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Measurement of total antioxidant capacity of human plasma: Setting and validation of the CUPRAC–BCS method on routine apparatus ADVIA 2400

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ABSTRACT

Background: Quantification of Total Antioxidant Capacity (TAC) of human plasma is an important clinical target, since many diseases are suspected to be related with oxidative stress. The CUPRAC–BCS (BCS=Bathocuproinedisulfonic acid) method was chosen since it works using the photometric principle, with stable and inexpensive reagents and at physiological pH.
Methods: The method is based on the complex equilibria between Cu(II)–BCS (reagent) and Cu(I)–BCS. Cu (I)–BCS complex is formed by reducing ability of the plasma redox active substances. The photometric signal is achieved at 478 nm and calibration is performed using urate as a reference substance.
Results: Linearity, linear working range, sensitivity, precision, LoD, LoQ, selectivity and robustness have been considered to validate the method. Absorbance at 478 nm was found linear from 0.0025 up to 2.0 mmol L^{-1} of urate reference solution. Precision was evaluated as within-day repeatability, $S_r = 4$ μmol L^{-1}, and intermediate-precision, $S_{I(T)} = 15$ μmol L^{-1}. LoD and LoQ, resulted equal to 7.0 μmol L^{-1} and 21 μmol L^{-1} respectively while robustness was tested having care for pH variation during PBS buffer preparation. Tests on plasma (80 samples) and on human cerebrospinal fluid (30 samples) were conducted and discussed.
Conclusions: By the analytical point of view, the photometric method was found to be simple, rapid, widely linear and reliable for the routine analysis of a clinical laboratory. By the clinical point of view, the method response is suitable for the study of chemical plasma quantities related to redox reactivity.

1. Introduction

The free radical theory was introduced in 1956 by Harman [1]. Free radicals—namely Reactive Oxygen Species (henceforth ROS) and Reactive Nitrogen Species (henceforth RNS)—are highly reactive species due to the presence of unpaired electrons. During its short existence free radical is driven to capture an atom of hydrogen from other biomolecules, causing a chain reaction that alters irreversibly the chemical structure of the cellular components with which it comes into contact. These free radicals can be produced by endogenous causes, like metabolic products [2] and by exogenous causes, like ionizing radiations or smoke cigarette [3]. The most relevant ROS/RNS include: superoxide radical anion, hydroxyl radical, hydrogen peroxide (as radical precursor), alkoxyl radicals, peroxyl radicals, and nitrogen monoxide radical.

In the course of biological evolution an antioxidant defense system has been developed to protect human organism against ROS/RNS, in fact a sophisticated array of antioxidant molecules is found in biological systems [2]. This antioxidant defense system can be distinguished with respect to endogenous (enzymes and non-enzyme species) and exogenous (as some vitamins and polyphenols) sources.

When in the human organism there is a situation characterized by an imbalance between an increased production of oxidant species and/or a decreased efficacy of the antioxidant defense system, a condition called *oxidative stress* rise. Hence, the term *oxidative stress* identifies the change in the normal balance between oxidant substances and antioxidant system of detoxification. Moreover, the oxidative stress is involved in the pathogenesis and development of many human diseases such as cancer (lung cancer and prostate cancer) [4], diseases of the cardiovascular system (hypertension, atherosclerosis, ictus, and infarct) [5–7], and diseases of the central nervous system (Alzheimer's disease and Parkinson's disease) [8,9]. A literature search on the basis of information in the PubMed database revealed that the number of publications on antioxidant capacity (keyword: "antioxidant capacity") has grown nearly exponentially in the last 40 years (from 321 papers in 1970–1980 to 10,581 papers in 2000–2011 years).

This paper deals with the measurement of the Total Antioxidant Capacity (henceforth TAC) of human plasma.

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The method used for the measurement of the TAC was implemented and validated ("in-house" validation model) with special care for the application to routine analysis on the auto-analyzer of a clinical laboratory [10,11]. In fact, many papers show the interest of physicians in this topic but the analyst is currently not equipped to carry out such type of clinical test at a routine level with reliability. Moreover, measuring concentrations of individual antioxidants require complex and time-consuming analytical techniques; on the contrary, the determination of the TAC can be achieved by simple and fast methodology and might give more biologically relevant information than that obtained from measuring each antioxidant separately [12]. Many analytical methods have been developed to determine TAC in a wide range of matrixes such as biological fluids, food, beverages, plant extracts and several outstanding reviews about them have been discussed [13–21]. Depending upon the reaction involved, as reported by Prior et al. [21], these assays can be classified into: assays based on hydrogen atom transfer (HAT) reaction and assays based on electron transfer (ET). HAT assays involved a competitive reaction in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. ET assays involved the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced [21].

