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A new easy method for specific measurement of active myeloperoxidase in human biological fluids and tissue extracts

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ABSTRACT

The SIEFED ("Specific Immunological Extraction Followed by Enzymatic Detection") method already developed for the specific detection of the activity of equine myeloperoxidase (MPO) was adapted for the specific measurement of active human MPO in biological fluids or tissue extracts. The method consists of the extraction of MPO from aqueous solutions by immobilized anti-MPO antibodies followed by a washing (to eliminate the extraction medium and the biological fluid with their possible interfering molecules) and the measurement of the activity of MPO with a detection system containing a fluorogenic substrate, H_2O_2 and nitrite ions as reaction enhancer. The SIEFED was applied to study active MPO in human biological fluids (plasma, bronchoalveolar lavage fluid and supernatant from carotids extracts). The SIEFED for human MPO has a sensitivity limit of 0.080 mU/mL and showed good precision with intra- and inter-assay coefficients of variation below 10 and 20% respectively within a broad range of MPO activities establish from 0.156 to 473 mU/mL. The SIEFED for human MPO will be useful for the specific detection of active MPO in complex fluids and can be complementary to an ELISA to determine an active/total MPO ratio in healthy volunteers and patients especially in case of chronic or acute inflammatory diseases.

1. Introduction

Neutrophil myeloperoxidase (MPO) is involved in the intracellular bacterial destruction by producing potent oxidant molecules, mainly hypochlorous acid (HOCl) [1,2]. MPO is released in the extracellular milieu by dying or highly stimulated neutrophils in pathological conditions of acute and chronic inflammations [2–6]. In these conditions, MPO is able to exert a deleterious oxidant activity on neighbouring cells and tissues, and a therapeutic target should be to modulate its activity. Increased blood levels of MPO are now considered as markers of neutrophil activation and degranulation [7,8] and are measured by immunological methods (ELISA or RIA), which quantify the total concentration of the enzyme [9,10] without reflecting its true enzymatic activity. The activity of MPO is currently measured by the direct addition in the medium of H_2O_2 and a chromogenic or fluorogenic substrate as electron donors [11–13]. The oxidation of these electron donors is monitored by spectrophotometry or fluorimetry as a probe of the MPO activity [14,15]. Although these methods are suitable for measuring the activity of MPO *in vitro* in simple mixtures [13], they lack sensitivity, are cumbersome for cohort measurements and are not easily applicable to complex biological samples such as blood, plasma, or tissues, because of interferences with components endowed with peroxidasic activity (e.g. haemoglobin) [16,17], proteins (e.g. albumin, lipoproteins, ceruloplasmin) [18,19], reducing agents (e.g. ascorbic acid, glutathione) [16]. The physicochemical characteristics (pH, viscosity, etc.), the redox status or the colour of the biological fluids also interfere with the reading of the colorimetric or fluorescence response.

We designed a new technique, the SIEFED (for "Specific Immunological Extraction Followed by Enzymatic Detection") to measure the activity of an enzyme in biological fluids, and firstly developed it for equine MPO [20]. The major advantage of the SIEFED technique is its rapidity and an easy extraction of MPO out of the sample or reaction mixture by specific immobilized antibodies. The MPO binding to antibodies is followed by a washing

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step that eliminates the solution and its potential interfering capacity, before starting the detection of MPO enzymatic activity. In human, increasing evidence indicated that MPO plays important roles in the initiation and progression of arthritis, carcinogenesis, pulmonary, kidney, neurodegenerative, inflammatory bowel and especially cardiovascular diseases in which the enzyme is taken as an inflammatory marker and a prognostic agent [6]. In these diseases, an important clinical aspect is to know the active part of MPO, which is the real witness of the oxidant potential of the enzyme. We describe here the development and optimization of the SIEFED technique for the specific detection of the enzymatically active human MPO in complex media [plasma, bronchoalveolar fluids (BALs)] and tissue extracts (carotids). The SIEFED technique is based on the immunological capture of the antigen by antibodies. An interesting point of this study was to compare the active MPO content measured by SIEFED with the total MPO content measured by ELISA to determine an active/total MPO ratio for the tested samples.

2. Experimental

2.1. Reagents

Analytical grade phosphate salts, sodium and potassium chlorides, sodium hydroxide, sodium acetate, H_2O_2 (30%, w/v), ethanol and Tween 20 and human myeloperoxidase (Calbiochem) were purchased from Merck (VWR International, Leuven, Belgium). Bovine serum albumin (BSA) and sodium nitrite were purchased from Sigma–Aldrich (Bornem, Belgium). 96-Wells microtiter plates (Combiplate 8 EB) were from Thermo Scientific (Breda, The Netherlands). The fluorogenic substrate, Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine), was purchased from Molecular Probes (Invitrogen) (Merelbeke, Belgium).

