proteins can occur by electrophilic xenobiotics (or by their bioactivation metabolites) or by endogenously generated electrophiles resulting from oxidative stress, glycation, and related processes. Whereas the covalent modification by xenobiotic-derived electrophiles is a recognized molecular mechanism underlying toxic events induced upon exposure to toxic chemical agents, the modification of proteins by endogenously generated reactive intermediates is now considered a key element in the molecular pathology of multiple diseases [11]. Additionally, the characterization of these modifications can give insights into environmental agents and endogenous processes that may be contributing factors to human diseases and toxic effects. In fact, covalent protein adducts are often used as biomarkers of exposure to exogenous toxicants and as biomarkers of disease [12].

Protein covalent adducts are usually used as biomarkers for different reasons. They are very stable and accumulate over the lifespan of proteins, reducing variability and providing more accurate measures of long-term exposure. However, the adducted fraction of the proteins is very low, which hampers their detection in complex samples [13].

It has been demonstrated that 16α-hydroxyestrone, a steroid containing an acyloin group, can react with proteins' lysine residues, yielding a Schiff base, which can then by stabilized by Heyns rearrangement to form a very stable adduct [14, 15]. Thus, it was hypothesized that the measurement of protein adducts formed with aldosterone are superior as diagnosis/prognosis tools in RHTN and CKD, over the free aldosterone plasma levels currently used in clinic. Three main reasons are expected to contribute for this: 1) Since covalent adducts accumulate over the lifespan of proteins, their measurement could provide a more accurate measure of long-term exposure than the free aldosterone levels; 2) since aldosterone has to cross cell membranes to reach proteins such as hemoglobin, therefore the adducts formed with this protein are expected to better mirror the aldosterone tissue levels than the free plasmatic aldosterone levels; and 3) the possibility of accurately quantifying the covalent adducts formed by multiple functionally and metabolically related steroids is expected to enhance the differential diagnosis ability. Therefore, this work had three main objectives. The first was to identify, by LC-HRMS, the reaction products formed *in vitro* between lysine and corticoids that contain the acyloin functional group. The second objective was to investigate if this type of adducts could be enriched using resins containing a hydrazide functional group. Lastly, this work aimed to investigate whether histone's lysine residues could be targets for covalent modifications by aldosterone. For this, histones isolated from a cell line derived from epithelial line of renal proximal tubule (HK2) exposed to aldosterone were analyzed.

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich, unless specified otherwise, and used without further purification.

2.2. Reactions

2.2.1. Corticosteroids' Reaction with Lysine

a. Reaction with no other reactants

To a solution of lysine (0.67 mg, 4.58×10^{-6} mol, 5.0 eq) in 50mM ammonium bicarbonate buffer pH 7.4 (0.5

mL), a solution of aldosterone (0.33 mg, 9.16× 10^{-7} mol, 1.0 eq), prednisolone (0.33 mg, 9.16× 10^{-7} mol, 1.0 eq), dexamethasone (0.33 mg, 8.41× 10^{-7} mol, 1.0 eq), cortisol (0.33 mg, 9.10× 10^{-7} mol, 1.0 eq), or corticosterone (0.33 mg, 9.52× 10^{-7} mol, 1.0 eq) in ethanol (33 µL) was added. The resulting solution was incubated at 37° C in a thermomixer for 24 h. One aliquot was directly monitored by LC-HRMS and the other was subjected to the enrichment procedure (section 2.3.3).

b. Reaction in the presence of sodium cyanoborohydride

To a solution of lysine (0.67 mg, 4.58× 10^{-6} mol, 5.0 eq) in 50mM ammonium bicarbonate buffer pH 7.4 (0.5 mL), a solution of aldosterone (0.33 mg, 9.16× 10^{-7} mol, 1.0 eq), prednisolone (0.33 mg, 9.16× 10^{-7} mol, 1.0 eq), or dexamethasone (0.33 mg, 8.41× 10^{-7} mol, 1.0 eq) in ethanol (33 µL) and a solution of sodium cyanoborohydride (0.29 mg, 4.58× 10^{-6} mol, 5.0 eq) were added. The resulting solution was incubated at 37° C in a thermomixer for 24 h. One aliquot was collected and directly monitored by LC-HRMS.

c. Reaction in the presence of hydroxylamine

To a solution of lysine (0.67 mg, 4.58× 10^{-6} mol, 5.0 eq) in 50mM ammonium bicarbonate buffer pH 7.4 (0.5 mL), a solution of aldosterone (0.33 mg, 9.16× 10^{-7} mol, 1.0 eq), prednisolone (0.33 mg, 9.16× 10^{-7} mol, 1.0 eq), or dexamethasone (0.33 mg, 8.41× 10^{-} mol, 1.0 eq) in ethanol (33 µL) and a solution of hydroxylamine (0.031 mg, 9.25× 10^{-7} mol, 1.0 eq) were added. The resulting solution was incubated at 37° C in a thermomixer for 24 h. One aliquot was collected and directly monitored by LC-HRMS.