The "Oxygen Radical Absorbance Capacity" (ORAC) [22,23], the "Total Radical Absorption Potential" (TRAP) assay [24,25] and the Crocin Bleaching Assay [10,12,26] are some of the most commonly used HAT assays for the TAC determination of biological fluids. On the other hand, the "Trolox Equivalent Total Antioxidant Capacity" (TEAC) [11,18,27], the "Ferric Reducing Ability of Plasma" (FRAP) [20,28] and the "CUPric ion Reducing total Antioxidant Capacity" (CUPRAC) [13,29–36] are some of the most commonly used ET assays.

The CUPRAC–BCS method was chosen among those mentioned above. The advantages of the method were smartly emphasized in the papers of Apak and co-workers [31] and may be summarized as follows: (i) it works at a physiological pH (the redox reactivity tested remain that corresponding to the natural conditions occurring in vivo); (ii) chemical preparations and measurements steps are fast and simple; (iii) stability of reagents is satisfactory, especially with respects to that of the methods employing radicals; (iv) the analytical response (i.e., absorbance) vs. concentration of a proper standard is linear over a wide range; (v) it is able to include in the overall response the measure of thiol-type antioxidants; and (vi) it is inexpensive. Anyway, food chemistry is the field showing the greatest number of papers about this method while its application to biological matrixes is quite poor [13,30,31,35,36].

Although Apak and co-workers are exhaustive in their paper, no extensive validation studies of the method are reported, especially for biological matrixes and for automated clinical apparatus. A validation study of the CUPRAC–BCS method was performed by Campos and co-workers [13] by way of a micro-plate reader technology, while the results presented in this paper were collected and validated using the auto-analyzer ADVIA 2400 Chemistry System. Applicability of the method is tested on various types of healthy and pathological plasma, as well on to the cerebrospinal fluid.

2. Materials and methods

2.1. Chemicals

Uric acid sodium salt (purity ≥98%), Bathocuproinedisulfonic acid (BCS, purity ≥98%), Cu(II) sulfate pentahydrate (purity ≥98%), PBS (Phosphate Buffered Saline 0.1 mol L^{-1}), L-Ascorbic acid (purity ≥98%) and α-Glucose (purity ≥98%) were purchased from Sigma-Aldrich and were of analytical grade. Cu(II) reference solution was from Merck (1000 ppm ± 1).

All solutions were prepared using grade A glassware and distilled and deionized water from MilliQ apparatus. Uric acid for the calibration was dissolved in 30 mmol L^{-1} NaOH to attain a concentration of 2 mmol L^{-1}. Cu(II) reference solution was diluted in the range 0.2–2.0 mmol L^{-1}. Bathocuproinedisulfonic acid was dissolved in 10 mmol L^{-1} PBS (pH 7.40) to attain a concentration of 36 mmol L^{-1} (stock solution). Cu(II) sulfate was dissolved in MilliQ water to reach a concentration of 10 mmol L^{-1} (stock solution). L-Ascorbic acid was dissolved in MilliQ water to a concentration of 50 mmol L^{-1} (stock solution). Reagent R1 was prepared by diluting Bathocuproinedisulfonic acid stock solution to 900 μmol L^{-1} in PBS buffer (pH 7.40). Reagent R2 was prepared by diluting Cu(II) sulfate stock solution to 640 μmol L^{-1} in MilliQ water.

2.2. Spectrophotometric apparatus

The visible photometric determinations were carried out using ADVIA 2400 Chemistry System by Siemens. The kinetic of the reaction was measured by means of a spectrophotometer (cuvette optical path-length 6 mm) which performed a series of readings, one every 14 s at 14 wavelengths simultaneously (from 340 to 884 nm) for the analytical cycle under thermo stated condition at 25 °C.

2.3. Software

All data obtained were presented using the software Origin 6.1 (by OriginLab) and analyzed using SPSS Statistics 17.0 (by SPSS).

