

## A simple colorimetric assay to determine the concentration and proportion of human mercaptalbumin

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### ABSTRACT

**Objectives:** Human serum albumin can take on two forms, mercaptalbumin (HMA) or non-mercaptalbumin (HNA), depending on the redox status of its Cys34. The ratio of HMA and HNA is considered to be a novel biomarker of oxidative stress. While HPLC and mass spectrometry are established methods to measure HMA and HNA, a simple colorimetric assay was applied to measure this biomarker.

**Design and methods:** Michler's Hydrol (4,4'-Bis(dimethylamino)benzhydryl) is a blue dye with a maximum absorption at 612 nm, and its absorption decreases when it reacts with a thiol group. Concentrations of HMA in serum samples from 36 healthy subjects were measured based on absorption changes of Michler's Hydrol. The proportion of HMA (HMA%) in total albumin was also obtained by dividing the HMA concentration by total albumin concentration, which was obtained by a bromocresol purple (BCP) assay. The proportion of HNA (HNA%) was obtained by subtracting HMA% from 100%.

**Results:** HMA concentrations obtained by Michler's Hydrol assay were highly correlated ( $r^2 = 0.97$ ) with reference values obtained by HPLC (HMA%) and BCP assay (total albumin). The HNA % obtained by Michler's Hydrol and BCP assays combined also gave a good correlation ( $r^2 = 0.96$ ) and a small deviation (average 2.4%) with respect to HPLC as a reference method.

**Conclusions:** A colorimetric assay using Michler's Hydrol was optimized for a 96-well plate format so that it can be easily performed in a standard laboratory setting. This assay gives HMA concentrations and HNA proportions comparable to HPLC.

### 1. Introduction

Human serum albumin (HSA) is the most abundant protein in the blood, accounting for approximately 60% of all protein found in human serum [1–3]. Among its multiple functions, it is hypothesized that HSA acts as a regulator of the body's redox states by counteracting oxidative stress through Cys34, the only cysteine that does not form an intramolecular disulfide bond [2,4]. Reduced Cys34 buffers oxidative stress in the extracellular environment by reducing other thiols such as cysteine, glutathione, and homocysteine through disulfide exchanges, by reacting with two-electron oxidants such as hydroperoxides and hypohalous acids or by

**Abbreviations:** HSA, Human Serum Albumin; HMA, Human mercaptalbumin; HNA, Human non-mercaptalbumin; CKD, Chronic kidney disease; A612, Absorbance at 612 nm; BCP, Bromocresol purple.

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reducing reactive oxygen species via the formation of thiyl radicals and disulfide radical anions [1,2,5].

Depending on the redox state of Cys34, HSA may exist in the form of either human mercaptalbumin (HMA) or non-mercaptalbumin (HNA) [1,6]. In the presence of oxidative stress, the HMA/HNA ratio decreases. Thus, the use of the HMA/HNA ratio, which is a function of oxidation of Cys34, is considered a potential biomarker to assess the severity of chronic diseases for which oxidative stress is a significant etiological manifestation [6–10]. Such diseases include diabetes [11–13], chronic kidney disease [14], coronary artery disease [15,16], atherosclerosis [17], cirrhosis [18], Parkinson's disease [19] and Alzheimer's disease [20].

The majority of studies on the redox state of HSA utilize high performance liquid chromatography (HPLC) [6–10,12–14,16,19,20]. Several recent studies have also employed mass spectrometry, which yields a higher resolution, thus allowing for finer distinctions between molecular structures [4,11,18,21,22]. Both of these methods produce consistent results in routine analyses and are thus well established in the field. However, their throughput is limited, and specialized columns or instruments are required for both.

A handful of studies have explored alternative approaches using simple dye-based assays [3,23,24]. These can be adapted for high-throughput formats, such as multi-well plate readers and clinical chemistry analyzers. For example, Yoshihiro and co-workers developed a simple colorimetric assay to measure the percentage of HNA using bromocresol purple (BCP) dye [24]. This assay takes advantage of the fact that the BCP dye, traditionally used to measure total serum albumin concentrations, shows greater colorimetric absorbance when it reacts with HNA than with HMA [25].