2.2.2. Corticosteroids' reaction with HSA

A solution of HSA (0.5mg, 7.46× 10^{-9} mol, 0.07 eq) was prepared in 50mM sodium bicarbonate buffer pH 7.4 (0.5mL). A solution of aldosterone (0.1mg, 2.77× 10^{-7} mol, 1 eq), prednisolone (0.1mg, 2.77× 10^{-7} mol, 1 eq), dexamethasone (0.1mg, 2.54× 10^{-7} mol, 1 eq), cortisol (0.1mg, 2.76× 10^{-7} mol, 1 eq), or corticosterone (0.1mg, 2.89× 10^{-7} mol, 1 eq) in ethanol (10 µL) was then added. The resulting solution was incubated overnight, at 37° C, whereupon it was digested with Pronase E at a protease:protein ratio 1:10 (w/w) at 37° C overnight. One aliquot was directly analyzed by LC-HRMS and the remaining aliquots were subjected to the enrichment procedure (as described in section 2.3.3).

2.2.3. Enrichment procedure with hydrazide resin

Different combinations of coupling and elution buffers were tested. In total, seven different experiments were tested, and they are summarized in table 1. The procedure explained below was applied to all the experiments present in table 1.

Table 1 - Coupling and elution buffers used in each one of the seven experiments for Heyns adduct enrichment with hydrazide resin

Experiment	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7
Coupling Step	0.1M Sodium Acetate, 0.15M Sodium Chloride, 0.1M Aniline buffer, pH 5.5	0.1M Sodium Acetate, 0.15M Sodium Chloride, 0.1M Aniline buffer, pH 5.5	0.1M Sodium Acetate, 0.15M Sodium Chloride, 0.1M Aniline buffer, pH 5.5	0.1M Sodium Acetate, 0.15M Sodium Chloride buffer, pH 5.5	0.1M Sodium Acetate, 0.15M Sodium Chloride buffer, pH 5.5	0.1M Sodium Acetate, 0.15M Sodium Chloride buffer, pH 5.5	No enrichment
Elution Step	0.2M Hydroxylamine, 0.1M Aniline buffer, pH 5.5	0.2M Hydroxylamine buffer, pH 5.5	0.1M Sodium Acetate buffer pH 2.8	0.2M Hydroxylamine, 0.1M Aniline buffer, pH 5.5	0.2M Hydroxylamine buffer, pH 5.5	0.1M Sodium Acetate buffer pH 2.8	

100µL of *UltraLink Hydrazide Resin* (Thermo Fisher Scientific) were added to an *Eppendorf*. The resin was equilibrated using 5 resin-bed volumes of coupling buffer (500µL), which was then centrifuged for 2 minutes, and the supernatant removed. This procedure was repeated once more. 80µL of digested protein solution was added to the resin, followed by 250µL of coupling buffer. The resin was incubated overnight at 37° C.

The resin was centrifuged for 2 minutes, and the supernatant removed. 5 resin-bed volumes of coupling buffer were added, and the resin centrifuged for 2 minutes, and the supernatant discarded. This step was repeated twice.

To elute the modified amino acids attached to the resin, 5 resin-bed volumes of elution buffer were added to the resin, and it was left incubating overnight at 37° C. The next day, it was centrifuged for 2 minutes, the supernatant was collected. One aliquot was collected and monitored by LC-HRMS.

2.2.4. Histones isolation from cell pellets

2.2.4.1. Cell culture

The following protocol was performed in collaboration with two iBB collaborators, Cláudia Miranda and Tiago Fernandes.

A cell line derived from an epithelial line of renal proximal tubule cells (HK-2: ATCC CRL-2190) was obtained from American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12; 11320033, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, S 0616, Biochrom), 4mM L-Glutamine (25030-081, Gibco) and 1% antibiotic-antimycotic (15240062, AntiAnti, Invitrogen). Cells were kept at 37° C, and 5.2% CO_2, these conditions were established using NUAIRE DH AUTOFLOW incubator. Cell passaging was carried out at 70-80% confluence, every 3 to 4 days, using trypsin - ethylenediaminetetraacetic acid (EDTA) (0.05%) (25300-054, Gibco) for cell detachment.

For *in vitro* experiments, cell number was determined using Bruker counting chamber. Depending on the final application, the appropriate number of cells was seeded in DMEM/F-12 (10% FBS) for a 24h long period. After this, cells were deprived of FBS for a 16h long period (starvation) prior to establishing experimental conditions. In the following assays, unless specifically mentioned otherwise, DMEM/F-12 was supplemented with 10% FBS.

2.2.4.2. Experimental Conditions

Cells were seeded in two T-flasks. After 24 hours, in one of the T-flasks, the culture medium was replaced and 100nM of aldosterone were added. In the other T-flask, no aldosterone was added to the culture medium. 24 and 96 hours after aldosterone addition, sample were collected from both T-flasks.

2.2.4.3. Histone isolation procedure

To a 200µL cell pellet, 500µL of nuclei isolation buffer, NIB, (PBS 1X, 0.1% Triton X-100) supplemented if the following inhibitors 0.5mM PMSF, 5mM sodium butyrate, 5µg/mL Aprotinin (AppliChem), and 5µg/mL Leupeptin, were added. The cells were resuspended with the micropipette tip, and another 500µL of NIB were added, and the cells resuspended again. The suspension was placed on ice for 10 minutes. During this period, every 30 seconds, the suspension was lightly agitated. After the 10 minutes, the suspension was centrifuged (3,250× g at 4° C for 30 minutes), and the supernatant discarded. 500µL of ice-cold PBS buffer were added to the pellet and used to resuspend it. After resuspending with the micropipette tip, another 500µL of iced cold PBS buffer were added, and the suspension was resuspended again. The suspension was centrifuged (3,250× g at 4° C for 20 minutes), and the supernatant discarded.

