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Simultaneous measurement of protein-bound 3-chlorotyrosine and homocitrulline by LC–MS/MS after hydrolysis assisted by microwave: Application to the study of myeloperoxidase activity during hemodialysis

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ABSTRACT

A high degree of uremia is common in patients with end-stage renal disease and has been linked to the development of chronic inflammation and cardiovascular diseases. In conditions where transplantation is not possible, uremia can be reduced by hemodialysis although the repeated interventions have been implicated in loss of renal function, partially as a result of chronic inflammation and/or oxidative stress processes. In this context, it has been suggested that myeloperoxidase (MPO) can contribute to the oxidative stress during hemodialysis and to the cardiovascular risk. Protein damages due to MPO activity have never been assessed during hemodialysis although two of its reaction products, 3-chlorotyrosine and homocitrulline, are of interest. Indeed, the first one is a specific product of MPO activity and the formation of the second one could be catalyzed by MPO. In order to analyze these products in plasma proteins, a total hydrolysis method followed by liquid chromatography mass spectrometry analysis was developed. Different conditions of hydrolysis were tested and the optimized procedure was assessed for complete hydrolysis and artifactual chlorination. Finally, the method was used for analyzing 3-chlorotyrosine and homocitrulline in plasma proteins during a hemodialysis session in fifteen patients and data were related to measurements of MPO concentration and activity. Both increases in MPO activity and protein-bound 3-chlorotyrosine were observed, highlighting the involvement of MPO in oxidative stress during hemodialysis and further demonstrating the link between hemodialysis and cardiovascular diseases.

1. Introduction

End-stage renal disease (ESRD) is associated with high cardiovascular risk, poor survival rate and considerable long term morbidity. In this context, uremia has been linked to chronic inflammation and cardiovascular disease. Furthermore, 50 percent of premature deaths in ESRD patients are due to cardiovascular events [1,2]. It has also been proposed that hemodialysis

Abbreviations: AOPPs, advanced oxidation protein products; BSA, bovine serum albumin; Cl-Tyr, 3-chlorotyrosine; ESI, electrospray ionization; ESRD, end-stage renal disease; FA, formic acid; Hcit, homocitrulline; HD, hemodialysis; LDL(s), low-density lipoprotein(s); Lys, lysine; MoxLDL(s), myeloperoxidase modified low-density lipoprotein(s); MPO, myeloperoxidase; MS/MS, tandem mass spectrometry; QTOF, quadrupole time-of-flight; RRLC, rapid resolution liquid chromatography; SIEFED, specific immunological extraction followed by enzymatic detection; Tyr, tyrosine

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(HD) triggers inflammation as a result of exposure of blood to the bioincompatible system stimulating monocyte and macrophage cells [3,4]. These processes induce the production of proinflammatory cytokines and contribute to the maintenance of oxidative stress. The latter has been linked to activation of neutrophils and production of interleukins, leading to a release of reactive oxygen species (ROS). In this context, enzymes such as myeloperoxidase (MPO) have been implicated in the development of cardiovascular and chronic kidney diseases [5–7]. MPO is a member of the superfamily of mammalian heme peroxidase enzymes secreted by activated phagocytes, which catalyzes hypochlorous acid (HOCl) formation in the presence of hydrogen peroxide (H_2O_2) and chloride anion (Cl^-) [8]. The MPO/H_2O_2/Cl^- system can oxidize DNA, RNA, lipids or proteins generating, for example, modified/oxidized (lipo-) proteins [8–10]. MPO can also target the kidneys through HOCl production [11,12]. Wu et al. [13] reported that MPO concentration could serve as a marker of oxidative stress during HD and Himmelfarb et al. [14] showed that MPO concentration increases during HD. As a matter of fact, many studies focused on MPO during HD, pointing out the potential deleterious effect of this enzyme related to cardiovascular complications and loss of renal function. However, to the best of our knowledge, proteins damages due to MPO activity during HD session have never been investigated.

The oxidation of proteins and amino acid residues such as tyrosine (Tyr), leads to 3-chlorotyrosine (Cl-Tyr), di-tyrosine and peptide cross-linking which are called advanced oxidation protein products (AOPPs). AOPPs are formed by ROS and notably by the chlorinated oxidant, HOCl derived from the chlorination activity of MPO [15]. In addition, it has been shown that AOPPs are increased in both patients with coronary artery disease [16] and receiving HD [17]. These data suggest that AOPPs could play a role in the physiopathology of inflammation in chronic renal disease especially as mediators of inflammation [18–20]. We therefore focused on the specific and non-specific modifications of proteins produced by MPO during HD in order to better document the deleterious effects of MPO activity. The MPO reaction products selected were Cl-Tyr and homocitrulline (Hcit). Cl-Tyr is a specific product of oxidation of Tyr by MPO and Hcit is produced by the reaction of lysine (Lys) residues and cyanate resulting from urea decomposition and possibly from thiocyanate oxidation by MPO [21].

