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Technical note

Determining myeloperoxidase activity and protein concentration in a single assay: Utility in biomarker and therapeutic studies

Muir Russell, Nina Prokoph, Neil Henderson, Susanna Eketjäll, Clare A. Balendran, Erik Michaëlsson, Mark Fidock, Glen Hughes *

Innovative Medicines and Early Development Biotech Unit, AstraZeneca, United Kingdom

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ABSTRACT

Myeloperoxidase (MPO) is predominantly expressed by neutrophils and is an important enzyme used by the immune system for the neutralisation of bacteria and other microorganisms. The strong oxidative activity of MPO has been linked to pro-inflammatory responses in surrounding cells and tissues with implication in the pathophysiology of cardiovascular, neuroscience and inflammatory diseases. This broad disease association has made MPO an attractive biomarker and therapeutic target. Here we describe the construction and validation of a single combined MPO activity and protein concentration assay using commercially available reagents. This method offers the investigative laboratory the ability to generate results from blood plasma samples in a single analytical run using the same sample aliquot.

1. Introduction

Myeloperoxidase (MPO) is an oxidative enzyme predominantly synthesised and stored in the azurophilic granules of neutrophil cells. It plays an important role in the innate immune system during microbial phagocytosis, where it catalyses the formation of reactive oxidative species, such as hypochlorous acid, which have a strong antibacterial activity (Klebanoff, 2005). These toxic oxidative species are not confined to the neutrophil and can diffuse outside the cell where they can cause tissue inflammation and damage (Klebanoff, 2005). MPO activity and its role in oxidative stress has been implicated in the pathophysiology of neurodegenerative conditions such as Alzheimer's and Parkinson's disease (Lefkowitz and Lefkowitz, 2008) as well as cardiovascular diseases such as coronary artery disease (Zhang et al., 2001) and heart failure (Anatoliotakis et al., 2013).

With a widespread disease linkage, MPO is an important therapeutic target for drug development (Malle et al., 2007) and a useful disease biomarker (Pulli et al., 2013). Recent clinical studies have been reported (www.clinicaltrials.gov) including AstraZeneca's drug candidates AZD3241, a selective and irreversible MPO inhibitor, currently in clinical development for multiple system atrophy (NCT02388295) and AZD4831 in Heart Failure with preserved Ejection Fraction (NCT02712372). Clinical biomarker studies in coronary artery disease (NCT01239979) and cardiac risk in oncology (NCT02496260) have also been described. These studies, and others, can help provide the data needed to clinically validate MPO as both a therapeutic target, mechanistic and disease biomarker.

Importantly, biomarker studies will rely on the use of robust fit-for-purpose validated assays (Lee et al., 2006) to measure MPO protein and activity levels. Previously, it has been shown that antibody capture of MPO is required before determining MPO activity in order to remove assay interfering substances (Pulli et al., 2013). Additionally, it has been published that an immunocapture step can be used for the combined measurement of both the total MPO protein level and enzyme activity from the same sample (Franck et al., 2015). This assay consisted principally of two parts. Firstly, measurement of activity from MPO captured from the biological sample, using an antibody immobilised to a microtiter plate. Secondly, application of a wash step to the sample to remove the activity substrate reagents, followed by total protein determination using a second anti-MPO antibody in a sandwich immunoassay format. This approach has distinct advantages including speed and sample usage, but unfortunately the bespoke polyclonal reagents used for this published assay are not commercially available. To increase the availability and widespread usage of this combined approach we evaluated commercially available assay kits to incorporate into a dual use assay without depending on a diverse range of suppliers for components.

* Corresponding author at: Respiratory Companion Diagnostic Unit, Personalised Healthcare and Biomarkers, Innovative Medicines and Early Development Biotech Unit, AstraZeneca, da Vinci Building, Melbourn Science Park, Cambridge Road, Melbourn, Royston SG8 6HB, United Kingdom.
E-mail address: glen.hughes@astrazeneca.com (G. Hughes).

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2. Materials and methods

2.1. Subjects and sample collection

Evaluation of the analytical performance of the combined MPO method was carried out using EDTA-plasma samples obtained from 40 healthy volunteers (20 males and 20 females) obtained from Seralab (UK). The study was conducted in accordance with the UK Human Tissue Act (2004).

2.2. Evaluation of MPO immunocapture plates and MPO activity assay

The performance of the MPO immunocapture microtiter plates was evaluated using ELISA plates from commercial suppliers: Merck-Millipore (Cat. Number CBA024), Oxis (Cat. number BC-1129), Hycult (Cat. number HK324) and Mercodia (Cat. number 11-1176-01). MPO was titrated from 0 to 1000 ng/mL on each plate and the peroxidase activity assessed using the Innozyme assay (Merck-Millipore, Cat. Number CBA024), according to the manufacturer's protocol.