The resulting pellet was resuspended in 500µL of H_2SO_4} with the tip of the micropipette and then another 500µL were added and the pellet resuspended again. The resuspended pellet was placed at 4° C for 3h and manually agitated every 30 minutes.

Finished this period, a centrifugation (16,000× g for 10 minutes) was performed, the supernatant collected, 4 volumes of acetone added and placed in the fridge at 4° C overnight. The next day, it was centrifuged (3,458× g for 25 minutes) and the supernatant removed. The pellet obtained was left air drying for one hour and resuspended with the least possible amount of distilled water (in this case, 100µL). An aliquot was collected to measure protein concentration by Bradford protein assay.

A 10µg aliquot solution was digested with trypsin at a protease:protein ratio 1:10 (w/w) at 37° C for 2h. The digestion was quenched by addition of formic acid. One aliquot was collected and analyzed by LC-HRMS. The remaining histone solution was digested with Pronase E at a protease:protein ratio 1:10 (w/w) at 37° C for 2h. One aliquot was collected and analyzed by LC-HRMS.

2.2.4.4. *In vitro* histone modification with aldosterone

To an aqueous solution (100µL) of histone's octamer (0.1mg, 9.26×10^{-10} mol, 1.0eq), histone H2A (0.1µg, 7.15×10^{-9} mol, 1.0eq), histone H2B (26.1µg, 7.25×10^{-8} mol, 10.0 eq), H3 (23.6µg, 6.54×10^{-8} mol, 10.0 eq) or histone H4 (0.1mg, 8.85×10^{-9} mol, 1.0eq), an ethanolic solution of aldosterone [3.3µg, 9.26×10^{-9} mol, 10.0 eq in ethanol (1µL)] or [33.4µg, 9.26×10^{-8} mol, 100.0 eq in ethanol (26µL)] solution was added. The resulting solution was incubated at 37° C in a thermomixer for 2 h. One aliquot was collected and monitored by LC-HRMS.

2.3. Methods

2.3.1. Mass Spectrometry

Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS)

HRMS analyses were conducted by liquid chromatography (Ultimate 3000 RSLCnano system, Thermo Fisher Scientific, Bremen, Germany) interfaced with a Bruker Impact II quadrupole time-of-flight mass spectrometer equipped with a CaptiveSpray (nanospray) source (Bruker Daltoniks, Bremen, Germany).

Protein digested to peptides Chromatographic separation was performed on an Acclaim PepMap C18 column (75 µm× 150 mm, 3 µm particle size; Thermo Fisher Scientific). The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile:water (80:20) containing 0.1% formic acid (B). The elution conditions were as follows: 2% B for 5 min, 2–50% B over 45 min, 50–60% B over 10 min, 60–65% B over 5 min, 95–2% B over 3 min, and 2% B for 27 min. The injection volume was 1µL, the flow rate was 300 nL/min, and the

column was maintained at 40° C. Quality control samples (a tryptic peptide digest of bovine serum albumin) were analyzed along with the analytical runs (after every 10 samples) in order to check the consistency of analysis regarding signal intensity and retention time deviations. A Lock Mass (HP-121 Calibration Standard, *m/z* 1221.9906; Agilent Technologies, Santa Clara, CA, U.S.A.) was used during the analysis for spectrum calibration. Data were acquired in positive mode from *m/z* 100 to 2200 at an acquisition rate of 5 spectra/sec, using a data-dependent auto-MS/MS method to select the 10 most abundant precursor ions per cycle for fragmentation. The MS source parameters were set as follows: dry gas heater temperature, 150° C; dry gas flow, 3 L/min; and capillary voltage, 1600 V.

Data Processing

The acquired MS data files of the samples digested to peptides were converted to *.mgf format using the Compass DataAnalysis software (v4.1 Bruker Daltonics). MaxQuant (v.1.6.17.0, Cox and Mann, 2008) search engine was used for peptide identification. Search parameters included precursor ion mass tolerance = 15 ppm, fragment ion mass tolerance = 30–40 ppm, number of missed-cleavages ≤4 and variable amino acid modifications = aldosterone incorporation (mass increment of 342.1831 Da) and aldosterone incorporation followed by reduction (mass increment of 344.1987 Da) at the amino acid lysine. The acquired MS/MS spectra was searched against an in-house compiled human histones database. All human histones sequences were obtained from Uniprot (UniProt Consortium, 2007).