Total protein hydrolysis followed by liquid chromatography–tandem mass spectrometry analysis (LC–MS/MS) is a common and easy method to detect and quantify amino acids from proteins. Heating the protein at 110 °C for 12–24 h in hydrochloric acid (6 N) has frequently been used for this purpose [22]. The development of microwave ovens resulted in more rapid procedures using higher temperatures (about 160 °C) for shorter periods of time (about 20 min) [23,24]. This technique takes profit of microwave energy and of the vapor phase of HCl to hydrolyze proteins and does not influence the effectiveness of the method [25]. Unfortunately, both methods can lead to the production of Cl-Tyr as a result of the presence of HCl and are also able to attack some residues such as Hcit.

The present study is devoted to the development of an easy and rapid hydrolysis method for the detection by LC–MS/MS of both Cl-Tyr and Hcit from plasma proteins. Acid and alkaline hydrolyzes were tested and the acid method was optimized and validated. This method was thereafter applied to measure Cl-Tyr and Hcit during HD session in 15 patients and data were compared to the concentration and the activity of MPO in plasma during HD.

2. Materials and methods

2.1. Solvents and reagents

Formic acid (FA), high-purity LC–MS methanol (MeOH), potassium cyanate (KOCN), sodium hydroxide and HOCl were purchased from Merck (Darmstadt, Germany). Butanolic-HCl (3 M), [^{13}C, ^{15}N] lysine and [$^{13}C_9$] tyrosine, bovine serum albumin (BSA) and a standard mix of amino acids were obtained from Sigma-Aldrich (St Louis, MO, USA). Hydrochloric acid was purchased from VWR (Leuven, Belgium). Water was purified using a Milli-Q purification system from Millipore (Bedford, MA, USA).

2.2. Samples, subjects and ethics statement

Plasma samples from healthy donors were used for development and optimization of the method. For the measurement of plasma protein oxidation and plasma MPO activity/antigen, 15 patients on chronic maintenance HD therapy were studied. Blood was drawn just before dialysis (T0) after access cannulation, and from efferent dialyzer lines at 30 (T1) and 60 min (T2) after initiation of dialysis and at the end of treatment (T3). The median age of the patients was 77 (73–81) years. The etiology of ESRD was diabetic nephropathy in 3 patients, polycystic kidney disease in 2 patients, nephroangiosclerosis in 6 patients, IgA nephropathy in 1 patient and mixed nephropathy (diabetes and nephroangiosclerosis) in 3 patients. Table 1 reports detailed characteristics of HD and of each patient. This study conforms with the Declaration

Table 1
Characteristics of HD and patients.

Patient number	Gender	Age (Years)	HD duration (hours/week)	Dialyzer	Access	Smoking status	Diabetes
1	Male	80	10.5	Nephral 300	FAV	NS	No
2	Female	64	9	FB 190	Hemocath	S	Yes
3	Male	73	9	FX 80	Hemocath	NS	Yes
4	Female	88	9	HF 17	Hemocath	NS	Yes
5	Female	77	10.5	FX 80	FAV	NS	No
6	Female	62	9	FB 190	Hemocath	S	No
7	Male	81	9	HF 17	Hemocath	NS	No
8	Female	87	6	FB 190	Hemocath	NS	No
9	Male	76	9	FX 80	FAV	NS	No
10	Female	95	9	FB 190	Hemocath	NS	No
11	Male	73	10.5	Nephral 300	Hemocath	NS	Yes
12	Male	82	9	FX 80	FAV	NS	No
13	Male	76	9	HF 17	FAV	NS	No
14	Female	81	9	FX 80	Hemocath	NS	Yes
15	Male	77	9	FX 80	FAV	S	Yes

Nephral (AN69ST): High flux, FB 190 (Cellulose triacetate): Low flux, FX 80 (Polysulfone): High flux, HF 17 (Polyphenylene): High flux.
Hemocath: long-term tunneled catheter, FAV: arterio–venous graft. NS: non-smoker and S: smoker.

of Helsinki and its protocol was approved by the Ethics Committee of the ISPPC (“Intercommunale de Santé Publique du Pays de Charleroi”) Hospital. Finally, all subjects gave their written informed consent.

2.3. Preparation of labeled internal standard

[^{13}C, ^{15}N] Hcit was synthesized by overnight mixing at 37 °C, 2 mM of [^{13}C, ^{15}N] Lys in PBS buffer pH 7.4 with 6 mM of KOCN. The quantity of [^{13}C, ^{15}N] Hcit was calculated by interpolating the resulting concentration of [^{13}C, ^{15}N] Lys on a standard curve after derivatization of both [^{13}C, ^{15}N] Lys and [^{13}C, ^{15}N] Hcit. [$^{13}C_9$] Cl-Tyr was synthesized by mixing [$^{13}C_9$] Tyr (2 mM in a solution of phosphoric acid 50 mM and NaCl 100 mM) and 2, 4 or 6 mM of HOCl added progressively under gentle agitation. The quantity of [$^{13}C_9$] Cl-Tyr was calculated by interpolating the resulting concentration of [$^{13}C_9$] Tyr on a standard curve after derivatization of both [$^{13}C_9$] Tyr and [$^{13}C_9$] Cl-Tyr.