Another colorimetric approach is to approximate the concentrations of HMA with total thiol concentrations [3], based on the notion that HMA accounts for the majority of thiol in human serum or plasma [1,2,26]. 4,4'-Bis(dimethylamino)benzhydrol (hereafter Michler's Hydrol) is a blue dye with an absorption maximum at 612 nm, and its absorption decreases when it reacts with free thiols [27]. An assay using Michler's Hydrol to measure HMA has been briefly described [3]. While 5,5'-dithiobis(2-nitrobenzoic acid) (hereafter DTNB) is often used to measure thiol groups, its absorption spectrum (max at 412 nm) overlaps with that of bilirubin (max at 451 nm), and small-molecular-weight materials in serum interfere with direct measurement of HMA in serum by DTNB [3]. Michler's Hydrol is an alternative dye for the direct measurement of HMA, as it is less likely to be accompanied with interference. In addition, Michler's Hydrol can be purchased from global chemical suppliers at a relatively low price, which makes it an attractive candidate for industrial development.

In this paper, we introduce a simple colorimetric assay using Michler's Hydrol to determine the HMA concentration, which correlated well with reference HMA concentrations obtained by HPLC and BCP. The proportion of HMA was also determined by dividing the HMA concentration from the Michler's Hydrol assay by the total albumin concentration from the BCP assay, and it also correlated well with the reference values obtained by HPLC. We optimized the originally-described procedure [3] so that it can be easily performed in a 96-well plate format. The assay accuracy was also significantly improved by eliminating the contribution of non-thiol components to absorption changes of Michler's Hydrol. The assay described here is also scalable for high-throughput analysis and can potentially serve as an alternative to HPLC and mass spectrometry, for which redox states of albumin are used as biomarkers in clinical practice.

## 2. Materials and methods

### 2.1. Chemicals

4,4'-Bis(dimethylamino)benzhydrol and guanidine-HCl were purchased from Tokyo Chemical Industry (Tokyo, Japan). Sodium iodoacetate and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), bromocresol purple, and Triton X-100 were purchased from FUJIFILM-Wako Chemicals (Tokyo, Japan).

### 2.2. Human serum

Normal human serum (Consera-1-gou, freeze-dried form) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Standardized serum for the BCP assay (TP/ALB standard) was purchased from Nittobo Medical (Fukuyama, Japan). Eighteen and ten (i.e., a total of 28) human serum samples were purchased from BioIVT (Westbury, NY, USA) and BIOPREDIC International (Parc d'affaires, France), respectively. Donors of these samples were all healthy individuals comprised of 19 males and 9 females with ages ranging from 18 to 69 years. Informed consent (in writing) was obtained from all donors by the suppliers. An additional eight human serum samples were also obtained from healthy individuals comprised of four males and four females with ages ranging from 28 to 45 years. For these eight additional samples, serum was prepared within 30 min after the blood was drawn and immediately stored at  $-80^{\circ}\text{C}$ . Informed consent (in writing) was obtained from all donors of these additional eight samples, and donor ID was kept confidential for ethical purposes. This study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine, the University of Tokyo (No. 10964-9)).

### 2.3. Michler's hydrol assay to measure HMA concentrations of serum samples

All serum samples from the individual subjects were diluted two-fold by 50 mM NaCl in 100 mM sodium citrate pH 5.0 (hereafter "citrate buffer"), as doing so stabilizes HMA by preventing spontaneous oxidation [28]. The calibration standard (normal human serum, Consera-1-gou in section 2.2) was serially diluted in the citrate buffer to produce HMA concentrations ranging from 30 to 300  $\mu\text{M}$ . Michler's Hydrol was first dissolved in dimethyl sulfoxide at 10 mM as a stock solution and then diluted down to 35  $\mu\text{M}$  in 6 M guanidine-HCl, 250 mM sodium acetate, pH 5.0.

All the samples and calibration standards were divided into two sets of 15  $\mu\text{L}$  aliquots on a 96-well plate. To one set of the aliquots, 10  $\mu\text{L}$  of 200 mM iodoacetamide in 250 mM Tris-HCl pH 8.0 was added (alkylation set) and incubated at 37 °C for 15 min. To another set of the aliquots, 10  $\mu\text{L}$  of 250 mM Tris-HCl pH 8.0 (non-alkylation set) was added instead. 225  $\mu\text{L}$  of 35  $\mu\text{M}$  Michler's Hydrol in 6 M guanidine-HCl, 250 mM sodium acetate, pH 5.0 was then added to all the samples. After thorough mixing, the solution was incubated further at room temperature for 20 min. A612 was measured by a 96-well plate reader (Nivo 3F microplate reader, PerkinElmer, Waltham, MA, USA). For every sample, including the calibration standard, measurements were performed in triplicate using three wells, and average A612 was taken for the calculation.