2.2. Purification of human MPO and rabbit anti-human MPO polyclonal antibodies

MPO was purified from human neutrophils by detergent enhanced extraction followed by two steps of chromatography, and antisera were obtained by rabbit immunization against the pure human MPO emulsified with complete Freud adjuvant as previously described [9,21]. Polyclonal antibody (IgG), purified from rabbit antisera by affinity chromatography on Protein A sepharose (GE Healthcare, The Netherlands), was characterized for its specificity and stability and firstly used to develop a radioimmunoassay for total MPO assay in human plasma [9].

2.3. Methodology of the SIEFED technique

Three buffers were used. The coating buffer was 20 mM K and Na phosphates (pH 7.4), 137 mM NaCl and 2.7 mM KCl. The blocking buffer was obtained by addition of 5 g/L BSA to the coating buffer. The diluting buffer was the blocking buffer added with 0.1% Tween 20. The washing solution was 154 mM NaCl with 0.1% Tween 20. Microtitration wells were coated overnight, at 4 °C with 100 μL/well of serial dilutions of a rabbit IgG solution (62.5–500 ng/well) diluted with the coating buffer to determine the optimal IgG concentration to be used in the SIEFED assay. After removing the coating solution, the blocking buffer was added, the plates were incubated for 150 min at room temperature and washed three times. Serial dilutions of a standard MPO solution (pure human MPO) or unknown samples were added (100 μL/well) and incubated for 1 or 2 h either at 22 °C or at 37 °C or for 20 h at 4 °C. After three washings, the peroxidasic activity of MPO was detected by adding 100 μL of a 40 μM Amplex Red solution freshly prepared in phosphate buffer (50 mM) at pH 7.5 and added with 10 μM H_2O_2 and 10 mM nitrite. After incubation in the darkness (30 min, 37 °C), the fluorescence was read with a Fluoroscan Ascent (Thermo Scientific) with excitation and emission wavelengths set at 544 and 590 nm, respectively. Controls (blank) were performed with the dilution buffer. The fluorescence value was directly proportional to the quantity of active MPO present in the sample.

2.4. Validation of the SIEFED technique

The stability of the reference human MPO (from Calbiochem) was tested after its dilution in phosphate buffer saline (PBS) or PBS added with 5 g/L BSA and 0.1% Tween 20 (dilution buffer) and either incubated for 2 h at 37 °C or submitted to several freezing–thawing cycles.

The cross-reactivity of the anti-MPO antibodies with albumin, haemoglobin, elastase or eosinophil peroxidase was measured as previously described [9]. The detection limit was defined as the lowest active MPO concentration (mean value ± SD) that was statistically different from the value obtained with buffer alone. The standard curve was calculated and plotted point to point by using the Ascent Software for Multiscan Ascent (Thermo Labsystems). The precision and reproducibility were estimated by the coefficients of variation (CV) within or between assays calculated for several determinations of the same sample in the same assay or in different assays performed on different days. Accuracy was estimated by the recovery of known amounts of purified MPO added to the samples (plasma, BALs and carotid extracts).

To assure that the SIEFED technique could be reproduced with other anti-MPO antibodies, the immunoreactivity of our antibody was compared with that of the primary MPO-antibody (polyclonal antibody from rabbit) used in the MPO-ELISA kit (ELIZEN MPO) purchased from the Zentech company (Belgium).

2.5. Sampling technique

To test the stability of the neutrophils in blood after sampling, blood was drawn from five healthy donors without anticoagulant and on three anticoagulants with centrifugation (10 min, 22 °C, 1000 × *g*) 1 or 3 h after sampling. The anticoagulants were EDTA (1.8 mg/mL blood, in 3.5 mL vacutainer tubes; Venosafe, Terumo, Belgium), lithium heparin (15 IU/mL blood), or citrate (3.2% Na-citrate solution). Blood in plain tubes was allowed to clot (3 h) at room temperature (22 °C) before serum collection. For all the other studies with healthy and pathological subjects, venous peripheral blood samples were obtained in vacutainer tubes on EDTA (1.8 mg/mL blood), and centrifuged within 30 min after drawing.