2.4. Modification of plasma for method development and optimization

Plasma from healthy donors (control) was modified in vitro by cyanate and/or HOCl (each 100 μM) for 4 h at 37 °C, producing Hcit and Cl-Tyr respectively.

2.5. Protein hydrolysis for Hcit and Cl-Tyr detection

2.5.1. Alkaline hydrolysis

Hydrolysis in alkaline conditions was tested as the method is known to avoid artifactual chlorination of tyrosine during sample preparation. Hydrolysis was performed by heating 20 μL of modified plasma in 200 μL NaOH 4 M at 120 °C during 24 h such as previously described [26,27]. 10 μL of internal standard (^{13}C–^{15}N lysine, 3.4 μM and $^{13}C_9$ tyrosine 34 μM, final concentration) were also added before the hydrolysis. Samples were then brought to room temperature, evaporated to dryness under nitrogen stream and derivatized as described below.

2.5.2. Acid hydrolysis

An acid hydrolysis method was developed and optimized for the detection of both Hcit and Cl-Tyr in plasma proteins. The latter was assisted by a microwave oven. To evaluate the operating conditions, several durations of hydrolysis were tested on control and modified plasma samples. 20 μL of samples were placed in 3 mL hydrolysis quartz vial. 10 μL of internal standard ([^{13}C–^{15}N] Lys, 3.4 μM and [$^{13}C_9$] Tyr 34 μM, final concentration) and 200 μL HCl 6 N supplemented with phenol 0.05% (to prevent the halogenation of Tyr) were added to the vial. Acid hydrolyzes using a StartS microwave oven and a protein hydrolysis reactor (Milestone, Italy), were carried out by heating to 110 °C over 5 min and maintaining the temperature at 110 °C during 5, 10, 20, 30 min, 1 or 4 h. A temperature sensor was connected to the microwave software in order to set the power of microwave to ensure that the temperature was reached or maintained. Maxima of power were set at 800 W during the increase phase (the first 5 min to reach 110 °C) and at 500 W to maintain the temperature. After cooling (30 min), sample solutions were transferred from vials to 2 mL centrifugation tubes, vials were washed with 300 μL methanol and solutions were dried under nitrogen.

2.6. Assessment of the total protein hydrolysis by the optimized method

In order to assess the completion of hydrolysis, the optimized acid method was compared to the method developed by Damm et al. [25] who validated a microwave-assisted hydrolysis at 160 °C. First, 20 μL of 5 independent samples of BSA at 70 mg/mL were placed with 200 μL of HCl 6 N supplemented with phenol 0.05% during 30 min at 110 °C. Five other samples were hydrolyzed at 160 °C during 5 min for comparison. After cooling, the 10 samples were dried, derivatized, evaporated and reconstituted as mentioned below. In the meanwhile standard curves of amino acids were constructed between 10 and 1000 nM. Briefly, 10 μL of the standard mix were evaporated under gentle stream of nitrogen before derivatization.

2.7. Assessment of the recovery of Cl-Tyr and Hcit

The recoveries of Hcit and Cl-Tyr were assessed by spiking 20 μL of plasma from healthy donors with either [^{13}C, ^{15}N] Lys and [$^{13}C_9$] Tyr or [^{13}C, ^{15}N] Hcit and [$^{13}C_9$] Cl-Tyr. An amount corresponding to 50 picomoles of each labeled standard was added to 20 μL of plasma. Samples were hydrolyzed by the optimized acid method (30 min at 110 °C in HCl 6 N supplemented with phenol 0.05%).

2.8. Amino acids derivatization

Dried samples were derivatized by butanolic-HCl at 60 °C for 15 min to obtain butyric esters of amino acids. Excess of butanolic-HCl was evaporated under nitrogen [28,29]. The samples were diluted in 1 mL FA 0.1% and 10 μL were injected into LC–MS/MS.