A612 from the non-alkylation set was subtracted from A612 from the alkylation set, and this value was taken as  $\Delta\text{A612}$ .  $\Delta\text{A612}$  of the calibration standards were plotted against their HMA concentrations. Linear regression was applied to create a standard curve. HMA concentrations of the samples were obtained from  $\Delta\text{A612}$  of the samples and the standard curve (Fig. 1). (Below, data analysis based on  $\Delta\text{A612}$  is called "corrected analysis".)

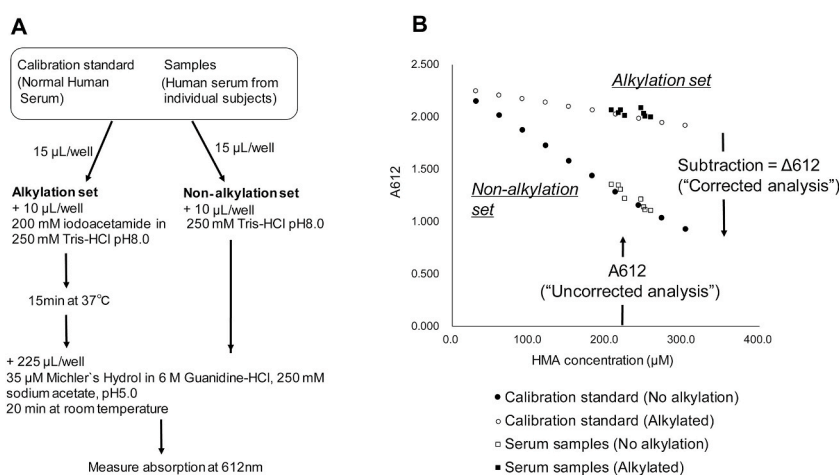
To test the improvement by canceling out the contribution of non-thiol components, a standard curve was created from A612 from the non-alkylation set of the calibration standard, and HMA concentrations were obtained from A612 of the samples and the standard curve instead (Fig. 1). (Below, data analysis based on A612 from the non-alkylation set is called "uncorrected analysis".)

To evaluate the performance of the assays, the correlation was determined by a linear regression between the HMA concentrations obtained by Michler's Hydrol assay and corresponding reference concentrations obtained by HPLC and BCP assays (section 2.4 and Fig. 2). Deviations of HMA concentrations by Michler's Hydrol assay from their reference values were also determined by taking the absolute values of differences between the values from Michler's Hydrol assay and corresponding reference values.

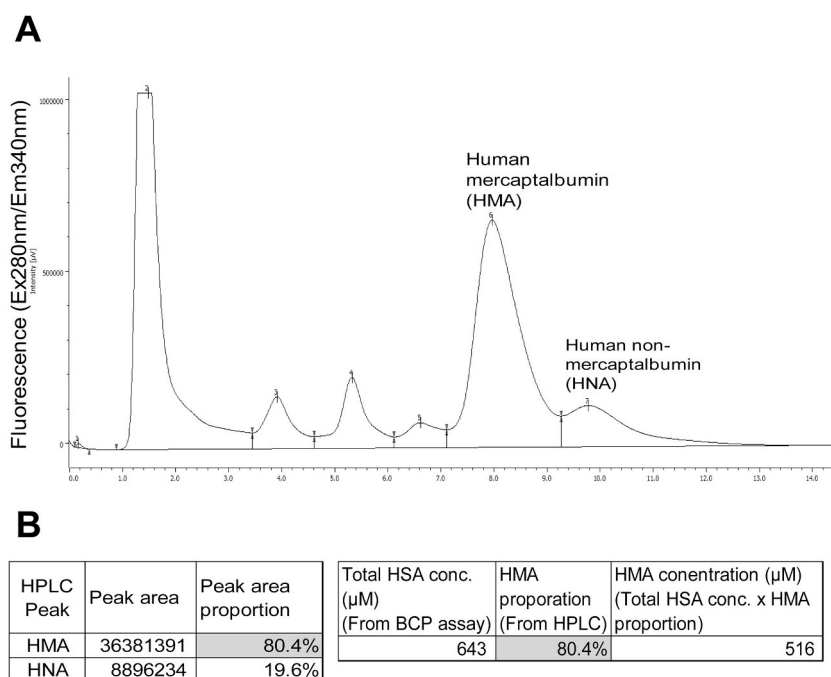
The proportion of HMA (HMA%) was calculated by dividing the HMA obtained from Michler's Hydrol assay by the total HSA concentration obtained from the BCP assay (i.e.  $\text{HMA}\% = [\text{HMA}]_{\text{Michler's Hydrol assay}} / [\text{HSA}]_{\text{BCP assay}}$ ). The proportion of HNA (HNA%) was then obtained by subtracting the proportion of HMA from 1 (i.e.  $1 - \text{HMA}\%$ ). HNA% from Michler's Hydrol and BCP assays (combined) was also evaluated based on their correlation and deviation with respect to HNA% from HPLC, which is considered to be a reference value by the standard method.