2.4. Sample collection and preparation

Blood from hospital subjects was collected into tubes containing lithium–heparin. Plasma was obtained by withdrawing supernatant of centrifuged blood at 3500 rpm for 6 min at 15 °C. For the validation of the method a pool of plasma was obtained, stored frozen in aliquots at −20 °C and thawed out before use. Cerebrospinal fluid was collected into sterile tubes after spinal tap.

2.5. TAC measurement

The CUPRAC–BCS method [13,29–36] uses Cu(II)–BCS complex as an oxidant. The model reaction of the chemical test is:

$$Ox_1(Cu(II)–BCS)+Rid_2 \rightarrow Rid_1(Cu(I)–BCS)+Ox_2$$

where the generic couple Rid_2/Ox_2 represents the whole of the antioxidant equipment of the plasma under test able to react with the oxidant Cu(II)–BCS complex at pH 7.4. Then, the neo-formed Cu(I)–BCS complex, whose absorption maximum is in the range 470–490 nm [13], is detected and quantified by the photometric measurement. The quantification is achieved through the calibration curve obtained with Cu(I)–BCS as reference species, in the range of concentrations 0.2–2.0 mmol L^{-1}. The extent of formation of Cu(I)–BCS is the measure of TAC. However, calibration can also be referred to an antioxidant. Urate was selected since the most of the antioxidant capacity of human plasma is due to this molecule and it is able to simulate conveniently the behavior of plasma. The redox reaction between Cu(II)–BCS and urate was preliminarily studied and the number of electrons exchanged was used as the redox factor to express the final result of the analysis. Since 1 mol of urate can reduce 2 mol of Cu(II)–BCS complex, and is therefore equivalent to 2 mol of electrons (see Results section), the result can be express as μmol L^{-1} of Cu(I)–BCS reducing equivalent by multipling the urate concentration obtained from the calibration curve for 2000.

2.6. Study of the redox reaction between Cu(II) and urate

This study was subdivided into two steps: (i) estimation of the molar absorption coefficient of the Cu(I)–BCS complex (ΔA_{478} vs. $C_{Cu(I)–BCS}$) and (ii) estimation of the redox relationship between Cu(II)–BCS and urate, being the slope of the related straight-line the electronic exchange sought. The molar absorption coefficient of Cu(I)–BCS is achieved by reducing Cu(II)–BCS to Cu(I)–BCS using L-Ascorbic acid as reducing molecule in excess. A selected volume of diluted Cu(II) reference solution (concentrations: 0.2, 0.5, 1.0, 1,5 and 2.0 mmol L^{-1}) was added to reagent R1 (final concentration 700 μmol L^{-1}) and incubated for 1 min. Absorbance at 478 nm was then measured (A_0). Following, L-Ascorbic acid at concentration of 10 mmol L^{-1} was added and after incubation for 3 min [19] the absorbance at 478 nm was measured (A_1). The ΔA_{478} was calculated for each standard and used to estimate the molar absorption coefficient sought. Then, the redox relationship between Cu(II)–BCS and urate is estimated reducing the Cu(II)–BCS to Cu(I)–BCS using urate freshly prepared (five points in three replicates per three days—urate range 0.2–2 mmol L^{-1}) as reducing molecule and effective amount of Cu(I)–BCS in cuvette is obtained by way of the molar absorption coefficient Cu(I)–BCS achieved in the first step and plotted vs. effective urate concentration in cuvette. The slope of the straight-line ($C_{Cu(I)–BCS}$ vs. C_{urate}) corresponds to the electronic exchange sought.

2.7. Automated CUPRAC method on ADVIA 2400

A selected volume of either urate standard solution or plasma was added to reagent R1 (final concentration 700 μmol L^{-1}) and incubate for 1 min. Absorbance at 478 nm was then measured (A_0). Thereafter, reagent R2 (final concentration 128 μmol L^{-1}) was added and after incubation of 3 min [19] the absorbance at 478 nm was measured (A_1). The change in absorbance (ΔA_{478}) between the final reading (A_1) and the first reading (A_0) was calculated for each sample and reported on the calibration curve. The TAC results are expressed as μmol L^{-1} of Cu(I)–BCS reducing equivalent by using the electronic exchange between Cu(II)–BCS and urate as a converting factor.