For adducted amino acids Chromatographic separations were performed on: (1) an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) using a Luna C18 column (3.0µm, 2.0× 150 mm; Phenomenex) and an elution gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at a flow rate of 170µL/min. The elution conditions were as follows: 5–50% B for 6 min; 50–100% B for 4 min; isocratic elution with 100% B for 5 min; 100–5% B for 4 min; and finally, 5% B for 9 min; or (2) an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) using a HypersilGold C18 column (2.1×150 mm, 1.9µm particle size; Thermo Fisher Scientific) at a flow rate of 200µL/min. The elution conditions were as follows: 5% B for 2.4 min; 5–25% B for 2.1 min; 25–70% B for 4.1 min; 70–100% B for 3 min; 100% B for 3 min; 100–5% B for 2 min; and finally, 5% B for 6 min. In either instance, the injection volume was 10µL. The column and the autosampler were maintained at 40 and 8° C, respectively. The mass spectrometric parameters were set as follows: end plate offset, 500 V; capillary voltage, 4.5 kV; nebulizer, 40 psi; dry gas, 8 L/min; heater temperature, 200° C. Spectra were acquired in the positive electrospray ionization mode ESI (+). Internal calibration was performed for sodium formate cluster, with a sodium formate solution introduced to the ion source via a 20µL loop at the beginning of each analysis using a six-port valve. Calibration was then performed using high-precision calibration mode (HPC). Acquisition was performed in the *m/z* 50–1,000 range and in a data-dependent MS/MS mode with an isolation window of 0.5, acquisition rate of 3 Hz and a fixed cycle time of 3s. Precursor ions were selected for auto MS/MS at an absolute threshold of 153, with the active exclusion mode set at three spectra and released after 1 min, but precursor ions with intensities in the range of 5× the previous intensities were reconsidered.

Data Processing

The acquired MS data files of the samples digested to peptides were converted to *.mgf format using the Compass DataAnalysis software (v4.1 Bruker Daltonics). MaxQuant (v.1.6.17.0, Cox and Mann, 2008) search engine was used for peptide identification. Search parameters included precursor ion mass tolerance = 15 ppm, fragment ion mass tolerance = 30–40 ppm, number of missed-cleavages ≤4 and variable amino acid modifications = aldosterone incorporation (mass increment of 342.1831 Da) and aldosterone incorporation followed by reduction (mass increment of 344.1987 Da) at the amino acid lysine. The acquired MS/MS spectra was searched against an in-house compiled human histones database. All human histones sequences were obtained from Uniprot (UniProt Consortium, 2007). For adducted amino acids the acquired data was processed by Compass DataAnalysis software (v4.1 Bruker Daltonics). Extracted ion chromatograms (EIC), with a mass window of ±0.02 ppm, were performed for searching the protonated molecule of the expected metabolites in the full scan spectra. All spectra corresponding to metabolites were then manually checked. The mass deviation from the accurate mass of the identified metabolites remained below 5 ppm for the precursor and below 10 ppm for product ions.

2.3.1.1. Liquid Chromatography Mass Spectrometry (LC-MS)

LC-MS analyses of adducted peptides were conducted on an HPLC Dionex Ultimate 3000 system coupled in-line to an LCQ Fleet ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher Scientific, Waltham, MA). Chromatographic separation was performed by the same condiction used for HRMS analysis. The mass spectrometer was operated in the ESI positive mode, with the following optimized parameters:

ion spray voltage, 4.5 kV; capillary voltage, 16 V; tube lens offset, 58 V; sheath gas (N_2), 80 arbitrary units; auxiliary gas (N_2), 5 arbitrary units; capillary temperature, 270° C. Spectra typically corresponded to the average of 20–35 scans and were recorded in the 100-1000 Da range. Tandem mass spectra (MS/MS) were obtained with an isolation window of 1 or 6 m/z units, 28-35% relative collision energy and an excitation time of 30 ms.

Data Processing

Data acquisition and processing were performed using the Thermo Xcalibur software (v2.2, Thermo Fisher Scientific).

3. Results and Discussion

3.1. Covalent Modification of Proteins

To see if corticosteroids containing an acyloin group, such as aldosterone, have the ability to covalently modify proteins yielding stable covalent adducts, aldosterone was incubated with HSA, at 37° C overnight, in 50mM ammonium bicarbonate buffer pH 7.4. To monitor the formation of covalent adducts, a mass spectrometry-based adductomics approach was used, which involved the digestion of the adducted protein to amino acids (by digestion with Pronase E) followed by LC-ESI-HRMS upon comparison with standard adduct. These standard adducts were prepared by incubating aldosterone and lysine.

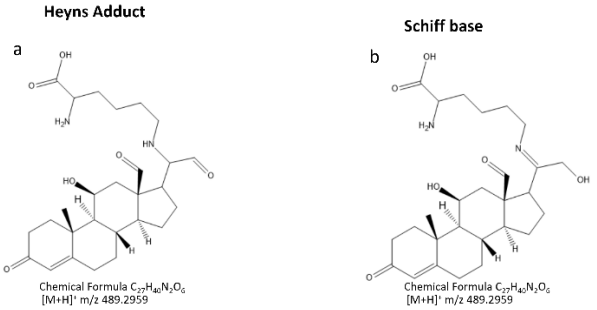

Figure 1. 1a- aldosterone-lysine Heyns adduct; 1b – aldosterone-lysine Schiff base.

In the extracted ions chromatograms obtained from lysine and HSA incubations, for the *m/z* corresponding to Heyns adduct and Schiff base (489.2959), it was possible to detect multiple signals with distinct retention times. In fact, Heyns adduct and Schiff base are isomeric structures that will provide isobaric ions (figure 1), which can explain the spectra obtained. The similarity of the tandem mass spectra obtained for the different signals prevented the distinction of these two adducts by MS (figure 2).