Bronchoalveolar lavages (BALs) were performed in intensive care patients for therapeutic purpose (bacterial agent determination) via the endotracheal tube, with four aliquots of 50 mL warm sterile 0.9% NaCl solution as previously described [22]. The first aliquot was discarded and the second used for bacterial studies. The aspirated liquids of the third and the fourth aliquots were strained through sterile gauze and centrifuged (10 min, 4 °C, 300 × *g*); the supernatant was used for SIEFED assay.

Carotid atherosclerotic plaques were collected during surgery for carotid thrombo-endatherectomy and rinsed with 0.9% NaCl solution before their freezing at −20 °C. After thawing, the carotid plaques were homogenised on ice in 20–25 mL of cold 10 mM Tris-buffer, pH 7.4. The extract was centrifuged (20 min, 4 °C, 17,000 × *g*) and the supernatant was used for SIEFED assay.

Plasma samples, supernatants from BALs and carotid extracts were freshly used or kept frozen in small aliquots at −20 °C. Immediately before the SIEFED assay, samples were thawed and diluted with the diluting buffer before loading into the wells.

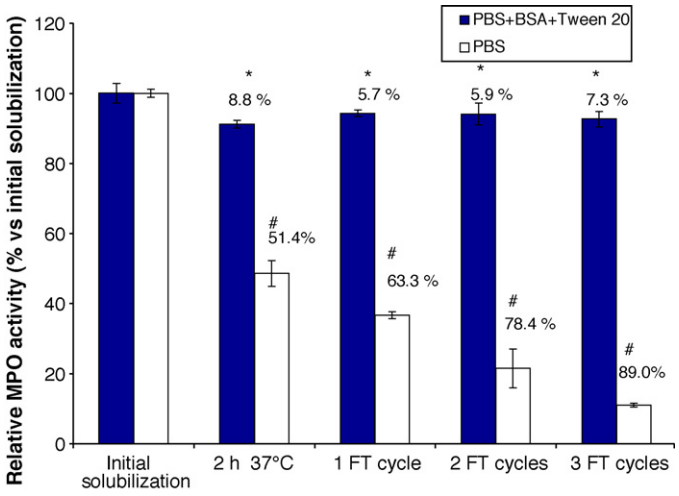

Fig. 1. Stability of human MPO activity in PBS buffer or PBS buffer + BSA + Tween 20 after an incubation period of 2 h at 37 °C or after several freezing–thawing (FT) cycles. The test performed directly after MPO dilution was taken as 100%. Mean ± SD, $n = 3$, ANOVA $^{*}p < 0.05$ versus initial solubilization in PBS + BSA + Tween 20; $^{\#}p < 0.05$ versus initial solubilization in PBS.

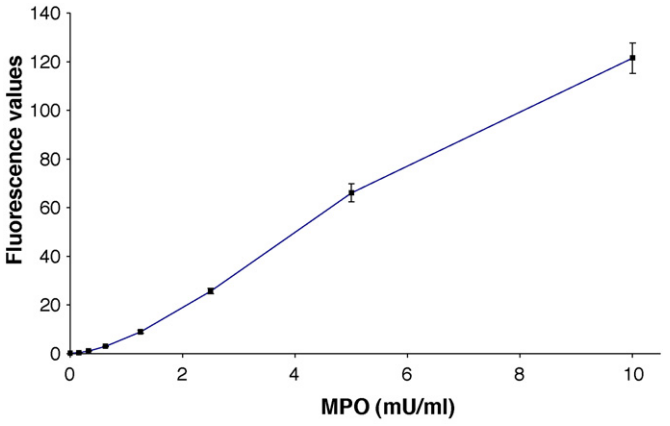

Fig. 2. Standard curve for the measurement of the activity of human MPO by the SIEFED technique (mean ± SD, $n = 5$).

Optimal dilution was determined by testing serial dilutions (1:1, 1:2.5, 1:5, 1:10, 1:20, 1:40, 1:80) of the samples in the dilution buffer.

2.6. Total MPO assay by a sandwich ELISA

To compare active MPO to the total content of the enzyme, total (active and non-active) MPO was measured with a sandwich human myeloperoxidase ELISA kit (ELIZEN MPO, Zentech SA, Belgium). Before the ELISA, plasma samples, and carotid extracts were diluted 20 times and BAL samples 50 times with the diluting buffer furnished by the ELISA kit.

2.7. Statistical analysis

SigmaStat 3.5 software (Systat, San Jose, CA) was used for the analysis. Data are presented as the mean ± SD and were evaluated by a one-way ANOVA.