2.9. LC–MS/MS analysis

Analyses were performed using a rapid resolution liquid chromatography (RRLC) system 1200 series from Agilent Technologies (Santa Clara, CA, USA). Amino acid separation was performed on a Fused Core Ascentis® Express C18 column (100 × 2.1 mm I.D., 2.7 μm particle size, 160 Å) from Supelco (Bellefonte, PA, USA) using a FA/MeOH gradient. A 6520 series electrospray ionization (ESI)-quadrupole time-of-flight (QTOF) from Agilent Technologies was used for the MS/MS analyses. ESI-QTOF target-MS/MS parameters were: Positive mode; extended dynamic range mode (2 GHz); gas temperature of 350 °C; drying gas of 9 L/min; nebulizer pressure of 45 psig; capillary voltage of 4500 V; fragmentor 175 V; fixed collision energy (CE) were optimized for each residue and were: Lys and Lab-lys: 18 V (precursor ion mass-to-charge (m/z), 203.1754 and 205.1758 respectively); Hcit and Lab-Hcit: 17 V (precursor ion m/z, 246.1812 and 248.1879 respectively); Tyr and Lab-Tyr: 17 V (precursor ion m/z, 238.1438 and 247.1740 respectively); Cl-Tyr and Lab-Cl-Tyr: 17 V (precursor ion m/z, 272.1048 and 281.1350 respectively); MS scan range and rate: 50–1000 at 8 spectra/s; MS/MS scan range and rate: 50–500 at 3 spectra/s; isolation width: medium mode (± 4 m/z). Data were acquired using MassHunter Acquisition® software (Agilent Technologies) and analyzed by MassHunter Qualitative Analysis® software (Agilent Technologies). The analyzed transitions were m/z: 203.1754 → 84.0800, 130.0870, 186.1490 for Lys; 205.1758 → 85.0840, 131.0900, 187.1522 for Lab-lys; 246.1812 → 127.0850 for Hcit; 248.1879 → 128.0850 for Lab-Hcit; 238.1438 → 136.0757 for Tyr; 247.1740 → 144.1025 for Lab-tyr; 272.1048 → 170.0367 for Cl-Tyr; and 281.1350 → 178.0636 for Lab-Cl-Tyr. The precursor masses correspond to the butanolic esters of the amino acid residues.

2.10. Cl-Tyr and Hcit analysis of plasma proteins from patients

Acid hydrolysis of plasma samples from the 15 patients was performed using the optimized conditions described earlier.

2.11. Activity of MPO and total MPO assay

Activity of MPO was measured using the "Specific Immunological Extraction Followed by Enzymatic Detection" (SIEFED) method as described by Franck et al. [30] for human fluids. Total MPO content in plasma was measured using a sandwich human MPO ELISA kit (ELIZEN MPO, Zentech SE, Belgium). These analyses make possible to calculate the active MPO fraction from the total MPO and the specific activity of MPO (activity/antigen ratio).

2.12. Statistics

Data were analyzed using the SigmaStat® 3.5 software (Systat®, San Jose, CA). Median values with range (25–75%) were given because the data were not normally distributed (Kolmogorov–Smirnov). Non-parametric tests with the Friedman Repeated Measures Analysis of Variance on Ranks test, which includes a chi-square test, were the most appropriate for our study design. This test displays the results of the chi-square, degrees of freedom and p value. Outcomes were considered as statistically significant with a two-tailed $p < 0.05$.

3. Results

3.1. Hcit and Cl-Tyr analysis optimization and measurement

After chemical protein hydrolysis, amino acid analysis by LC/detection is a common method to quantify amino acid residues. Derivatization is not required in the context of mass spectrometry but our experiments showed that the signal and the resolution of amino acid on the LC column could be improved by derivatization (data not shown). Therefore, derivatization was always performed before amino acids analysis. Alkaline hydrolysis was selected for the hydrolysis of tyrosine residues as it avoids artifactual chlorination of this amino acid [27]. However, the alkaline medium is not a good choice for the simultaneous detection of both Cl-Tyr and Hcit, as Hcit residues are hydrolyzed in corresponding Lys residues such as observed in our experiments (see supplementary data, Fig. S1). For this reason, a method using acid hydrolysis with microwave heating at 110 °C was developed and optimized. Kinetics of appearance of Hcit and Cl-Tyr were determined by measuring both Hcit/Lys and Cl-Tyr/Tyr ratios in control and modified plasma samples. An alternative hydrolysis method was developed by using a temperature of 110 °C instead of 160 °C as previously described by Damm et al. [25] in order to avoid the formation of Cl-Tyr during hydrolysis. Indeed, the production of Cl-Tyr frequently occurs by heating proteins at high temperature in presence of HCl. Acid hydrolysis was carried out by heating modified plasma samples to 110 °C over 5 min and maintaining the temperature at 110 °C during 5, 10, 20, 30 min, 1 or 4 h. Fig. 1 shows the data for Cl-Tyr detection from HOCl-modified plasma (A) and for Hcit in cyanate modified plasma (B). These results indicate that both Cl-Tyr/Tyr and Hcit/Lys ratios reached a maximum after 30 min, whereas an incomplete hydrolysis of peptide bonds occurs before this time. At 1 and 4 h, variability of both ratios increased and the Hcit/Lys ratios decreased at 4 h.

3.2. Assessment of the total protein hydrolysis by the optimized method

Damm et al. [25] reported that the protein hydrolysis was not efficient when decreasing the temperature from 160 °C to 110 °C within a runtime of 5 min. In the present study, the time of hydrolysis at 110 °C was longer, using 30 min instead of 5 min. Nevertheless, the assessment of the total protein hydrolysis was performed on BSA. Fig. 2 gives the comparison of the theoretical number of amino acids from BSA with the number obtained by the method of Damm (stated as the conventional method, CONV) and by our optimized method. The results were normalized to the Val residues (CONV) as previously described [25]. Although the variability of both methods was high, no statistical difference was found between the two methods confirming that the optimized hydrolysis method is as efficient as the methods previously validated for microwave hydrolysis.