#### 2.4. Determination of the HMA/HNA ratio and HMA concentration by HPLC (reference method)

HPLC was performed by following the procedure described by Yasukawa and co-workers [29] as a reference method to measure the HMA/HNA ratio. Polyvinyl alcohol cross-linked gel conjugated with diethyl amine was packed in a stainless column (50  $\times$  7.6 mm I. D.). The column was equilibrated with 60 mM sodium sulfate in 25 mM sodium phosphate, pH 6.0 (hereafter, Buffer-A), and 1 M  $\text{MgCl}_2$  solution was used as an eluent (hereafter, Buffer-B). Immediately after injecting 5  $\mu\text{L}$  of the samples, a linear gradient program from 100% Buffer-A to 75% Buffer-A/25% Buffer-B was performed by an HPLC system (LC-Net II/ADC System, JASCO Corporation, Tokyo, Japan) consisting of a degasser (DG-2080-54 Plus), an auto-sampler (AS2057 Plus), two pumps (PU-2080 Plus), a mixer (MX-2080-32 Plus), a fluorescence detector (FP-2025 Plus), and a column oven (CO-2060 Plus). The linear gradient was completed in 7.5 min followed by an isocratic flow of 75% Buffer-A/25% Buffer B for 2.5 min at flow rate of 1 mL/min, and the column was maintained at 40 °C throughout the run. The fluorescence intensity of the eluate was monitored with excitation and emission wavelengths of 280 nm and 340 nm. Two main peaks eluted toward the end of the chromatography were identified as HMA and HNA, and their peak areas were



**Fig. 1.** Assay procedure. A) Assay workflow. All the calibration standards and samples are divided in two sets, an alkylation set and non-alkylation set. The former is incubated with iodoacetamide before the addition of Michler's Hydrol reagent. Buffer is added to the latter to adjust volume. Michler's Hydrol reagent is then added. Finally, absorbance at 612 nm is measured from all wells. B) Sample data from the calibration standards and eight serum samples. For the "corrected analysis", absorbances at 612 nm (A612) of the non-alkylated control were subtracted from corresponding absorbances at 612 nm of the alkylation set. This value was used as  $\Delta\text{A612}$  to obtain the HMA concentration. For the "uncorrected analysis", A612 of the non-alkylation set alone was also used to obtain HMA concentrations for comparison.



**Fig. 2.** Determination of reference HMA proportion and concentration. An example of a serum sample is shown. A) Reference HMA proportion was determined by HPLC as described in section 2.4. B) Reference HMA concentration was obtained by multiplying the proportion as in (A) and the total HSA concentration given by the BCP assay as described in section 2.5.

used to calculate the HMA/HNA ratio (i.e.  $\%HMA = \frac{HMA_{\text{peak area}}}{HMA_{\text{peak area}} + HNA_{\text{peak area}}}$ ). Reference values of HMA concentrations were obtained by multiplying the proportion of HMA (%HMA obtained by HPLC) by the total HSA concentration obtained by the Bromocresol Purple (BCP) assay (as described in section 2.5 below) (Fig. 2 & [29])