3. Results

3.1. Stabilization of the reference human MPO

According to the supplier (Calbiochem), human MPO specific activity ranged from 180 to 220 U/mg proteins: we took the mean value, 200 U/mg proteins, as MPO specific activity. Fig. 1 shows that MPO diluted in PBS was not stable: the enzyme lost 51.4 ± 3.7% of its activity after 2 h at 37 °C and 89.0 ± 0.5% after three freezing thawing cycles. In PBS with 5 g/L BSA and 0.1% Tween 20, MPO remained stable, with only a slight loss of activity of 8.8 ± 1.1 and 7.3 ± 2.2% respectively after 2 h at 37 °C or three freezing thawing cycles.

3.2. Calibration curve and detection limit of the SIEFED technique

The standard curve was obtained by plotting the fluorescence values as a function of the known activity of the reference MPO, from 0.156 to 10 mU/mL (Fig. 2). Table 1 shows the fluorescence data measured for a typical active MPO reference curve (mU/mL) with the corresponding reference concentrations of the pure human MPO in ng/mL. The intra-assay CVs did not exceed 8%. The detection limit of the test (0.080 mU/mL) was determined as the lowest active MPO concentration (mean value ± SD) statistically different from the value obtained with buffer alone.

3.3. SIEFED method for MPO measurement in biological samples

Based on the detection limit and the slope of the standard curve, we chose 250 ng per well (or 2.5 μg/mL) as the optimal concentration of the coating antibody. After blocking and washing, the coated plates could be stored at 4 °C under dry conditions for up to 4 weeks. The binding of purified MPO to the coated antibodies increases with time and is similar at 37 °C and room temperature (RT) (Fig. 3). The activity of the samples measured after the incubation at RT are generally lower than after the incubation at 37 °C but, interestingly, the activity obtained after an overnight incubation at 4 °C or after 1 or 2 h incubation at 37 °C are similar (CV < 10%) (results not shown). The incubation at 4 °C does not disturb the activity of MPO and its binding to the antibody. The rapidity of the test being a priority factor, a 2 h incubation time at 37 °C was chosen for further studies.

As previously described [20], to reach a high sensitivity of the SIEFED technique for its application to biological samples, we used as peroxidase substrate, the Amplex Red molecule, which is transformed into the fluorescent resorufin upon its peroxidase-

Table 1
Standard curve values for the measurement of active human MPO by the SIEFED technique.

Human MPO (mU/mL)	Human MPO (ng/mL)	Mean fluorescence value ($n = 5$)	SD	CV (%)
0	0	0.149	0.002	1.3
0.156	0.78	0.441	0.025	5.7
0.3215	1.56	1.067	0.069	6.5
0.625	3.125	3.003	0.107	3.6
1.25	6.25	8.976	0.656	7.3
2.5	12.5	25.737	1.123	4.4
5	25	66.083	3.701	5.6
10	50	121.429	6.213	5.1

Mean ± SD, $n = 5$. The detection limit of the test was 0.080 mU/mL.

Table 2
Effect of the delay between blood collection and centrifugation on the activity of MPO measured by SIEFED in anticoagulated plasma and serum.

Collecting tube	Time between blood collection and centrifugation (h)	Mean MPO activity (mU/mL)	SD (mU/mL)	CV (%)
Citrate	1	1.4	0.3	23.2
	3	2.5	1.0	39.0
EDTA	1	1.9	0.2	11.7
	3	3.4	0.3	7.9
Li-heparin	1	2.9	0.7	23.4
	3	6.3	2.1	32.4
Dry (serum)	3	19.9	5.1	25.8

Mean ± SD of five healthy donors, $n = 5$.

catalyzed oxidation by H_2O_2 [23]. The optimal working conditions to measure the activity of MPO were reached with a freshly prepared 40 μM Amplex Red solution and 10 μM H_2O_2. The addition of 10 mM sodium nitrite ($NaNO_2$) in the reaction mixture increased 4–5-folds the sensitivity of the SIEFED [20].

3.4. Sampling conditions: effects of the anticoagulant and the delay in blood centrifugation

When centrifugation was done 1 h after blood sampling, the lowest mean MPO activity was observed for blood drawn on citrate and the highest one for blood drawn on Li-heparin (Table 2). After a delay of 3 h before centrifugation, the MPO activity values increased 1.8 times for EDTA and citrate and 2.1 times for Li-heparin. Interestingly, the lowest CVs, both after 1 and 3 h before blood centrifugation, were obtained for the EDTA plasmas what suggested that the neutrophils remained stable with time. For citrate and Li-heparin samples, the high CVs were unacceptable. A high mean value of MPO activity was observed for serum (Table 2). On the basis of these results, it was decided to use preferably EDTA anticoagulated blood for all the other studies; citrated samples obtained after blood centrifugation within 1 h can also be used, but we excluded the serum samples.