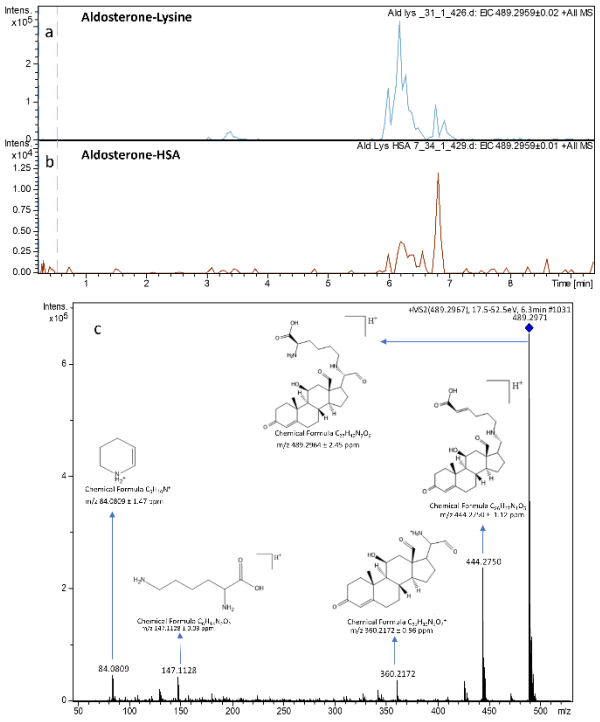

Figure 2. - extracted ion chromatogram at m/z 489.2959 collected by positive electrospray ionization of the sample containing aldosterone and lysine; 1b - extracted ion chromatogram at m/z 489.2959 collected by positive electrospray ionization of the sample containing aldosterone and HAS; 2c - MS/MS spectra of MS/MS 489.2964±2.4 ppm with a retention time of 6.3 minutes, corresponding to the aldosterone-lysine Heyns adduct, obtained from the extracted ion chromatogram at m/z 489.2959 collected by positive electrospray ionization of the sample containing aldosterone and lysine.

Therefore, two methodologies were used to distinguish the Heyns adducts from the Schiff bases: i) addition of hydroxylamine to form the respective oxime from the Heyns adducts; and ii) addition of sodium cyanoborohydride to selectively reduce the Schiff bases. Since the aldosterone-lysine Heyns adduct has two aldehydes, if a product corresponding to the Heyns adduct modified by two hydroxylamine is detected, then Heyns adduct formation can be confirmed. However, after performing the extracted ion chromatogram of *m/z* corresponding to the possible oxime-modified aldosterone-lysine Heyns adduct, no evidence was obtained for its formation. Although this was an unexpected result, possible steric restraints could explain the difficulty of the reaction of hydroxylamine with the aldehyde groups of these adducts.

Considering this last result, to attest the formation of aldosterone-lysine Heyns adducts, a selective reduction of the amide bond of the Schiff base adduct with sodium cyanoborohydride was performed. This allowed to identify the signal *m/z* 491.3130±3.1 ppm, compatible with the protonated aldosterone-lysine reduced Schiff

base. The formation of this reduced adduct was accompanied by the disappearance of the signal with longer retention time observed in the extracted ion chromatogram at *m/z* 489.2959, suggesting that the signal with shorter retention time, observed in reaction with and without reduction, corresponds to the aldosterone-lysine Heyns adduct (figure 3).

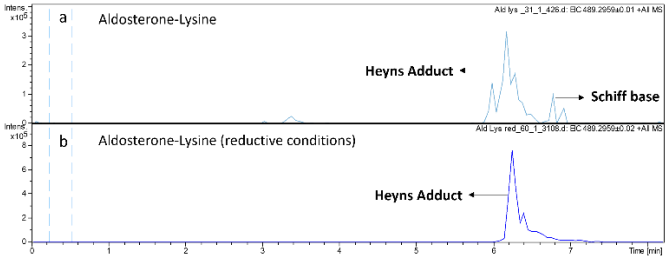

Figure 3. 3a -Extracted ion chromatogram at m/z 489.2959 collected by positive electrospray ionization of the sample containing aldosterone and lysine; 3b - extracted ion chromatogram at m/z 489.2959 collected by positive electrospray ionization of the sample containing aldosterone, lysine and sodium cyanoborohydride, evidencing the disappearance of the signal with longer retention time.

The reaction mixtures obtained from the incubation reaction between lysine and aldosterone were more complex than anticipated. Therefore, it was investigated if other products besides Schiff base and Heyns products could be formed during this reaction. Considering the aldosterone-lysine Heyns adduct's structure, it is possible for a bis adduct and a ring closure adduct to form. The first can occur intermolecularly, upon Heyns adduct reaction with one additional lysine, while the ring closure adduct can occur intramolecularly, upon Heyns adduct reaction with an α-amine group of the lysine (figure 4).