### 2.5. Bromocresol purple (BCP) assay and determination of total HSA concentration

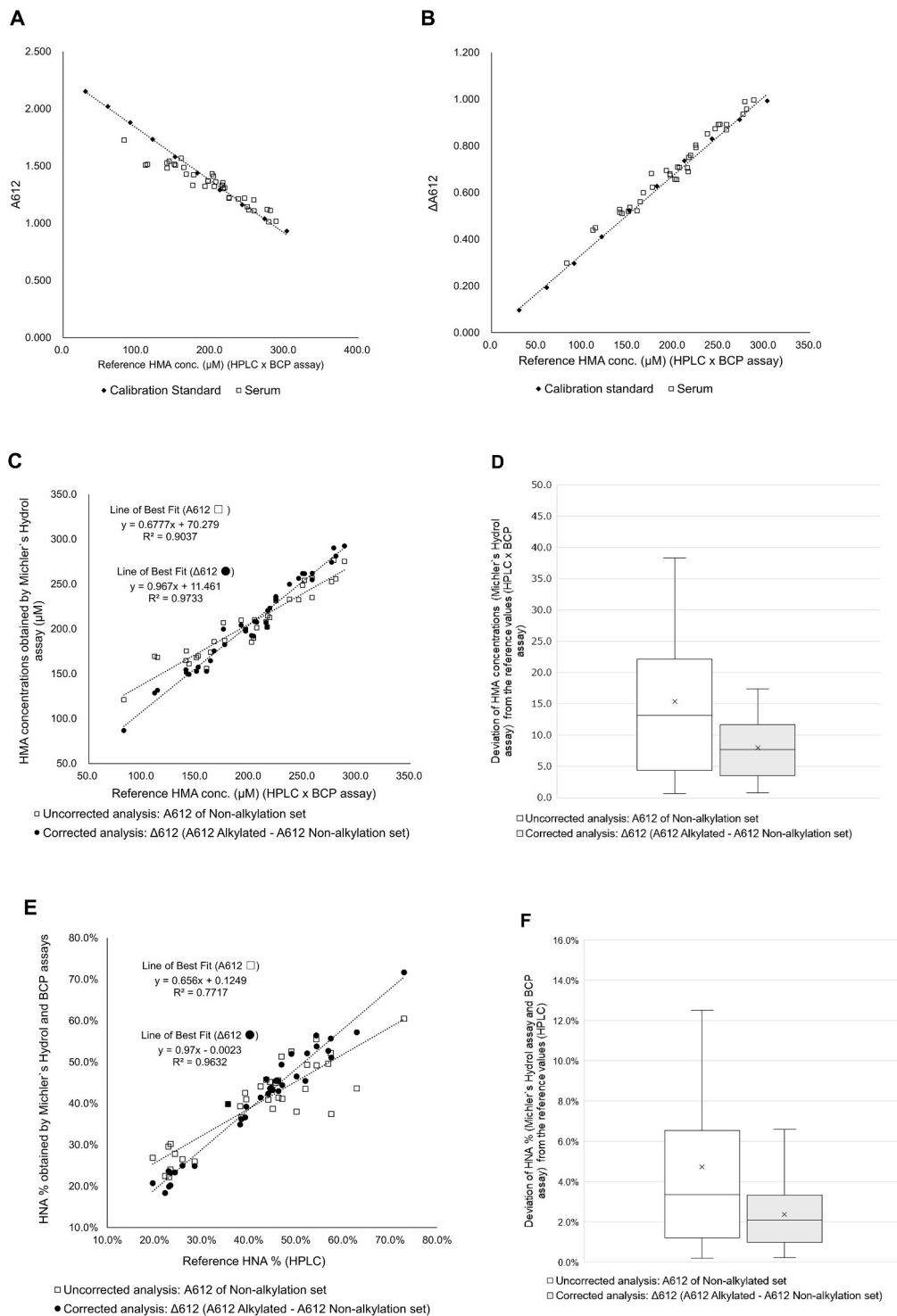
The BCP assay was performed by slightly modifying the method described previously [25,30]. Volume ratio of the sample to the first reagent was increased since manual operation on 96-well plate was more prone to pipetting errors. The composition of the first reagent was also slightly changed from those used for measurement by clinical chemistry analyzers [30] by increasing DTNB concentration to maintain more than five fold molar excess of DTNB over total albumin as sample volume was increased.

15 μL of samples or serially diluted TP/ALB standards were placed in each well of a 96-well plate. 85 μL of the first reagent (588 μM DTNB, 0.038% (w/v) SDS in 29.4 mM Tris HCl, pH 8.0) was added to each well and incubated at 37 °C for 10 min. 150 μL of the second reagent (240 μM bromocresol purple, 0.3% (v/v) Triton X-100 in 250 mM succinate-NaOH, pH 5.5) was then added and incubated at 37 °C for 10 min. Absorbance at 600 nm was measured by a plate reader (Nivo F3 microplate reader, PerkinElmer) and was used to determine the concentration of total HSA.