3. Results

3.1. Study of the redox reaction between Cu(II) and urate

Starting from the estimation of the molar absorption coefficient of the complex Cu(I)–BCS (Table 1) the diagram $C_{Cu(I)–BCS}$ vs. C_{urate} was built. The slope of the straight-line corresponds to the electronic exchange sought and resulted equal to 1.999 with a standard deviation of 0.009 (Table 2), under the mentioned experimental

Table 1
Weighted least squares regression on the data used for estimation of the molar absorption coefficient Cu(I)–BCS.

Summary of the coefficients					
	Value	Standard deviation		*t*-Value	Significance
Slope	10,983.704	146.574		74.936	0.000
Intercept	−0.002	0.001		−1.556	0.127
Summary of ANOVA test					
	Sum of squares	Degrees of freedom	Variance	*F*	Significance
Model	2.457×10^9	1	2.457×10^9	5.615×10^3	0.000
Prediction error	1.881×10^7	43	437,457.332		
Total	2.457×10^9	44			
Summary of the model					
	R	*R*-square	Adj. *R*-square	ROOT-MSE	*N*
Model	0.996	0.992	0.992	661.405	45

Note:
- Predictor: $C_{Cu(I)–BCS}$,
- Dependent variables: ΔA_{478}, and
- Weighted least square regression: weight for $C_{Cu(I)–BCS}$ is $w = C_{Cu(I)–BCS}^{-2.0}$.

Table 2
Summary of the study of the redox reaction between Cu(II)–BCS and urate.

Summary of the coefficients						
	Value	Standard deviation	*t*-Value			Significance
Slope	1.999	0.009	227.223			0.000
Intercept	0.002	8.518×10^{-5}	27.663			0.000
Summary of ANOVA test						
	Sum of squares	Degrees of freedom	Variance	*F*		Significance
Model	82.735	1	82.735	4.393×10^4		0.000
Prediction error	0.081	43	0.002			
Total	82.816	44				
Summary of the model						
	R	*R*-square	Adj. *R*-square	ROOT-MSE		*N*
Model	1.000	0.999	0.999	0.043		45

Note:
- Predictor: C_{urate},
- Dependent variable: $C_{Cu(I)–BCS}$, and
- Weighted least square regression: weight for C_{urate} is $w = C_{urate}^{-2.0}$.

conditions, indicating an electron exchange equal to two for the redox reaction.

3.2. Stability of the reference molecule

Starting from urate stock solution stored in the dark at 4 °C, stability of the calibration curve was monitored during the days. Stability was evaluated using the value of the calibration curve slope as marker. Each useful diluted solution was measured during 9 days in four repetitions of the calibration curve *per* day. Regularly decreasing trend of the slope related to time was observed. The urate stock solution remains stable for 3–4 days maximum (Fig. 1).

3.3. Linearity, linear working range and sensitivity

Thirteen separate series of urate (0.0025, 0.005, 0.01, 0.02, 0.03, 0.05, 0.10, 0.15, 0.20, 0.50, 1.0, 1.5 and 2.0 mmol L^{-1}) were prepared by diluting urate stock solution freshly prepared and analyzed in three replicates. Absorbance at 478 nm was found linear from 0.0025 up to 2.0 mmol L^{-1} of urate reference solution (Table 3). Linear working range and sensitivity were investigated using urate reference solution in the range of 0.2–2.0 mmol L^{-1} in three replicates *per* day for 6 days (Table 4).

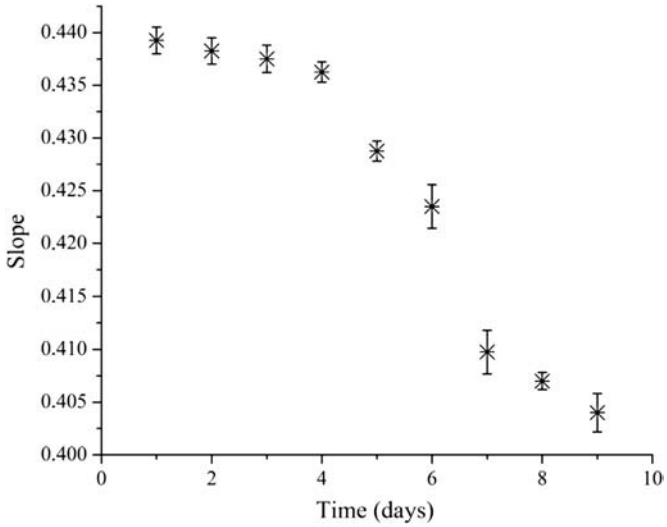

Fig. 1. Evaluation of calibration curve stability: a urate stock solution was tested daily for 11 days using the slope value as marker. Each point represents the mean ± SD of four replicates *per* day.