3.5. Application of the SIEFED technique to biological samples

The dilution of the sample improved the MPO activity till the dilution 1:10, undoubtedly by improving the binding between MPO and the primary antibody (Fig. 4). At higher dilutions, the measured values were stabilized and reached a plateau for most samples at the dilution 1:20 and for some samples at the dilution 1:40. But at this dilution, the limit of sensitivity is reached for samples with low content of active MPO. Linearity of the assay was determined by evaluating dilutional parallelism. Percentages of recovery calculated from observed-to-expected values for two plasmas, two BALs and two carotid extracts at two dilution factors (1:10 and 1:20) ranged from 94.1 to 130.0% (mean ± SD: 113.4 ± 12.2%; Table 3).

The accuracy of the assay was evaluated by determining the spiking recovery in two plasmas, two BALs and two carotid extracts. Percentages of recovery for seven spiking concentrations of active MPO added to the samples ranged from 72.6 to 113.1% (mean ± SD: 93.5 ± 9.2%; Table 4). A lack of recovery above 20% was observed for the highest active MPO concentrations (2.5 and 5 mU/mL) added to the plasma samples, but not to BALs and carotid extracts.

The intra-assay precision of the SIEFED technique estimated by repeated measurements within the same run on three different types of biological fluids with MPO activities ranging from 2.6 to 473.0 mU/mL showed CVs that did not exceed 10%. The mean CV value for all the samples ($n = 45$) was 3.8 ± 2.7% (Table 5).

Inter-assay precision was estimated by duplicate or triplicate assays of the biological fluids performed in different runs over a period of 4, 3 or 2 days. The MPO activities ranged from 1.8 to 118.1 mU/mL and the CVs did not exceed 20%. The mean CV value for all the samples ($n = 49$) was 10.2 ± 4.8% (Table 5).

In the plasma of 19 healthy donors, the total and active MPO contents were measured by ELISA (ELIZEN MPO, Zentech, Belgium) and by SIEFED respectively after checking that the primary antibody used in the kit had a similar response for the capture of active MPO than our antibody, and by diluting the samples in the same manner for the ELISA and the SIEFED. The mean total MPO value was 42.0 ± 15.0 ng/mL, while the mean active MPO value was 15.1 ± 5.2 ng/mL corresponding to 3.0 ± 1.0 mU/mL (Table 6). The active/total MPO ratio indicates that about 40 ± 9% of total plasmatic MPO was active in the healthy donors. The values of active and total MPO in five pathological plasmas, ranged from 80.5 to 210.7 ng/mL for active MPO and from 87.1 to 235.4 ng/mL for total MPO, were higher than the mean value obtained in healthy donors

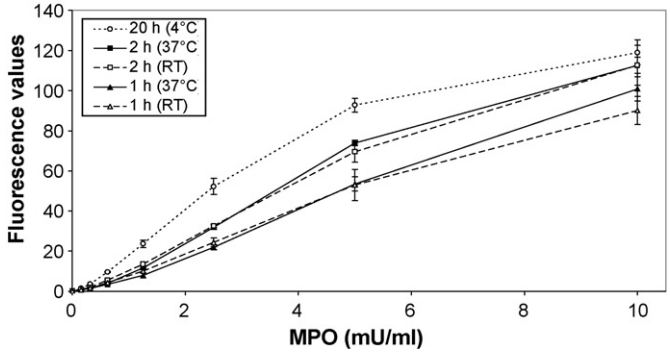

Fig. 3. Effects of incubation time and temperature on the MPO binding to its antibodies (mean ± SD, $n = 3$).

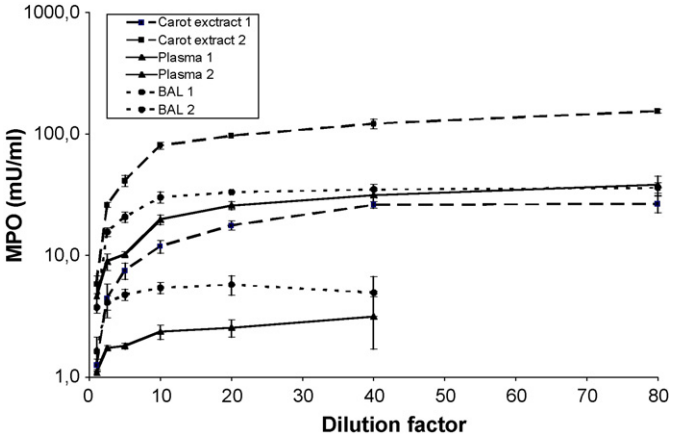

Fig. 4. Curves obtained with serial dilutions of plasma, carotid extracts and BALs (mean ± SD, $n = 5$).