## 3. Results

In our initial trials with the Michler's Hydrol assay based on its original description [3], we noticed sample-to-sample variability in deviation from calibration curve and absorption changes per moles of HMA. We hypothesized that a source of such variability is non-thiol components that contribute to the absorption in both the samples and calibration standards to varying degrees. In order to reduce the sample-to-sample variability, we cancelled out the contribution of non-thiol components by taking the differences ( $\Delta A_{612}$ ) between  $A_{612}$  of alkylated samples (alkylation set) and untreated samples (non-alkylation set) by subtracting  $A_{612}$  of the latter from the former. To show improvement by this subtraction scheme, an analysis was also conducted based on  $A_{612}$  of the non-alkylation set alone. Here, we call the former (based on  $\Delta A_{612}$ ) the "corrected analysis" and the latter (based on  $A_{612}$  of the non-alkylation set) the "uncorrected analysis".

As the uncorrected and corrected analyses,  $A_{612}$  of the non-alkylation set and  $\Delta A_{612}$  were respectively plotted against reference concentrations of HMA from established methods: HPLC for the proportion HMA in total HSA, and BCP for total HSA concentration. Since Michler's Hydrol loses its color when it reacts with thiol,  $A_{612}$  of the non-alkylation set decreased proportionally with HMA concentration (Fig. 3A). As the non-alkylation set induces much larger decreases of  $A_{612}$  than the alkylation set,  $\Delta A_{612}$  ( $A_{612}$  of alkylation – non-alkylation set) showed a positive correlation with HMA concentrations (Fig. 3B). The calibration curves of both uncorrected and corrected analyses showed linear relationships between the HMA concentration and  $A_{612}$  of the non-alkylation set or



**Fig. 3.** Performance of Michler's Hydrol assay with respect to the reference values. The average of each A612 measurement (three replicates) is represented by each datapoint. A) Uncorrected analysis: A612 of the non-alkylation set from all the samples plotted against their reference HMA concentrations as determined by HPLC × BCP assays. B) Corrected analysis: ΔA612 from all samples plotted against their reference HMA concentrations as determined by HPLC × BCP assays. C) Correlation of HMA concentrations obtained by Michler's Hydrol assay (uncorrected and corrected analysis) and their reference values. D) Deviation of HMA concentrations obtained by Michler's Hydrol assay (uncorrected and corrected analysis) from their reference values. E) Correlation of HNA proportion determined by  $1 - (\text{Michler's Hydrol assay (HMA conc.)} / \text{BCP assay (Total HSA conc.)})$  and their reference values from HPLC (i.e., absolute value of  $[\text{HMA}]_{\text{Michler's Hydrol}} - [\text{HMA}]_{\text{HPLC} \times \text{BCP}}$ ). F) Deviation of HNA proportion determined by  $1 - (\text{Michler's Hydrol assay (HMA conc.)} / \text{BCP assay (Total HSA conc.)})$  from the reference values obtained by HPLC (i.e., absolute value of  $\text{HNA}\%_{01 - ([\text{HMA}]_{\text{Michler's Hydrol}} / [\text{HSA}]_{\text{BCP}}) - \text{HNA}\%_{\text{HPLC}}}$ ). (For the boxplot in D & F: upper end of the vertical line ("whisker") = maximum, lower end of the vertical line ("whisker") = minimum, upper edge of the box = 75% percentile, lower edge of the box = 25% percentile, X = average, horizontal line in the box = median.).

$\Delta 612$  ( $R^2 > 0.99$  for both).

Regarding data points of the serum samples, the uncorrected analysis appeared to show slightly more sample-to-sample variability than the corrected analysis. The difference became evident when HMA concentrations were calculated from the standard curve. HMA concentrations from the corrected analysis (i.e. based on  $\Delta A612$ ) gave a significantly better correlation with their reference values as compared to the uncorrected analysis (i.e. based on  $A612$  of the non-alkylation set) (Fig. 3C). The results were also evaluated based on their deviation from the reference values (Fig. 3D). While HMA concentrations given by the uncorrected analysis deviated from the reference values by  $15 \mu\text{M}$  on average (average of absolute values of  $[\text{HMA}]_{\text{Michler's Hydrol}} - [\text{HMA}]_{\text{HPLC} \times \text{BCP}}$ ), the average deviation was reduced to  $8 \mu\text{M}$  in the corrected analysis (Fig. 3D). Variability of the deviation was also reduced in the corrected analysis (Fig. 3D).