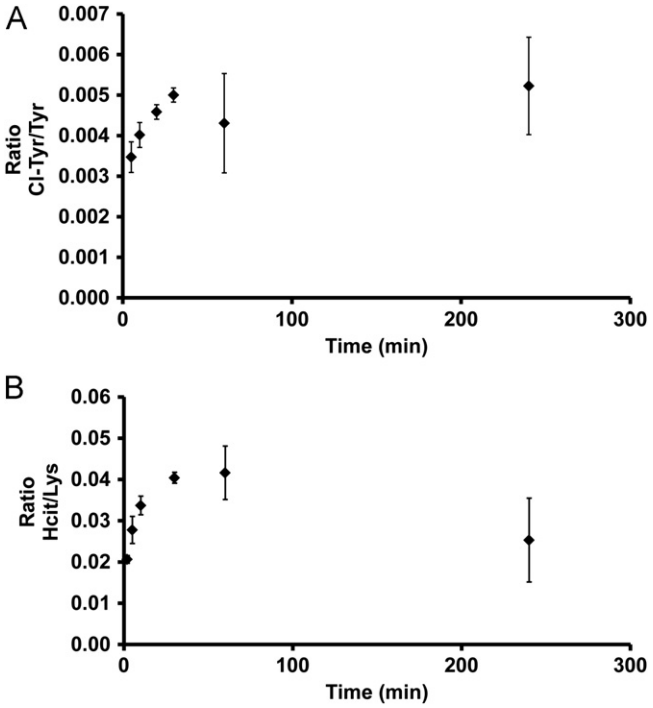

Fig. 1. Cl-Tyr/Tyr (A) and Hcit/Lys (B) ratios as a function of acid hydrolysis time (Mean ± SD, $n=3$). Plasma modified by HOCl or cyanate was hydrolyzed by heating and keeping samples at 110 °C during 5, 10, 20, 30 min, 1 or 4 h using a microwave oven. Cl-Tyr, Tyr, Hcit and Lys were then determined in the samples after derivatisation and using LC–MS/MS.

3.3. Assessment of possible artifactual Cl-Tyr formation

In order to assess that no Cl-Tyr was artifactually produced, [$^{13}C_9$] Cl-Tyr was monitored in BSA and control plasma. No labeled Cl-Tyr was detectable in any sample at any time of hydrolysis. Figs. S2 and S3 in supplementary data show the chromatograms of [$^{13}C_9$] Cl-Tyr at the different time of hydrolysis and the chromatogram of a standard of [$^{13}C_9$] Cl-Tyr and the spectrum with its product ion. Furthermore, Cl-Tyr was monitored in control plasma (data not shown) and none was detected at 30 min of hydrolysis. These results show that no artifactual Cl-Tyr is produced using the optimized method of hydrolysis.

3.4. Recoveries of Cl-Tyr and Hcit

The recoveries of Hcit and Cl-Tyr were assessed by spiking 20 μL of plasma from healthy donors with labeled standards. Samples were submitted to hydrolysis by the optimized method described above and compared to samples containing the same amount of labeled residues in PBS buffer but which were directly evaporated before derivatization. Although the variability was quite high in these experiments, the quantity of [^{13}C, ^{15}N] Hcit after hydrolysis did not differ as compared to standard curves in PBS (recovery of 96 ± 15%). Concerning [$^{13}C_9$] Cl-Tyr, 53 ± 10%

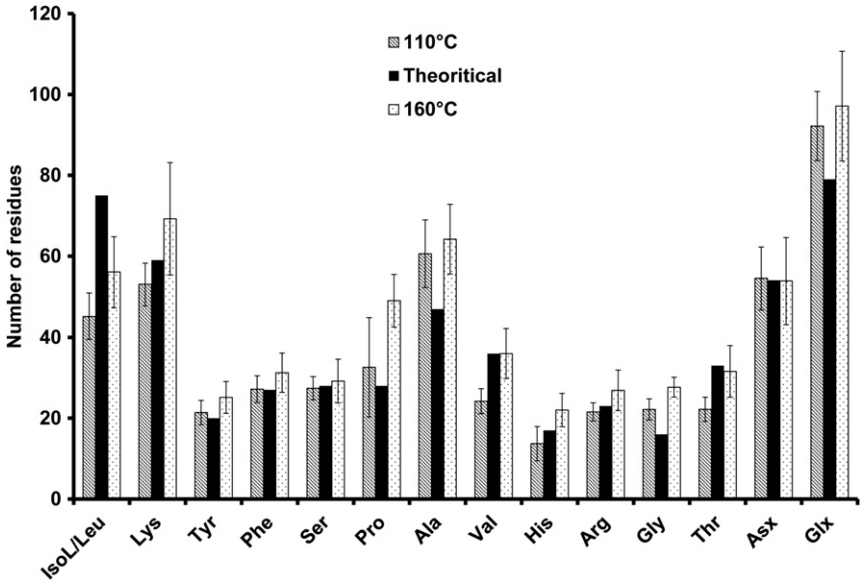

Fig. 2. Comparison of amino acid compositions of BSA using an hydrolysis method at 160 °C for 5 min (this method was used as our conventional method, CONV, light gray bars), or the optimized method developed in the present study (dark gray bars). The theoretical number of residues is shown in black. The results were normalized to the Val residues (CONV) and were expressed as mean ± SD on 5 independent samples.

were recovered after hydrolysis. However, in the same sample, 45 ± 11% of [$^{13}C_9$] Tyr were also recovered after hydrolysis. The sensitivity of the method decreased but the ratio Cl-Tyr/Tyr was not affected by hydrolysis (127 ± 32%).

