

Short Communication

An Automated Panel for Assessing Pro-Oxidant and Antioxidant Status in Human Serum

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Abstract

Background: The implied role of reactive oxygen species in diabetes, cardiovascular disease and some forms of cancer suggests that the measurement of pro-oxidant and antioxidant biomarkers may be a useful addition to current routine tests.

Methods: Validation procedures were undertaken to establish performance characteristics for glutathione peroxidase, glutathione reductase, superoxide dismutase and total antioxidant status assays on the ILab Aries analyser. Blood samples from thirty healthy participants enabled the determination of preliminary reference ranges for these tests in addition to the calculated antioxidant gap.

Results: The performance of all the tests was favourably comparable with that stated by the manufacturer. Preliminary reference ranges were similar to those in previous publications. There was a requirement for separate male and female ranges for all the panel tests apart from gamma-glutamyltransferase.

Conclusions: An automated panel for selected serum pro-oxidant and antioxidant biomarkers is feasible in terms of test performance, turnaround time and costs. Additional studies on larger groups of individuals with known disorders will determine the clinical usefulness of this panel.

Keywords: Antioxidants; Glutathione peroxidase; gamma-Glutamyltransferase; Glutathione reductase; Oxidative stress; Uric acid

Introduction

Reactive oxygen species (ROS), namely the hydroxyl radical (OH[•]), the superoxide ion (O₂⁻) and hydrogen peroxide, serve as signalling molecules for gene expression, cell growth, inflammation and apoptosis by altering protein function via the covalent modification of cysteine residues [1-3]. ROS are generated intracellularly in mitochondria, as by-products of oxidative phosphorylation and fatty acid oxidation, in cytosol, exosomes and phagosomes via enzymatic action on endogenous and exogenous substances and extracellularly by release from cells via aquaporins or exosomes [1]. Both enzymatic actions, namely superoxide dismutase, glutathione peroxidase and glutathione reductase, and the non-enzymatic antioxidant roles of mainly

albumin and uric acid maintain ROS concentrations within suitable limits. Overproduction of ROS, for instance in local hypoxia or depletion of intra-/extracellular antioxidant capacity, results in ROS concentrations that have been implicated in the development and progression of diseases including diabetes, cardiovascular disease and cancer [4,5].

The volume of recent publications on ROS linked diseases prompts the question of whether automated profiles to reflect pro-oxidant/antioxidant balance should be included as part of the routine Blood Sciences test provision. The aims of this preliminary study were to evaluate the performance of selected serum pro-oxidant and antioxidant biomarkers on an automated system, to compare pilot study healthy participant reference ranges with manufacturer and previously published values, to obtain a preliminary reference range for the calculated antioxidant gap [6] and to assess the feasibility of adding a pro-oxidant and antioxidant panel to current routine testing.

Materials and Methods

Participants

Non-fasting venous blood samples were obtained from thirty healthy participants (14 males and 16 females, aged 23 to 62) following informed consent.

Sample processing

Blood samples collected in serum separator and plasma separator tubes were centrifuged at 3000 rpm for 10 minutes, stored at 4°C and analysed within 24 hours.

Analytes

All tests were performed on the Instrumentation Laboratory ILab Aries, benchtop clinical chemistry analyser (supplied by Werfen UK) at 37°C using application parameters specified by the reagent manufacturers. Instrumentation Laboratory reagents, calibration sera and control sera for albumin, gamma-glutamyltransferase and uric acid were purchased from Werfen UK. Biokit quantex C-reactive protein reagents, standards and control sera were purchased from Werfen UK. Glutathione peroxidase, glutathione reductase and total antioxidant status reagents, standards and control sera were purchased from Randox Laboratories Ltd.