Table 3
Active MPO values measured by SIEFED for dilutional parallelism of plasma, carotid extracts and BALs (mean ± SD, $n = 3$).

Samples	Dilution factor	MPO observed value (mU/mL)	MPO expected value (mU/mL)	Recovery (%)
Plasma 1	1:10	0.53		
	1:20	0.30	0.27	111.1
Plasma 2	1:10	1.71		
	1:20	1.04	0.85	122.3
Carotid 1	1:10	9.04		
	1:20	5.88	4.52	130.0
Carotid 2	1:10	0.42		
	1:20	0.23	0.21	110.0
BAL 1	1:10	10.23		
	1:20	5.77	5.12	112.6
BAL 2	1:10	0.34		
	1:20	0.16	0.17	94.1

Table 4
Recovery of spiked active human MPO measured by SIEFED in plasma, carotid extracts, and BALs.

Samples	Initial active MPO (mU/mL)	Added active MPO (mU/mL)	MPO expected (mU/mL)	MPO observed (mU/mL)	Recovery (%)
Plasma 1 (dilution 1:20)	0.843	0.078	0.92	0.90	97.8
		0.156	1.00	0.96	96.0
		0.312	1.16	1.18	101.7
		0.625	1.47	1.38	93.9
		1.25	2.09	1.86	89.0
		2.5	3.34	2.79	83.5
		5	5.84	4.33	74.1
Plasma 2 (dilution 1:10)	2.73	0.078	2.81	2.80	99.6
		0.156	2.89	2.82	97.6
		0.312	3.04	2.87	94.4
		0.625	3.36	3.02	89.9
		1.25	3.98	3.41	85.7
		2.5	5.23	4.12	78.8
		5	7.73	5.61	72.6
Carotid 1 (dilution 1:20)	5.8	0.078	5.84	6.24	106.9
		0.156	5.91	6.21	105.1
		0.312	6.07	6.22	102.5
		0.625	6.38	6.58	103.1
		1.25	7.01	6.82	97.3
		2.5	8.26	7.95	96.3
		5	10.76	9.73	90.4
Carotid 2 (dilution 1:10)	0.53	0.078	0.61	0.69	113.1
		0.156	0.69	0.69	100.0
		0.312	0.84	0.86	102.4
		0.625	1.16	1.31	112.9
		1.25	1.78	1.84	103.4
		2.5	3.03	2.94	97.0
		5	5.53	5.11	92.4
BAL 1 (dilution 1:20)	0.53	0.078	0.61	0.56	91.8
		0.156	0.69	0.66	95.7
		0.312	0.84	0.80	95.2
		0.625	1.16	1.02	87.9
		1.25	1.78	1.47	82.6
		2.5	3.03	2.42	79.9
		5	5.53	4.48	81.0
BAL 2 (dilution 1:10)	1.565	0.078	1.64	1.55	94.2
		0.156	1.72	1.56	90.8
		0.312	1.88	1.75	93.5
		0.625	2.19	2.01	91.6
		1.25	2.82	2.44	86.6
		2.5	4.07	3.50	86.1
		5	6.57	6.00	91.4

(Table 6). The active/total MPO ratio was also higher than in healthy controls, with a range from 0.5 to 0.9 (Table 6). In the five carotid and three BAL samples, the ratio of active versus total MPO showed that the active part of the enzyme varied from 20 to 90% of total MPO amount (Table 6), but no comparison was possible with healthy carotid and BAL samples.

4. Discussion

Owing to our experience with equine MPO [20] and RIA assay of human MPO [21], we developed a SIEFED technique for the specific and rapid measurement of the enzymatically active form of human MPO in complex biological samples. This technique has five inter-

Table 5
Intra- and inter-assay reproducibility of the SIEFED technique for active MPO measurements in human plasma, carotid extracts and BALs.