2.3. Combined MPO activity and total protein assay

Duplicate aliquots (100 μL) of calibration and plasma samples were added to the 96-well ELISA plate, pre-coated with anti-human MPO monoclonal antibody (Mercodia). The plate was sealed and incubated at RT for 60 min, with shaking at 400 rpm, to allow capture of the MPO protein. Unbound material was removed by 4 successive washes of the plate with 1× sample buffer (350 μL/well) and then filled with 100 μL/well 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, both provided in the Innozyme assay. TMB substrate was prepared according to manufacturer's recommendations and contained hydrogen peroxide (H_2O_2, 0.001% v/v). The plate was sealed and incubated in the dark at 37°C without shaking for 30 min, then the absorbance read at 650 nm on a plate reader (SpectraMax, Molecular Devices).

The TMB substrate solution was then removed by 6 successive washes with 1× wash buffer (350 μL/well, Mercodia). The plates were then incubated with 100 μL/well anti-human-MPO peroxidase conjugated antibody (Mercodia) at RT for 90 min, with shaking at 400 rpm. Following a further 6 washes with 1× wash buffer the plates were developed by the addition of 200 μL/well TMB substrate (Mercodia), the plate sealed and incubated in darkness at RT for 15 min. The reaction was stopped using 50 μL 0.5 M sulphuric acid, then the absorbance read at 450 nm on a plate reader.

A single calibration curve was prepared, from 0.78 to 200 ng/mL, using the MPO standard and 1× sample buffer (Innozyme). Activity and total protein curves were fitted using a 4-parameter logistic fit with $^1/y$ weighting and sample values determined by interpolation of the results at 650 nm and 450 nm using SoftmaxPro (Molecular Devices).

3. Results

3.1. Evaluation of MPO immunocapture plates

All of the immunoplates tested captured MPO and provided dose dependent activity curves (Fig. 1). The Mercodia plate was chosen as the basis of our assay based on the amplitude and dynamic range of the curve. In the initial comparison of the activity measurements using different ELISA capture plates, a stop solution was used. As expected, we found it crucial for the success of the combined assay that no sulphuric acid stop solution is added at the end of the activity assay measurement. Acidification denatures and uncouples the MPO-antibody interaction leading to a loss of signal prior to the total protein measurement by ELISA (data not shown). Instead, the plate is read at a specified time point.

3.2. Performance of the combined activity and total protein assay

We further defined the analytical characteristic of the Mercodia-Innozyme assay (Table 1). Calibration curve verification showed reproducibility across the tested dynamic range, with acceptable precision defining a lower and upper limit of quantification (LLOQ/ULOQ), for activity and protein, of 3.00 ng/mL and 200 ng/mL. Intra- and inter-assay precision for both assays were acceptable, although it's noteworthy that the enzyme assay component of the assay trends to larger coefficients of variation (CV) and particular attention should be paid to rapid and efficient sample handling to keep assay performance CVs below 20%. Dilutional linearity could not be established for the assay and results of neat plasma analysis will not be comparable to data generated with dilution.

Where results for a target population are expected to exceed the range, all samples must be analysed at the same dilution to obtain comparable data. As proof of concept, plasma samples from 20 male and 20 female healthy volunteers were analysed in 9 independent analysis runs, 3 by the combined method and 3 each by the Innozyme activity method and Mercodia ELISA, both according to the kit inserts. Using the coefficient of determination (R^2), from linear regression analysis, we established the commutability between the different assay formats (Fig. 2) with R^2 greater than 0.90. The number of data points in the activity assessment was lower due to some results being below the LLOQ when measured with using the Innozyme activity kit. This prevented a more rigorous statistical comparison however the slopes of the activity and protein regression lines were consistent with swapping out the kit supplied immunocapture plate and kit supplied standard respectively.

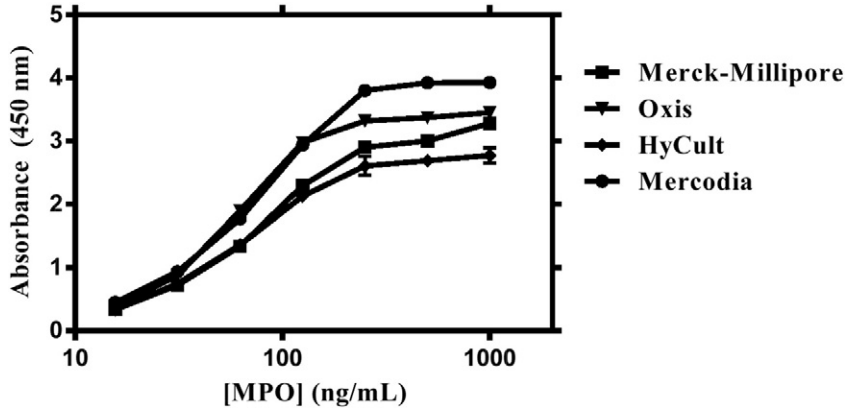

Fig. 1. Selection of immunocapture plates. Comparison of the activity calibration curves obtained for the measurement of MPO activity using commercial ELISA capture plates.