3.4. Precision

According to the ISO 5725-3:1994 standard [37], precision was estimated by studying the repeatability under different experimental conditions using a pool of plasma stored in aliquots at −20 °C and thawed out before use. A pool of plasma was analyzed during 6 days in 10 repetitions *per* day (Table 5). The Cochran test for homogeneity of the variance of the dataset was first applied. Then, the within-day repeatability (S_r) was estimated as follows (d is the number of days, S_{rj}^2 is the variance of each group of data):

$$S_r^2 = 1/d \sum_{j=1}^{d} S_{rj}^2 = 13.10\left(\mu\text{mol L}^{-1}\right)^2$$

and

$$S_r = 4\ \mu\text{mol L}^{-1}$$

The degrees of freedom ($d=6$, $n=10$) for S_r^2 are: $\nu_r = d(n-1) = 6(10-1) = 54$.

Estimation of the intermediate-precision $S_{I(T)}$ requires calculation of the average variance $S_{IM(T)}^2$ representing the variation between different groups of measure results. The average variance is estimated from ($\overline{X}_j$ is the mean of day j, $\overline{X}$ is the general mean)

$$S_{IM(T)}^2 = \left[1/(d-1)\right] \sum_{j=1}^{d} (\overline{X}_j - \overline{X})^2 = 207.47\left(\mu\text{mol L}^{-1}\right)^2$$

and the degrees of freedom for the $S_{IM(T)}^2$ are: $\nu_{IM} = d-1 = 5$. The relationship between $S_{IM(T)}^2$ and S_r^2 is

$$S_{IM(T)}^2 = S_{IL(T)}^2 + S_r^2/n \quad (1)$$

where $S_{IL(T)}^2$ is the variance representing the variation due to the effect of time between different groups of measures. *F*-test was conducted to check whether $S_{IL(T)}^2$ is greater than zero. Then, through Eq. (1) the variance $S_{IL(T)}^2$ was estimated giving the intermediate-precision $S_{I(T)} = 15$ μmol L^{-1} by way of the root square of the following equation:

$$S_{I(T)}^2 = S_{IL(T)}^2 + S_r^2 = 219.11\left(\mu\text{mol L}^{-1}\right)^2 \quad (2)$$

The repeatability of the method expressed as a coefficient of variation (henceforth CV) resulted equal to 0.004 and 0.013 for within-day repeatability and intermediate-precision respectively. Then, three pools of plasma with TAC values of 416 (low level), 910 (intermediate level), and 1990 (high level) μmol L^{-1} were considered

Table 3
Weighted least square regression on the data used to estimation of the linearity.

Summary of the coefficients				
	Value	Standard deviation	*t*-Value	Significance
Slope	0.457	0.001	335.679	0.000
Intercept	0.027	0.039×10^{-2}	68.376	0.000

Summary of ANOVA test					
	Sum of squares	Degrees of freedom	Variance	*F*	Significance
Model	3.556	1	3.556	1.126×10^{6}	0.000
Prediction error	0.001	37	3.156×10^{-5}	–	–
Total	3.557	38	–	–	–

Summary of the model					
	R	*R*-square	Adj. *R*-square	ROOT-MSE	*N*
Model	1.000	0.999	0.999	0.006	39

Note:
- Predictor: C_{urate},
- Dependent variables: ΔA_{478}, and
- Weighted least square regression: weight for C_{urate} is $w = C_{urate}^{-0.5}$.

Table 4
Weighted least square regression on the data used to estimation of the linear working range.

Summary of the coefficients					
	Value	Standard deviation	*t*-Value		Significance
Slope	0.461	0.001	346.621		0.000
Intercept	0.026	0.001	25.140		0.000
Summary of ANOVA test					
	Sum of squares	Degrees of freedom	Variance	*F*	Significance
Model	9.444	1	9.44	1.201×10^5	0.000
Prediction error	0.007	88	0.786×10^{-4}	–	–
Total	9.451	89	–	–	–
Summary of the model					
	R	*R*-square	Adj. *R*-square	ROOT-MSE	*N*
Model	1.000	0.999	0.999	0.0089	90

Note:
- Predictor: C_{urate},
- Dependent variables: ΔA_{478}, and
- Weighted least square regression: weight for C_{urate} is $w = C_{urate}^{-1.0}$.