Samples	n^a	Mean MPO activity (mU/mL)	SD (mU/mL)	CV (%)
Intra-assay				
Plasma 1	4	16.0	0.2	1.3
Plasma 2	4	9.5	0.1	1.1
Plasma 3	3	6.3	0.0	0.6
Plasma 4	3	16.3	0.5	3.1
Plasma 5	2	5.9	0.3	5.1
Plasma 6	2	15.3	0.4	2.6
Carotid 1	4	119.5	11.9	10.0
Carotid 2	4	167.3	12.0	7.2
Carotid 3	3	4.2	0.1	2.4
Carotid 4	2	61.7	1.7	2.8
Carotid 5	2	473.0	26.0	5.5
BAL 1	4	4.3	0.1	2.3
BAL 2	4	30.9	2.2	7.1
BAL 3	2	17.6	0.4	2.3
BAL 4	2	2.6	0.1	3.8
Samples	n^b	Mean MPO activity (mU/mL)	SD (mU/mL)	CV (%)
Inter-assay				
Plasma 7	4	2.9	0.5	17.2
Plasma 8	4	15.5	0.5	3.2
Plasma 9	3	10.7	1.1	10.2
Plasma 10	3	24.4	2.4	9.8
Plasma 11	2	6.9	0.6	8.7
Plasma 12	2	19.8	3.2	16.1
Carotid 6	4	1.8	0.3	16.7
Carotid 7	4	54.7	2.4	4.3
Carotid 8	3	16.8	2.3	13.7
Carotid 9	3	88.4	9.5	10.7
Carotid 10	2	118.1	4.5	3.8
Carotid 12	2	4.4	0.4	9.1
BAL 5	4	5.5	0.9	16.3
BAL 6	4	31.0	2.8	9.0
BAL 7	3	29.0	2.9	3.4
BAL 8	2	50.8	5.6	11.0

CV = coefficient of variation, SD = standard deviation.
[a] Number of sample measurement during the same assay.
[b] Number of assays with the sample measured in duplicate or triplicate in each assay.

esting characteristics: (i) the easy immuno-capture of MPO out of a biological sample (or a reaction mixture containing MPO) without any preparation of the sample (no partial purification or precipitation of the enzyme), which is simply diluted, loaded into the antibody-coated wells and discarded after the incubation period during which MPO binding has occurred, (ii) the washing after the immuno-capture of MPO to eliminate the sample with its compounds potentially able to interfere with the enzymatic detection step, (iii) the specificity since the specific antibodies will only capture human MPO so that the detection system will disclose only the MPO activity, (iv) the high sensitivity of the test reached by the combined used of Amplex Red, a stable fluorogenic substrate for detection of peroxidases [12], and nitrite as an enhancer of the reaction, (v) the measurement of the activity by referring to a calibration curve made with pure human MPO.

As previously described, the success of the SIEFED technique depends on a good compromise between a strong binding of MPO and a low inhibition of its activity by the antibodies [20]. We obtained the best conditions to bind active MPO by coating the wells with 250 ng of rabbit anti-MPO IgG. The use of a polyclonal antibody increased the possibilities to capture MPO by different epitopes, but also the risk to inhibit the enzyme as observed in different models with MPO in solution [24,25]. We thus expected an inhibitory effect of immobilized antibodies on the MPO activity, but the MPO activities measured in the tested samples are calculated from a calibration curve, in which the reference human MPO is submitted to the same inhibitory effect of the antibody.

Good conditions for MPO incubation with the primary antibody were established at 37 °C for 2 h. Interestingly, the values obtained after an overnight incubation at 4 °C or a 2 h incubation at 37 °C were similar (CV < 10%): the overnight incubation at 4 °C can thus be used as an alternative condition for the SIEFED technique.

A good sensitivity of the test was obtained with the fluorogenic substrate Amplex Red, which is transformed during the peroxidasic cycle of MPO into the fluorescent resorufin in the presence of H_2O_2 [12,23]. We succeeded to greatly increase the sensitivity of the reaction by adding nitrite to the reaction mixture [20]. This increasing effect can be firstly explained by a cooperation of nitrite and Amplex Red for the turn over of the peroxidasic reaction of MPO. Burner et al. [26] showed that nitrite is a good substrate to reduce the compound I of MPO provided that a good electron donor is present to reduce the compound II. Amplex Red could play the role of the second electron donor for compound II. But, the oxidation of nitrite by the compound I mediates nitrogen dioxide, a reactive radical which can react with fluorescent probes [27]. Thus a second way to explain the amplification role of nitrite is the reduction of nitrogen dioxide by Amplex Red allowing both the formation of resorufin and the recycling of nitrite for the turnover of the peroxidasic cycle.

The study of the blood sampling conditions revealed that consistently higher MPO activity was measured in samples collected on heparin and in serum than in samples on EDTA or citrate. The delay between blood sampling and centrifugation increased the MPO activity values for all the anticoagulants, but the increase

Table 6
Concentration (ng/mL) of active and total MPO measured by SIEFED and ELISA and active/total MPO ratio in plasma of 19 healthy donors (mean ± SD) and in 5 plasmas, 5 carotid extracts and 3 BALs of pathological subjects.