3.5. Effects of HD on plasma Cl-Tyr/Tyr and Hcit/Lys ratios

Cl-Tyr and Hcit were measured during HD using the optimized method described above. The Cl-Tyr/Tyr ratio increased after 30 min [Chi-square (3)=9.8; $p=0.02$] before returning to basal levels after 60 min (Fig. 3A). Such data have never been previously reported and are of particular interest regarding the inflammatory status of the patient. As far as the carbamylation profile is concerned, the Hcit/Lys ratio continuously decreased during HD, and the difference compared to baseline reached statistical significance after 60 min [Chi-square (3)=29.6; $p<0.001$] (Fig. 3B).

3.6. Effects of HD on plasma MPO concentration and activity

To investigate the effects of MPO during HD session in patients, MPO concentration and activity were measured and correlated to Cl-Tyr and Hcit concentrations in plasma proteins during HD. Antigenic concentrations of plasma MPO, measured using the commercial ELISA kit, increased during HD to reach a maximum after 30 min and then progressively decreased [Chi-square (3)=38.6; $p<0.001$], but at the end of HD, the level of total MPO remained significantly higher than the one at T0 (Fig. 4A). Plasma MPO enzyme activity showed a similar profile (Fig. 4B). A peak of activity was observed after 30 min [Chi-square (3)=30.9; $p<0.001$] then the activity decreased till the end of HD. Interestingly, MPO activity returned to the basal level (T0) at the end of HD. However, the specific activity [the ratio between the activity (mU/ml) and the antigenic concentration (ng/ml) of MPO] decreased after 30 min [Chi square (3)=18.4; $p<0.001$] and returned to basal levels (T0) at the end of HD (Fig. 4C). Although the quantity of MPO was multiplied by a factor of 3.4, the modification in enzyme activity was actually not in the same range and increased only by a factor of 2. The results suggest that the newly released MPO was intrinsically less active although the total activity increased after 30 min.

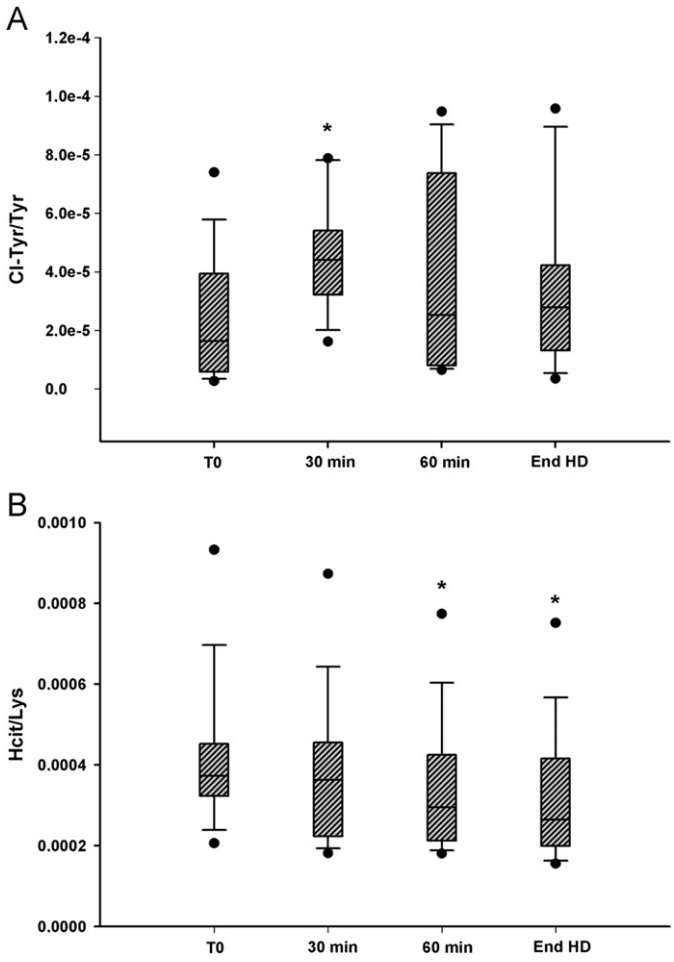

Fig. 3. Effects of HD on (A) Cl-Tyr/Tyr ratio and (B) on homocitrulline/lysine (Hcit/Lys) ratio. Ratios were determined in plasma samples taken 0, 30 and 60 min after HD initiation and at the end of HD. Values are shown as medians (25–75% range). Data comparisons with T0 were performed using a Friedman Repeated Measures Analysis of Variance on Ranks ($p=0.02$) and a Dunn's post-hoc test ($n=15$). Significant difference versus T0 ($p<0.05$).