Since the proportions of HMA and HNA are readouts of HPLC and mass spectrometry that are currently used to establish the clinical utility of albumin's redox state, we attempted to obtain the proportions of HMA and HNA (hereafter %HMA and %HNA). HMA% was first obtained by dividing the HMA concentrations from Michler's Hydrol assay by total HSA concentrations from the BCP assay. HMA% was then subtracted from 100% to obtain HNA%, as expression by HNA% is more commonly used. For HNA% too, the subtraction of absorption of alkylated samples significantly improved both the correlation (Fig. 3E) and the deviation (Fig. 3F).

#### 4. Discussion

The use of Michler's Hydrol assay to determine HMA concentrations was described more than 30 years ago [3], but to best of our knowledge, no other studies have employed this approach. While we could reproduce the negative correlation between  $A612$  and HMA concentrations as it was described, we found it laborious and not easy to reproduce. First, the original assay uses purified HMA as a calibration standard, and we had difficulty purifying HMA with consistent yield. However, we found that normal human serum can also be used as a calibration standard, which is often used as a calibration standard for many *in vitro* diagnostics. Second, we optimized the protocol to adapt it to a 96-well plate format, while the original assay requires  $50 \mu\text{L}$  of the sample and  $2 \text{ mL}$  of Michler's Hydrol reagent, as it was designed for a spectrophotometer. The 96-well plate format allowed us to process multiple samples at once. Third, we noticed that the absorption changes per mole of HMA and the deviation from the calibration curve varied from sample to sample. This can be due to variability of both non-HMA thiols and non-thiol components that affect the absorption. While we could not address the possible effects of non-HMA thiols, we found that even non-thiol components affected the absorption. By eliminating thiols by alkylation with iodoacetamide, we were able to cancel out the contribution of non-thiol components to the absorption, and this procedure significantly improved assay accuracy.

The readout of Michler's Hydrol assay is the HMA concentration, while the readouts of HPLC and mass spectrometry are the proportion of HMA, HNA, or species of HNA. Multiple studies have shown that an increased proportion of HNA is associated with the severity of chronic diseases that are exacerbated by oxidative stress [7,10,12–14,16,19]. In contrast to the original assay [3], we demonstrated that HMA% close to the value given by HPLC can be derived by simply dividing the HMA concentration by total HSA concentration. As the proportion of HMA and HNA has been established as a biomarker using HPLC and mass spectrometry, this conversion is useful when adapting Michler's Hydrol assay to clinical practice.

The premise of Michler's Hydrol assay is that HMA concentrations can be approximated by colorimetric changes of Michler's Hydrol when it reacts with thiols in serum [3], which is supported by previous reports indicating that HMA is the predominant source of thiol in human serum or plasma [1,2,31]. In addition, we found that HMA induces more colorimetric ( $A612$ ) changes in Michler's Hydrol than small molecule thiols. During our initial search for calibration standards, we found that the  $A612$  decrease per mole of thiol given by cysteine is significantly smaller than that of HMA. A comparison of five major small molecule thiols in human serum and purified HMA also showed that HMA induces more absorption changes than small molecule thiols (Supplementary Fig. 1). Based on a one-to-one stoichiometric reaction between Michler's Hydrol and thiols as described previously [27], we could not find a possible explanation for these differences in  $A612$  decreases per mole of thiol. Nevertheless, this finding indicates that Michler's Hydrol favors the measurement of HMA concentrations in human serum by receiving a smaller contribution from small molecule thiols.

The combined concentrations of small molecule thiols are reported to be  $14.4\text{--}19.1 \mu\text{M}$  in human plasma, while the protein-bound thiol concentration in human plasma is  $452 \mu\text{M}$  [31]. From these values, as well as the  $A612$  decreases per mole of small molecule thiols and HMA (Supplementary Fig. 1), the contribution of small molecule thiols on  $A612$  is estimated to be  $1/30\text{--}1/40$  of the contribution by protein-bound thiols, assuming that the  $A612$  decrease per mole of thiol on HMA represents that of protein-bound thiols. Serum samples before and after dialysis also gave nearly the same absorption changes per mole of HMA (Supplementary Table 1), which also indicated little contribution of small molecules to the changes of  $A612$ .