- Albumin (Alb): An end-point spectrophotometric method using bromocresol green dye-binding, absorbance readings were taken at 620 nm. Albumin concentrations in g/L were converted to mmol/L with division by 66.437 for antioxidant gap (GAP) calculations.
- Gamma-glutamyltransferase (GGT): A kinetic spectrophotometric method using L- γ -glutamyl-3-carboxy-4-nitroanilide and glycylglycine substrates to form 5-amino-2-nitrobenzoate with absorbance readings taken at 405 nm.
- Uric acid (UA): An end-point spectrophotometric method using uricase/peroxidase, 4-aminoantipyrine and 2, 4, 6-tribromo-3-hydroxybenzoic acid, with the absorbance of the end product quinoneimine absorbance readings were taken at a primary wavelength of 510 nm and a blanking wavelength of 600 nm. Uric acid concentrations are expressed as mmol/L.
- C-reactive protein (CRP): An end-point turbidimetric polystyrene latex particle immunoassay, with absorbance readings taken at 577 nm.
- Glutathione peroxidase (GPx): A kinetic spectrophotometric method using reduced glutathione (GSH), cumene hydroperoxide and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) as reagents.
- Glutathione reductase (GR): A kinetic spectrophotometric

method using oxidised glutathione (GSSG) and NADPH as reagents. Absorbance readings were taken at a primary wavelength of 340 nm and a blanking wavelength of 405 nm.

- Superoxide dismutase (SOD): A fixed point spectrophotometric method using xanthine, xanthine oxidase and 2-(4-iodophenyl)-5-phenyltetrazolium chloride as reagents. Absorbance readings were taken at a primary wavelength of 510 nm and a blanking wavelength of 700 nm.
- Total antioxidant status (TAS): An end-point spectrophotometric method based on the method of Miller et al. [7], using metmyoglobin, ferrylmyoglobin and 2,2'-Azino-di-[3-ethylbenthiiazoline sulphate] (ABTS[®]) as reagents and absorbance readings taken at 620 nm.
- Antioxidant gap (GAP): GAP was calculated using the equation (1) from Miller et al. [7]. with the assumption of equivalence between trolox equivalent antioxidant activity (TEAC) and TAS [8]. $GAP \text{ (mmol/L of antioxidant activity)} = TAS - [(\text{albumin} \times 0.69) + \text{uric acid}]$ (1) Albumin and uric acid are expressed as mmol/L.

Method evaluations

Alb, CRP, GGT and UA method validations were undertaken for other studies and performance of each was within the desirable specifications [9]. For GPx, GR, SOS and TAS, repeat measurements using deionised water, a low concentration reference material, a control material and a set of reference material dilutions were used to determine the limit of detection (LOD), limit of quantitation (LOQ), between run imprecision expressed as percentage coefficient of variation (%CV), between run percentage bias, expanded uncertainty of measurement and linearity, respectively. Additionally, recovery experiments were undertaken on mixtures of standards and control materials. All methods were calibrated at the start of each daily run of experiments. The expanded uncertainty of measurement (U) was calculated using between run standard deviations (SD) of repeated measurements using equation 2: $U = \pm 2 \times SD$ (2) The number (n) of repeated samples was as follows: 10 for the LOD and LOQ, 20 for within run percentage CV, 5 for between run percentage bias and percentage CV, 4 sets of mixtures for the recovery experiments and 8 dilutions for the linearity experiments. Control materials were reconstituted as stated in the manufacturer's instructions. The control material manufacturer assigned values were as follows: GPx 860 U/L, GR 84 U/L, SOD 1.74 U/mL, TAS 1.75 mmol/L.

Participant samples

Alb, CRP, GGT, GPx, GR, TAS and UA concentrations were measured on the serum samples of each participant within 24 hours of sample collection. Samples not analysed on the same day were stored overnight at 4°C. Preliminary reference ranges

were calculated according to the data distribution. The GAP was calculated for each participant and from this a preliminary reference range was obtained.

Statistical analysis

Microsoft Excel 2016 was used for method evaluation calculations and GAP values. IBM SPSS Statistics Version 23 was used to analyse participant demographics and for the determination of reference ranges for all analytes. Normality of distributions was determined using the Shapiro-Wilk test. The Independent T-test was used to compare mean values for parametric participant data. The Mann-Whitney U-test was used to compare median values for non-parametric participant data. A p-value of <0.05 was considered significant for all statistical analysis.