Table 5
TAC data (μmol L^{-1} of Cu(I)–BCS reducing equivalent) of the pool of plasma analyzed during six days by 10 independent repetitions per day on apparatus ADVIA 2400 using urate as a calibrator.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
1	1101	1122	1120	1096	1089	1090	–
2	1110	1119	1120	1102	1089	1087	–
3	1102	1123	1120	1095	1095	1091	–
4	1113	1123	1120	1090	1092	1088	–
5	1118	1118	1126	1102	1088	1094	–
6	1108	1121	1118	1102	1095	1088	–
7	1114	1122	1121	1102	1085	1086	–
8	1110	1121	1122	1098	1082	1090	–
9	1109	1122	1123	1104	1088	1088	–
10	1112	1119	1117	1097	1093	1091	$\overline{\overline{X}}$[a]
$\overline{X}_j$[b]	1110	1121	1121	1099	1090	1089	1105
S_{rj}^2[c]	27	4	6	18	18	5	–

[a] general mean
[b] groups mean and
[c] groups variance.

to verify the dependence between TAC and within-day repeatability (S_r). These three levels of antioxidant capacity were tested by way of 10 repetitions *per* level in 1 day. It resulted a CV of 0.003, 0.004 and 0.003 for low, intermediate and high levels respectively. This result allows to exclude a concentration effect on the repeatability.

3.5. LoD and LoQ

Propagation of errors approach for the estimation of LoD and LoQ, as described by Long and Winefordner [38], was used as follows:

$$\mathrm{LoD} = 2k\frac{\sqrt{S_B^2 + S_a^2 + \frac{S_b^2(a-\overline{x}_B)^2}{b^2}}}{b} \quad (3)$$

$$\mathrm{LoQ} = 10\frac{\sqrt{S_B^2 + S_a^2 + \frac{S_b^2(a-\overline{x}_B)^2}{b^2}}}{b} \quad (4)$$

($k = 1.645$ as cover factor to ensure α and β errors equal to 0.05) referred to the general calibration curve y = bx+a, where:

- a is the intercept,
- b is the slope,
- $\overline{x}_B$ is the blank mean,
- S_a is the standard deviation of the intercept,
- S_b is the standard deviation of the slope, and
- S_B is the standard deviation of the blank mean.

Slope, intercept and their standard deviations were estimated from the dataset obtained to achieve the linearity. To define $\overline{x}_B$ and S_B, 10 independent blank of reagent measurements have been carried out ($\overline{x}_B = 0.0283$, $S_B = 0.027 \times 10^{-2}$ and $N = 10$). Through Eqs. (3) and (4) LoD and LoQ resulted 7.0 μmol L^{-1} and 21 μmol L^{-1} respectively.

3.6. Robustness

Robustness was tested having care for pH variation during PBS buffer preparation. The capacity of Cu(II)–BCS to be reduced to Cu (I)–BCS by urate was studied in a PBS buffered at pH 6.4, 7.4 and 8.4; no significant variations of the kinetic curves are observed.

3.7. Selectivity

As it is well known, glucose shows reducing ability [39] and it could act as interfering molecule. Since this work aims to include diabetic patients in the evaluation of TAC, the reducing ability of α-glucose under the experimental conditions was tested. A standard solution of α-glucose (500 mg/dL) was prepared and measured as a sample under the optimal working condition set (pH 7.4). No significant signal was recorded. Successively, the same solution was analyzed in a working condition of pH 13 (taking into account the pH value for the determination of reducing sugars) and a significant signal was instead recorded. Therefore, pH 7.4 in not alkaline enough for this undesired reaction: this ensures that the method can be used to estimate the TAC on plasma of hyperglycemic patients.

3.8. Clinical data collection

Plasma from different patients was analyzed. Table 6 reports the results of the TAC values (expressed as mean ± SD) obtained for blood donors, diabetic and dialysis patients respectively. Finally, the CUPRAC–BCS method was also tested on cerebrospinal fluid to enlarge its fields of application.