Serum levels of some small molecule thiols such as homocysteine and cysteine increase depending on diseases, nutritional status and aging [32–35]. We tested whether elevation of these thiols could influence Michler's Hydrol assay. Each of five small molecule thiols (discussed above) was added to a serum sample from a healthy subject at several concentrations that exceed normal concentrations of their reduced forms [31] by 5–50 folds. At all the additional concentrations, these thiols had no significant effect on the HMA concentrations determined by Michler's Hydrol assay (Supplementary Table 2), which led to no significant impact on HNA proportions (Supplementary Table 3). This suggests that the potential elevation of these small molecule thiols in clinical samples would have little effect.

A previous study showed that the majority of protein-bound thiol in human plasma comes from albumin [26]. By following the procedure described in this report, we also labeled protein-bound thiols in eight serum samples by a thiol-reacting fluorescent dye, ThioGlo1, and visualized them on an SDS-PAGE gel (Supplementary Fig. 2). One distinct fluorescent band corresponding with the mobility shift of HSA indicated that HMA was the predominant species of protein-bound thiol. Although there may be other

protein-bound thiols, they were not as intensely labeled as HMA. While we consider this experiment to be a rough estimate, it supports the notion that most thiols in human serum come from HMA.

One limitation of this study is that all the human serum samples came from healthy donors, and many of them were apparently oxidized. Most samples purchased from commercial suppliers had an HNA proportion of more than 30% (40–60% for the majority). Although this degree of HNA proportion occurs in chronic disease patients [8,11,12,14,17,20,22], HNA proportions for healthy subjects fall between 15% and 30% [7,22,28]. Without any stabilizing agents (e.g., citrate buffer, pH 5.0), HMA in human serum is oxidized at 4 °C [28] and –20 °C [36]. Therefore, most of the purchased samples in this study were perhaps oxidized to various degrees during preparation and storage. As such, we collected human serum from eight additional donors and immediately stored them at –80 °C, which maintained the HNA proportion at 20–25%. Although our samples overall covered the range of HNA proportions found in clinical samples, we cannot exclude the possibility that samples with a high HNA proportion due to oxidation *in vivo* (e.g., associated with chronic disease) and *in vitro* behave differently in Michler's Hydrol assay. Further evaluation of Michler's Hydrol assay is needed using clinical samples that are not significantly oxidized.

While we manually performed Michler's Hydrol assays in a 96-well plate in this study, this assay can potentially be performed by automated systems such as liquid handlers and clinical chemistry analyzers. Indeed, we are currently developing a new reagent composition specifically suitable for clinical chemistry analyzers. Such automation may yield higher throughput and allow for the processing of larger sample sizes, which is suitable for further evaluation and improvement of this assay by using a large number of clinical samples from subjects with various backgrounds. This would accelerate the clinical development HNA/HMA proportions as a biomarker with definitive clinical utility and also position Michler's Hydrol assay as a high-throughput method alternative to HPLC and mass spectrometry.

## 5. Conclusions

The concentrations of HMA in human serum can be measured by Michler's Hydrol assay, which is based on an approximation of HMA concentrations with Michler's Hydrol's colorimetric changes mainly due to thiols. The assay accuracy was significantly improved by preincubation of a subset of the samples with an alkylating agent (iodoacetamide) and subtracting their absorption from the absorption of corresponding samples without alkylation. Proportions of HMA and HNA were also obtained by combining Michler's Hydrol assay and the BCP assay (for total albumin concentration). The results correlated well with HPLC, which has been established as a standard method for HMA/HNA measurement.

## Authorship statement

### Category 1

Conception and design of study: S. Tada, K. Yasukawa, Y. Yatomi, T. Uchiki;

Acquisition of data: S. Tada, T. Uchiki;

Analysis and/or interpretation of data: S. Tada, T. Uchiki;

### Category 2

Drafting the manuscript: S. Tada, K. Yasukawa, Y. Yatomi, T. Uchiki;

Revising the manuscript critically for important intellectual content: S. Tada, K. Yasukawa, Y. Yatomi, T. Uchiki;

### Category 3

Approval of the version of the manuscript to be published (the names of all authors must be listed): S. Tada, K. Yasukawa, Y. Yatomi, T. Uchiki;

## Declaration of competing interest

The authors of this article have filed patent applications related to the “corrected analysis” by canceling out the non-thiol components' contribution to A612 with following details; Methods and kits to measure concentrations of reduced albumin or to determine proportions of reduced and oxidized form of albumin in serum or plasma, Japan Patent Office application No. 2021–178318.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plabm.2022.e00281>.

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