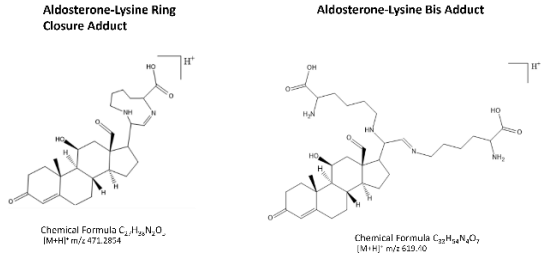

Figure 4 -Aldosterone-lysine ring closure adduct and bis adduct structures, formulas and $[M+H]^+$ m/z values.

After performing the extracted ion chromatogram of *m/z* corresponding to the aldosterone-lysine bis adduct, no evidence for its formation was obtained either in lysine or HSA incubations. However, in lysine/aldosterone incubations obtained upon stabilization with sodium cyanoborohydride, it was observed a signal consistent with the formation of a reduced bis adduct (figure 5). Regarding the aldosterone-lysine ring closure adduct, evidence for its formation under reductive and nonreductive conditions were searched, and both the ring closure adduct and the reduced ring closure adduct were detected under nonreductive and reductive conditions, respectively (figure 6).

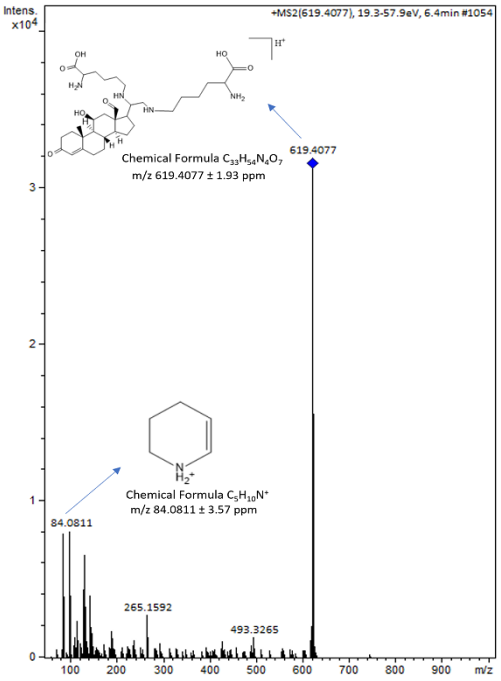

Figure 5. MS/MS spectra of MS/MS 619.4077±1.9 ppm with a retention time of 7.0 minutes, corresponding to the aldosterone-lysine bis adduct, obtained from the extracted ion chromatogram at m/z 619.4065 collected by positive electrospray ionization of the sample containing aldosterone, lysine, and sodium cyanoborohydride.

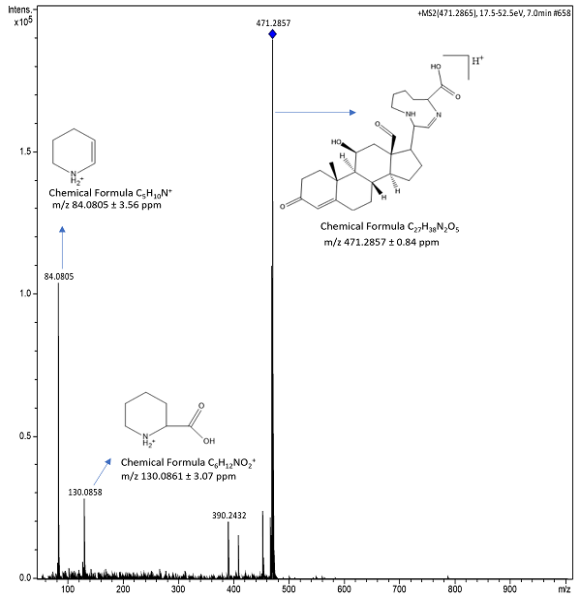

Figure 6. MS/MS spectra of MS/MS 471.2857±0.8 ppm with a retention time of 7.0 minutes, corresponding to the aldosterone-lysine ring closure adduct, obtained from the extracted ion chromatogram at m/z 471.2854 collected by positive electrospray ionization of the sample containing aldosterone and lysine.

These results suggest that aldosterone has the ability to covalently modify proteins, yielding an irreversible (stable) Heyns adduct. This can have repercussions in terms of the biomonitoring of this endogenous metabolite but also in its mode of action as a hypertensive metabolite. Therefore, it was investigated if the formation of Heyns adducts was a general ability of other hypertensive molecules bearing an acyloin group. Hence, prednisolone, dexamethasone, cortisol, and corticosterone, all corticosteroids containing an acyloin group, were incubated with lysine and HSA using exactly the same experimental conditions used for aldosterone incubations. Evidence for the formation of products consistent with Heyns adducts and Schiff bases were obtained for prednisolone, dexamethasone, cortisol and corticosterone incubations with lysine and HSA.

In the particular case of cortisol, these results constitute the first report based on MS of what was previously suggested by *Bucala et al. [14]*, that cortisol could covalently modify blood proteins to form Schiff base and Heyns adduct. As for aldosterone, cortisol and corticosterone, which are all important steroids related to elevated blood pressure, the demonstration of their ability to covalently modify proteins strongly supports the hypothesis previously made that this methodology can be used to monitor multiple HTN-related steroids, which is anticipated to increase the HTN differential diagnose ability of this method.