Results

The performance summary information for the GPx, GR, SOD and TAS methods on the ILab Aries and the participant demographics and results are provided in Tables 1 and 2 respectively. The between run coefficient of variation for each assay was compared with the manufacturers stated values, and found to be comparable, no values were available for TAS. Manufactures stated coefficients of variation for GPx, GR and SOD were 4.4%, 4.32% and 7.1% respectively.

The manufacturer's reference ranges from previous studies were noted for initial comparison of the data obtained here. The quoted serum or plasma ranges were 33 - 73 U/L for GR and 1.30 - 1.77 mmol/L for TAS. The U value for the calculated GAP is calculated by the combining U values for each component as shown in equation 3 [10]:

$$U_{(GAP)} = \sqrt{((U^2_{(TAS)} + 0.69^2 U^2_{(Alb)} + U^2_{(UA)}) = \pm 0.27 \text{ mmol/L (3)}$$

Discussion

This preliminary study has evaluated the performance of a selection of markers for the assessment of oxidative stress and antioxidant status on an automated system and compared reference ranges from a group of healthy participants to reagent manufacturer and previously published values. In addition, the markers have been used to calculate the antioxidant gap and a preliminary reference range for this established.

Method validations, Table 1, on the ILab Aries for GPx, GR, SOD and TAS demonstrated reliable performance with parameters comparable to those stated by the reagent manufacturers [11]. GPx and GR performances were more consistent and reliable when calibration was undertaken with a standard solution; alternative factor based applications were not reproducible on the ILab Aries.

Test	Limit of Detection	Limit of Quantitation	% CV Between run	% bias Between run	Expanded uncertainty of measurement (U)	Linearity (r ²)	% Recovery (range of values)
GPx	93 U/L	211 U/L	4.5	8.7	± 95 U/L	0.9917	95 - 97
GR	2.2 U/L	6.7 U/L	3.0	2.9	± 5.2 U/L	0.9968	100 - 101
SOD	0.08U/mL	0.35 U/L	8.4	- 4.2	± 0.35 U/mL	0.9703	102 -114
TAS	0 mmol/L	0.6mmol/L	7.5	0.4	±0.27mmol/L	0.9818	95 - 102
%CV, %bias and U values are expressed to two significant figures.							

Table 1: Performance summary for glutathione peroxidase, glutathione reductase, superoxide dismutase and total antioxidant status on the ILab Aries analyser.

Once confidence in method performance has been confirmed a general recommendation is that local reference ranges are established, to enable its use for clinical interpretative purposes [12]. Initial reference ranges were calculated from the results obtained from 30 non-fasting participants, Table 2. CRP concentration was measured to exclude the presence of conditions that may also influence the concentrations of antioxidant markers [1] and [13]. No values above 8 mg/L were obtained and therefore all the results were included. Serum GR may be used as a marker for oxidative stress and inflammatory conditions, which result in a decrease from reference values [14]. The GR reference ranges of 50-88 U/L and 50-72 U/L for males and females respectively were comparable to Melissinos et al. [15] 33-77 U/L and Goldberg et al. [16] 42-80 U/L and Delides et al. [17]. 47-79 IU/L.

	Male	Female	All participants	p-value
Number (n)	14	16	30	
Age, years, mean (range)	43 (23 - 62)	39 (23 - 59)	41 (23 -62)	0.425
Alb, g/L, mean (± 2 SD)	44 (40 - 48)	43 (40 - 45)	43 (40 - 47)	0.047
CRP, mg/L, median (IQR)	0.8 (0.6 - 2.1)	0.4 (0.1 - 1.0)	0.6 (0.2 - 1.7)	0.077
GGT, U/L, median (IQR)	24 (20 - 35)	16 (13 - 18)	19 (15 - 26)	0.000
UA, mmol/L, mean (± 2 SD)	0.310 (0.172 - 0.448)	0.257 (0.145 - 0.368)	0.282 (0.148 - 0.416)	0.026
GR, U/L, mean (± 2 SD)	69 (50 - 88)	61 (50 - 72)	65 (48 - 82)	0.006
TAS, mmol/L, mean (± 2 SD)	2.2 (2.0 - 2.4)	2.1 (1.8 - 2.3)	2.1 (1.9 - 2.4)	0.016
GAP, mmol/L, mean (± 2 SD)	1.4 (1.3 - 1.5)	1.4 (1.2 - 1.4)	1.4 (1.2 - 1.6)	0.207
All test values were normally distributed apart from CRP and GGT. Significant differences between males and females ($p < 0.05$) were observed for all participant data apart from age, CRP and GAP.				