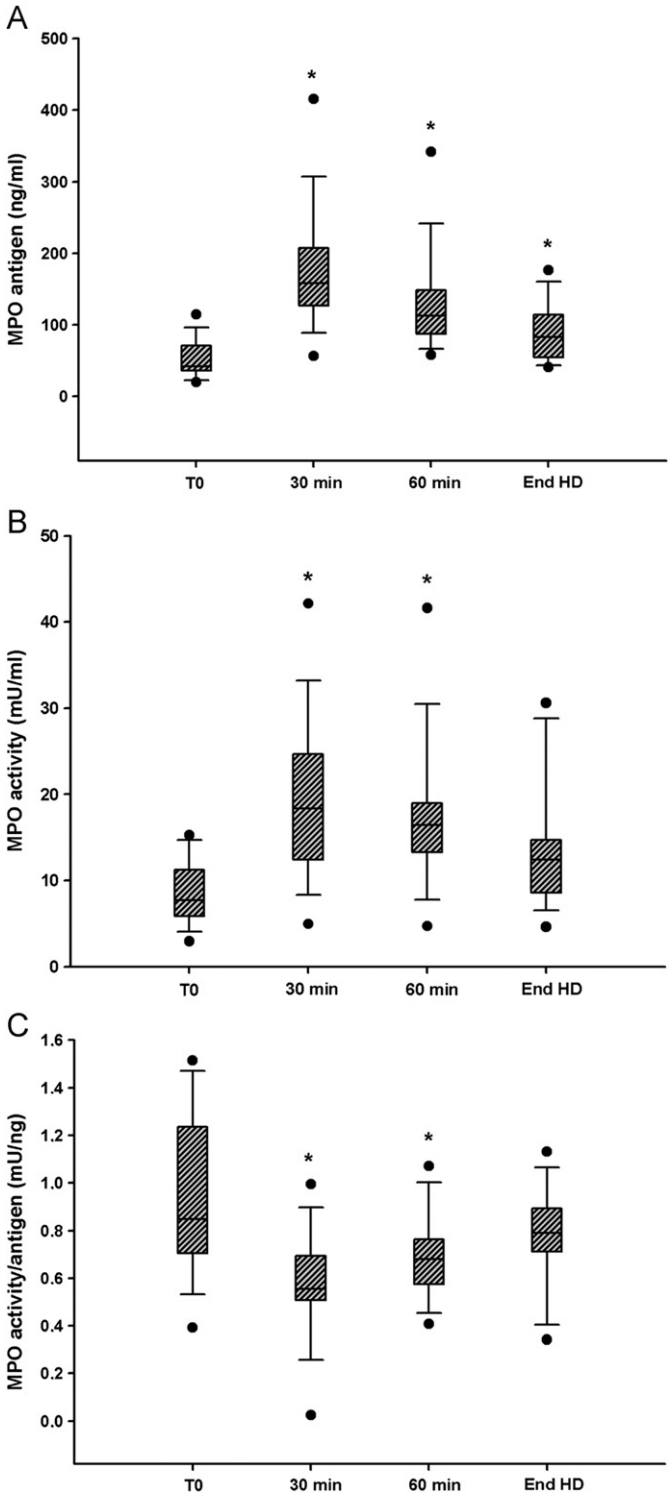

Fig. 4. Effect of HD on (A) plasma MPO concentration (ng/mL) (B) MPO activity (mU/mL) and (C) MPO specific activity (mU/ng antigen). Concentrations were determined in plasma samples taken 0, 30 and 60 min after HD initiation and at the end of HD. Values are shown as medians (25–75% range). Data comparisons with T0 were performed using a Friedman Repeated Measures Analysis of Variance on Ranks ($p < 0.001$) and a Dunn's post-hoc test ($n = 15$). Significant difference versus T0 ($p < 0.05$).

4. Discussion

Chronic uremia in patients with ESRD is related to oxidative stress and chronic inflammation, leading to ROS production. Treatment of patients with HD decreases uremia, but this intervention is less effective for reducing cardiovascular risk than kidney transplantation [31–33]. Furthermore, HD does not decrease the oxidative and inflammatory status of patients and inversely it seems to stimulate these processes in spite of the development of new dialysis membranes [3]. The complex inflammatory status and oxidative stress that occur during HD have not been fully characterized. Among the factors contributing to oxidative stress and cardiovascular risk during HD, the role of MPO has been emphasized [7], but MPO levels have never been correlated with increasing enzyme activity or with markers of MPO activity during HD.