3.2. Lysine Heyns adduct enrichment

As mentioned before, one of the key problems of monitoring the formation of covalent adducts *in vivo* is their relatively low concentration *in vivo*. In fact, the fraction of non-adducted protein is usually much higher when compared with the adducted fractions [13]. Therefore, for *in vivo* monitoring of covalent adducts, enrichment procedures are often used before their detection by MS methodologies.

Once the formation of lysine Heyns adducts formed with aldosterone, dexamethasone, prednisolone, cortisol, and corticosterone were evidenced both in HSA and lysine incubations, it was investigated if this type of adducts could be enriched using hydrazide-based chemistry. To test this hypothesis, *UltraLink Hydrazide* resin was added to lysine/aldosterone and HSA/aldosterone incubations (the latter following digestion to amino acids). The procedure involved 3 steps: 1) coupling step, where the reaction between the aldehyde and the hydrazine is promoted; 2) washing, where analytes that were not coupled in the previous step are going to be removed; and 3) elution step, where the parent aldehyde will be regenerated from the hydrazide resin, enabling the selective recovery of the aldehyde-containing molecule.

Since multiple experimental procedures are available in the literature, multiple conditions were tested in parallel (see table 1 in Materials and Methods). The enrichment of Heyns adduct was monitored by analyzing the elution solutions when compared to a solution that was not subject to this enrichment procedure.

After analyzing the elution solutions by LC-HRMS, it was not possible to detect any signals that could be from the Heyns adduct for any of the conditions tested. Considering the results obtained, the solutions collected after the coupling step were also analyzed by LC-HRMS where, once more, no signals that could be from the Heyns adduct were detected, allowing to conclude that the coupling step is efficient. However, since no Heyns adducts are being recovered from the resins, it is possible to conclude that the elution step is not being efficient. Given the reported advantages of using aniline catalysis to accelerate the oxime/hydrazone formation reaction [16], it is possible that this can also help on the elution step. However, to avoid aniline contamination in the HRMS system, an acidification step prior to analysis has to be tested.

Even though this enrichment procedure was not fully successful, the results suggest that it can be very useful for detecting Heyns adducts in complex samples, and further optimization is required.

3.3. Histone modification by aldosterone: *ex vivo* and *in vitro*

It has been proven that corticosteroids can modify proteins, such as HSA, by forming protein covalent adducts with lysine residues. This can significantly impact biomonitoring the long-term exposure to aldosterone and other corticosteroids, which can be endogenous metabolites or therapeutic drugs. However, the covalent modification of proteins can also be a molecular mechanism underlying the molecular mechanisms of the activity/toxicity of corticosteroids.

In the specific example of aldosterone, when bound to the MR, the resulting complex is translocated to the nucleus to act as a transcription factor [6]. In the nucleus are histones, which are proteins with lysine residues, making them susceptible to aldosterone modifications.

To investigate if histones can be modified by aldosterone, two experiments were performed: 1) human kidney HK2 cells were exposed to aldosterone, and then their histones were isolated to see if any modifications had occurred; 2) *in vitro* modifications of commercially available histones (H2A, H2B, H3, H4, and octamers) with aldosterone.

For *ex vivo* experiments, histones were isolated using acid extraction procedure, and following histone's quantification, it was possible to conclude that the isolation procedure was successful. However, it can still be optimized since low protein concentrations were

obtained in some of the samples. To investigate the aldosterone-derived modifications, both *ex vivo* and *in vitro*, histones were analyzed by LC-HRMS/MS by a proteomic bottom-up approach, following a 2h trypsin digestion. In the *ex vivo* samples, it was possible to identify canonical histone with good coverage; however, no aldosterone-derived modification was also identified. Very low coverages were obtained in the *in vitro* samples, suggesting that the conditions used for *in vitro* modification (namely, the volume of organic solvent used to dissolve aldosterone) might have promoted protein precipitation. Therefore, it was not surprising that no aldosterone-derived modification was identified in the *in vitro* samples.

4. Conclusion and Future Work

It has been proven by LC-HRMS that corticoids containing an acyloin functional group can form stable covalent adducts with proteins stemming from the stabilization of Schiff bases via Heyns rearrangement. This fact is expected to have repercussions not only for the biomonitoring of this class of compounds but also for their activity and toxic effects.

In view of the expected difficulties in detecting these adducts in levels expected *in vivo*, a methodology to enrich lysine-modified Heyns adduct derived from aldosterone prior to their analysis by LC-HRMS was tested. Whereas further optimization is required, the results obtained suggest that this enrichment procedure can be very useful for detecting these adducts in biologic samples.

As stated above, the covalent modification of proteins by aldosterone can also have a role in the activity and for the development of comorbidities associated with the high levels of this corticosteroid. In fact, aldosterone, when bound to the MR, is translocated to the nucleus and acts as a transcription factor [6]. Taking into consideration that histones are nuclear proteins very rich in lysine residues, it was hypothesized that histones could be prone to aldosterone modifications.

To test this hypothesis, a cell line derived from the epithelial line of the renal proximal tubule (HK2) was exposed to aldosterone. Following histone isolation by acid extraction, these proteins were trypsin digested and analyzed by LC-HRMS, following a bottom-up proteomics strategy. However, no aldosterone-derived modifications were identified. Despite this negative result by this strategy, this does not necessarily mean that the covalent modification of lysine residues of histones by aldosterone, via Heyns adduct formation, did not happen *ex vivo*. Therefore, aldosterone-derived modifications in histones should also be searched using the metabolomics-inspired strategy, which has proven to be more effective in identifying low abundant covalent adducts than the usual database search engines.