Table 2: Participant demographics and serum reference ranges.

TAS represents the contributions of enzymatic and non-enzymatic substances in the serum. Non-enzymatic components are predominantly albumin and uric acid; minor contributing substances include ascorbic acid, bilirubin, α -tocopherol, β -carotene and cysteine [7]. As with GR, a decrease is observed in conditions arising from or resulting in oxidative stress [18]. TAS reference ranges of 2.0-2.4 mmol/L and 1.8-2.3 mmol/L for males and females respectively were higher than those provided of 1.30-1.77 mmol/L. The upper limit values obtained were also close to the 2.5 mmol/L dilution limit of the assay. Other reported ranges include 1.07-1.89 mmol/L [19] and 1.2084-1.214 mmol/L [8]. The GAP calculation (equation 2) [6] represents antioxidant contributions from all serum components apart from ALB and UA and therefore may provide a useful additional indicator of the effect of substances that would not be feasible to measure routinely. For calculated algorithms the measurement uncertainty should be calculated from the measurement uncertainties of each component [10]. GAP initial reference ranges for males and females were 1.3-1.5 mmol/L and 1.2-1.4 mmol/L respectively with a measurement uncertainty of ± 0.27 mmol/L. The effect of measurement uncertainty when comparing healthy groups with patients with named disorders in future studies is necessary for accurate interpretation of findings.

GGT is included in this panel as an acknowledgment to the large recent body of work around its usefulness as an additional marker of inflammation and cardiovascular disease risk [20-22]. Reference ranges currently used are < 55 U/L for males and < 38 U/L, this study however obtained ranges of 3-40 U/L for males and

6-30 U/L for females. The use of GGT in this capacity is concerned with increases within the established reference ranges, further subdividing this into quartiles [21]. A larger number of participants will enable to establishment of a more accurate reference range which can then be subdivided into the quartiles for future studies.

Apart from GGT and GAP, all tests had significant differences between male and female reference ranges. It is recommended therefore that separate reference ranges are determined initially for all tests and a single range only used if there are non-significant differences. Even as an initial indication of reference ranges, the small sample size is a limitation to this work. Working reference ranges using a minimum of 120 male and 120 female subjects are necessary for further routine and research purposes, and will form part of our future work.

If considering the addition of tests onto the existing routine clinical biochemistry panel options, sample stability, turnaround time and costs must be considered. GR and TAS are completed in less than ten minutes and therefore do not delay the completion of the routine profile on serum samples. The GAP calculation can be easily incorporated into the ILab Aries Ratios functions and therefore appears on the completed panel. The cost of the reagents is comparable to other non-immunoassay routine methods, so the addition of the panel is feasible with respect to this. The Randox GPx and SOD assays are intended for whole blood use only and lengthy extraction procedures are required prior to measurement. They are therefore not suitable for incorporation in a serum test panel, but may be undertaken on the ILab Aries as standalone tests for routine and research purposes.

GR stability in stored serum samples is quoted as one week at 2-8°C and TAS serum stability as 36 hours at 2- 8°C by the reagent manufacturer. However, to take into account time between samples being taken and analysis in addition to possible add on requests to existing samples, stability studies are currently in progress to establish local criteria. Finally, the determination of intra-individual biological variation for TAS, GR and GAP will enable the establishment of reference change values and their subsequent use in determining clinically significant changes in individuals [23]. Continuing studies will establish the stability of the measured analysts under local storage conditions, intra-individual biological variation and antioxidant panel final reference ranges using a larger population.

Conclusions

This preliminary study has determined that an automated panel for assessing pro-oxidant and antioxidant status in human serum is feasible in terms of reliable method performance, ease of operation, turnaround time and costs. The panel may be undertaken simultaneously and on the same sample as current routine clinical chemistry tests. Additional studies on larger groups of individuals with known disorders will determine the clinical usefulness of this panel.

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