	Active MPO (mU/mL)	Active MPO (ng/mL)	Total MPO (ng/mL)	Active MPO/total MPO
Mean plasmatic value for 19 healthy donors	3.0 ± 1.0	15.1 ± 5.2	42.0 ± 15.0	0.4 ± 0.1
Plasma 1	19.8	99.1	139.6	0.7
Plasma 2	26.1	130.6	235.4	0.6
Plasma 3	42.1	210.7	415.2	0.5
Plasma 4	23.1	115.3	170.1	0.7
Plasma 5	16.1	80.5	87.1	0.9
Carotid 1	4.6	22.8	26.0	0.9
Carotid 2	63.0	315.1	530.3	0.6
Carotid 3	5.3	26.5	97.1	0.3
Carotid 4	5.6	27.9	131.9	0.2
Carotid 5	18.6	93.0	442.8	0.2
BAL 1	3.4	16.8	61.2	0.3
BAL 2	21.9	109.5	251.2	0.4
BAL 3	2.6	13.2	71.4	0.2

was similar for citrate and EDTA and more pronounced for heparin. These results are in agreement with previous studies showing higher total MPO contents in heparin plasma and serum than in citrate and EDTA plasma [28,29].

The SIEFED method allows reliable quantification of MPO activity from 0.156 to 10 mU/mL, what corresponds to 0.78–50 ng/mL pure MPO. The dilution buffer stabilizes the enzyme during the incubation time and freezing–thawing cycles, because BSA and Tween 20 are known to prevent adhesion and aggregation of proteins [30]. The intra-assay coefficients of variation of the reference curve do not exceed 8%. The detection limit of the assay (0.080 mU/mL) allows an easy measurement of active MPO in diluted biological fluids. Like for ELISA, the dilution of the samples increases the sensitivity of the test by reducing the interactions between MPO and other biomolecules of the sample and by improving the binding of MPO to the primary antibody. The polyclonal antibody could also recognize the precursor of MPO, which is active in human [31]. The dilutions of 1:10 to 1:20 are adequate for most of the samples, but we cannot exclude that higher dilutions will be necessary for pathological samples.

We calculated the mean normal value of active MPO measured in EDTA plasmas as 15.1 ± 5.2 ng/mL or 3.0 ± 1.0 mU/mL, but the number of tested normal plasmas will be increased by further studies. In pathological samples, we measured MPO activities ranging from 2.9 to 42.1 mU/mL for plasmas, 1.8 to 473.0 mU/mL for carotid extracts and 2.6 to 50.8 mU/mL for BALs with good intra-assay CVs (below 10%) and acceptable inter-assay CVs (below 20%). These preliminary results indicate that the SIEFED technique will be useful to measure the active MPO in biological fluids or extracts from patients suffering from inflammatory pathologies with neutrophil activation and infiltration [6,22,32,33]. In theses pathologies, the measurement of total MPO by ELISA is often taken as a marker of neutrophil activation, but we compared the total and active MPO contents in samples and found in plasma an active/total MPO ratio of 0.4 ± 0.1 in healthy donors and a higher but variable ratio of 0.5–0.9 in pathological subjects. A still higher variability of this ratio (from 0.2 to 0.9) was observed for carotid extracts and BALs from pathological subjects. These original results suggest an important variability of active MPO contents in pathological samples probably linked to the activation status of neutrophils, the inhibitory capacity of the fluids towards the enzyme or other disease conditions. The results obtained with the SIEFED technique emphasized the importance of measuring the active part of MPO, which is the real witness of the oxidant potential of the enzyme that is not taken into consideration by a classical ELISA. Indeed, the presence of free active MPO increases the risk of cytotoxicity, as the enzyme is taken up by cells or binds on the cell surface with an *in situ* production of oxidant species [34,35], which are involved in chlorination and nitration of proteins [2,6], and surely contribute to the pathogenesis of the disease [2,6] or to the modulation of the inflammation reaction [36].

In conclusion, the SIEFED technique that we developed for human MPO opens news perspectives for the study of pathologies accompanied by the release of active MPO. The SIEFED is a method of choice to measure easily, reliably, quantitatively, and specifically the active part of MPO present in biological samples or tissue extracts. Moreover, SIEFED and ELISA can be used as complementary immunological techniques to determine with accuracy the active/total MPO ratio in biological samples, what constitutes a new prospect in clinical research.

Acknowledgement

The SIEFED technique is protected by an international patent (PCT/BE2005/00017).

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