In these conditions, a method able to easily and rapidly detect both Cl-Tyr and Hcit in plasma proteins was of great interest. Three aspects have to be considered in the development of such a method: (i) Hcit and Cl-Tyr detections are optimal, (ii) there is no artifactual Cl-Tyr production, and (iii) the hydrolysis of proteins is complete. Heating the protein in acid or alkaline conditions at 110–120 °C has been widely used for amino acid characterization. With the development of microwave ovens, acid hydrolysis using HCl at higher temperature has been developed enabling to shorten the duration of the procedure. An alkaline method was also investigated as it has been reported to prevents artifactual chlorination [27]. Unfortunately, we observed that the latter leads to hydrolyze Hcit to Lys and that it should therefore not be recommended to this purpose. Furthermore, the process is long and cannot be transposed to rapid method using a microwave oven as NaOH is unable to form any vapor phase as HCl. A method using HCl 6 N was then investigated but many modifications had to be introduced to avoid artifactual Cl-Tyr formation. Phenol was added as a tyrosine protective agent and the temperature was reduced to 110 °C. The results showed that after 30 min hydrolysis at 110 °C, Cl-Tyr and Hcit reached a plateau, and that longer times did not improve hydrolysis. Higher variation was also observed after 30 min certainly due to leaking-out of solvents caused by the prolonged irradiation under high pressure and temperatures. Efficiency of hydrolysis (Fig. 2) was confirmed by further experiments which were in accordance with previous data [25]. Furthermore, monitoring of labeled Cl-Tyr confirmed that no artifactual chlorination appears during hydrolysis. The hydrolysis method was therefore efficient to ensure a total hydrolysis of plasma proteins for the analysis of both Cl-Tyr and Hcit and could be applied to investigate the MPO activity and its deleterious effects in patients undergoing HD.

ELISA measurements of MPO in plasma samples of 15 patients during HD revealed a rapid increase in MPO concentrations after 30 min of HD. These results agree with Himmelfarb et al. [14] and with Wu et al. [13] showing that plasma MPO increased after 15 min by a factor of 2 or 4 depending on the dialysis membrane. In the present study, non-parametric statistical analysis indicated an increase in the MPO concentration independent of the dialysis membrane type. Moreover, the values observed after 30 min were far over the plasma MPO concentration measured in healthy volunteers (42 ± 15 ng/mL)[30]. Such an increase could be explained by the neutrophil stimulation and degranulation by various factors including exposure to a bioincompatible system or a physical–mechanical stress. MPO activity from patient samples, measured using the specific immunological extraction followed by enzymatic detection (SIEFED) method, followed the same trend than MPO concentrations and increased by a factor of about 2 after 30 min of HD. Both results demonstrate for the first time that MPO released during HD is active and may contribute to the oxidative stress. Furthermore, plasma MPO activity measured in the present study was always above the mean value of healthy donors (3 ± 1 mU/mL) but in the range observed in subjects with several diseases as described by Franck et al. [30]. However, the specific activity decreased after 30 min because the increase of MPO concentration was actually not in the same range than the increase of MPO activity. The results suggest that the newly released MPO would be intrinsically less active.

As a consequence of the increase in the activity of MPO, we speculated that plasma proteins are liable to undergo oxidative damages. Similarly to MPO concentration or enzyme activity, Cl-Tyr increased after 30 min of HD and then decreased. These results disagree with those of Himmelfarb et al. [14] who did not observe an increase in Cl-Tyr after 15 or 60 min. This discrepancy may be attributed to several factors: (i) the number of patients: in their study only 5 patients were included whereas we analyzed Cl-Tyr in 15 patients; (ii) they did not measure Cl-Tyr after 30 min but only after15 min of HD. Interestingly, the peak in Cl-Tyr observed at 30 min and correlated with the increase in MPO activity during the HD session attests that the activity of MPO could contribute to oxidative stress during HD. The decrease in Cl-Tyr after 60 min and at the end of the session is quite surprising and suggests a rapid elimination and/or clearance of modified proteins or amino acid residues during HD. Nevertheless, the results highlight that MPO oxidative activity increased during HD, giving rise to oxidized proteins that can play roles in triggering oxidative stress, chronic inflammation and cardiovascular risk in patients with renal disease undergoing HD [34–36]. In contrast to the MPO activity curve, Hcit/Lys ratio decreased throughout the session. These results are in agreement with previous studies demonstrating that carbamylated residues decrease after HD [37,38]. The present data also show that MPO released during HD does not catalyze the formation of Hcit residues, indicating that Hcit is not suitable as a marker of MPO activity during HD. The decrease in Hcit after 1 h of HD also suggests that carbamylated proteins are low molecular weight proteins, which can be adsorbed on the dialysis membrane and/or pass through it to be eliminated [39,40].

In conclusion, a total protein hydrolysis method for the analysis of Cl-Tyr and Hcit was developed. This hydrolysis is rapid and avoids multiple steps of sample preparation before analysis. Using the latter, we were able to monitor two MPO reaction products (Cl-Tyr & Hcit) during HD session and corroborate the results with both the quantity and the activity of MPO. These data suggest a role of MPO in maintaining the chronic inflammatory status of patients with ESRD during HD.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2012.06.044

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