One of the shortcomings of this project was the unavailability of the HPLC equipment, not allowing to proceed with product isolation, and consequently to evaluate the extension of the pathway involving the Schiff base stabilization via Heyns rearrangement.

Taking into consideration one of the initial goals of this project, determining if aldosterone was capable of reacting with blood proteins with protein covalent adducts formation, even though it was possible to identify modification on HSA, it is still needed to determine the extension of this modification pathway, allowing to understand better the products formed during this reaction and learn the extent of the reaction. Therefore, a semi-preparative HPLC isolation step should be required for this evaluation.

Further optimizations of the hydrazine-based enrichment of aldosterone-derived Heyns adducts procedure are needed. The use of a hydroxylamine and aniline elution buffer must be tested, and/or the use of other hydrazine-based resins (with distinctive structures) need to be tested. Heyns adduct is very sterically hindered, which might prevent its attachment to the hydrazide resin. Thus, the enrichment procedure should be tested with another resin with a structure that might allow access to a very sterically hindered product.

After successfully developing an enrichment methodology, blood samples collected from hypertensive patients can be enriched, and the concentration of covalent adducts formed between aldosterone and hemoglobin measured, allowing to see if there is any correlation between aldosterone levels and HTN or resistance to treatment and/or CKD.

Lastly, it is important to study these modifications and their impact on biological pathways, their repercussions, and their involvement in disease development.

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6. Bibliography

[1] CDC. "Facts About Hypertension." https://www.cdc.gov/bloodpressure/facts.htm (accessed 09/09, 2021).

[2] WHO. "Noncommunicable diseases: Hypertension." https://cutt.ly/TxRdNec (accessed 24/03, 2021).

[3] F. H. Messerli, B. Williams, and E. Ritz, "Essential hypertension," (in eng), *Lancet,* vol. 370, no. 9587, pp. 591-603, Aug 18 2007, doi: 10.1016/s0140-6736(07)61299-9.

[4] J. J. Noubiap, J. R. Nansseu, U. F. Nyaga, P. S. Sime, I. Francis, and J. J. Bigna, "Global prevalence of resistant hypertension: a meta-analysis of data from 3.2 million patients," (in eng), *Heart,* vol. 105, no. 2, pp. 98-105, 01 2019, doi: 10.1136/heartjnl-2018-313599.

[5] R. M. Carey *et al.*, "Resistant hypertension: detection, evaluation, and management: a scientific statement from the American Heart Association," *Hypertension,* vol. 72, no. 5, pp. e53-e90, 2018.

[6] E. M. Freel, "Mechanisms of Hypertension: The Expanding Role of Aldosterone."

[7] C. Savoia, M. Volpe, G. Grassi, C. Borghi, E. Agabiti Rosei, and R. M. Touyz, "Personalized medicine—a modern approach for the diagnosis and management of hypertension," *Clinical Science,* vol. 131, no. 22, pp. 2671-2685, 2017.

[8] D. A. Calhoun, "Use of aldosterone antagonists in resistant hypertension," *Progress in cardiovascular diseases,* vol. 48, no. 6, pp. 387-396, 2006.

[9] R. Vardanyan and V. Hruby, "Chapter 27 - Steroid Hormones," in *Synthesis of Best-Seller Drugs*, R. Vardanyan and V. Hruby Eds. Boston: Academic Press, 2016, pp. 459-493.

[10] T. J. Wang *et al.*, "Multiple Biomarkers and the Risk of Incident Hypertension."

[11] D. C. Liebler, "Protein damage by reactive electrophiles: targets and consequences," *Chemical research in toxicology,* vol. 21, no. 1, pp. 117-128, 2008.

[12] G. W. Preston and D. H. Phillips, "Protein Adductomics: Analytical Developments and Applications in Human Biomonitoring."

[13] H. Carlsson, S. Rappaport, and M. Törnqvist, "Protein Adductomics: Methodologies for Untargeted Screening of Adducts to Serum Albumin and Hemoglobin in Human Blood Samples."

[14] R. Bucala, J. Fishman, and A. Cerami, "Formation of covalent adducts between cortisol and 16 alpha-hydroxyestrone and protein: possible role in the pathogenesis of cortisol toxicity and systemic lupus erythematosus," (in eng), *Proc Natl Acad Sci U S A,* vol. 79, no. 10, pp. 3320-4, May 1982, doi: 10.1073/pnas.79.10.3320.

[15] C. Charneira, J. Nunes, and A. M. Antunes, "16α-Hydroxyestrone: mass spectrometry-based methodologies for the identification of covalent adducts formed with blood proteins," *Chemical Research in Toxicology,* vol. 33, no. 8, pp. 2147-2156, 2020.

[16] J. Huang *et al.*, "Highly efficient release of glycopeptides from hydrazide beads by hydroxylamine assisted PNGase F deglycosylation for N-glycoproteome analysis," *Analytical chemistry,* vol. 87, no. 20, pp. 10199-10204, 2015.