4. Discussion

The redox reaction between Cu(II)–BCS and urate was investigated and the number of electrons exchanged. Stability of the

Table 6
Clinical data collection: TAC values.

	TAC[a] (mean ± SD)	*n*
Blood donors	1067 ± 141	20
Diabetic patients	1079 ± 184	20
Pre-dialysis patients	1470 ± 309	20
Post-dialysis patients	702 ± 84	20
Cerebrospinal fluid	318 ± 98	20

[a] TAC expressed as μmol L^{-1} of Cu(I)–BCS reducing equivalent.

urate reference solution was tested and statistical analysis was used to draw conclusions from the experimental data. One-way ANOVA on the dataset was conducted and the H_0 hypothesis was refused ($p < 0.05$). Then, the significant difference between the slope mean values obtained for each day was analyzed by the Tukey test. Both experimental evidence (regularly decreasing trend of the slope related to time) and statistical analysis are in good agreement; this allowed to conclude that the urate stock solution remains stable for 3–4 days maximum (stored in the dark at 4 °C). However, to avoid analytical artifacts a daily prepared urate solution was used. This decreasing trend is probably due to degradation of urate to allantoin under alkaline conditions, as reported by Grootveld [40].

From the analytical perspective, linearity, linear working range, sensitivity, precision, LoD, LoQ, robustness and selectivity have been considered to validate the method. Weight least squares regression equation was used to obtain the calibration curve function regression parameters (Table 4) and absorbance at 478 nm was found linear from 0.2 up to 2.0 mmol L^{-1} of urate. Therefore, the CUPRAC–BCS method on ADVIA 2400 shows a good linearity in agreement with the linear range found by Campos and co-workers [13] (absorbance at 490 nm linear from 0.0625 up to 2 mmol L^{-1} of Trolox and urate) obtained on F16 MaxiSorp microplates. Precision was evaluated paying particular attention to the within-day repeatability (S_r) and the intermediate-precision ($S_{I(T)}$). These standard deviations, under repeated measurements condition on plasma, resulted: $S_r = 4$ and $S_{I(T)} = 15$ μmol L^{-1} ($CV_{intraday} = 0.004$ and $CV_{interday} = 0.013$). The results are in good agreement with those found in the literature, although Campos and co-workers [13] estimated the precision of the method using different concentrations of urate, each batch in triplicate, and analyzed on 3 different days in order to obtain the intra- and inter-day repeatability (i.e., $CV_{intraday} = 0.009$ and $CV_{interday} = 0.056$). On the other hand, Apak and co-workers [31] reported $CV_{intraday} = 0.007$ and $CV_{interday} = 0.015$ without full explanation and referring to aqueous extracts of serum implying a loud pre-treatment of the sample. Both authors did not implement the method on automatic instrumentation. LoD and LoQ were estimated as described by Long and Winefordner [38]. According to the principles stated by these authors, if slope and intercept of the calibration curve are "not well defined because of nonlinearity in the calibration curve or a poor choice of calibration curve ranges, the result of the unknown determination, may be subject to considerable error". Hence, linearity was investigated from 0.0025 up to 2.0 mmol L^{-1} of urate reference solution in order to better define slope and intercept. LoD and LoQ resulted equal to 7.0 and 21 μmol L^{-1} respectively. Finally, robustness and selectivity were tested having care for pH variation during reagent preparation and the reducing ability of glucose as interfering molecule respectively.

From the clinical perspective, plasma from blood donors, patients with type 2 diabetes and patients under hemo-dialysis treatment (plasma samples collected from pre- and post-treatment) were analyzed. TAC values in patients affected by type 2 diabetes under therapy ($TAC_{mean} = 1079 \pm 184$ μmol L^{-1}) and in blood donors assumed as control group ($TAC_{mean} = 1066 \pm 141$ μmol L^{-1}) show no significant differences (two-sample *t*-test, $p < 0.01$) and underlines that plasma from diabetic patients under therapy shows the same redox reactivity of the plasma from non-pathological conditions. Moreover, TAC values in patients under hemo-dialysis treatment (sampling pre- and post-treatment) show significant differences in TAC values (two-sample *t*-test, $p < 0.01$) between pre- and post-dialysis ($TAC_{mean\ pre} = 1470 \pm 309$ μmol L^{-1} and $TAC_{mean\ post-} = 702 \pm 84$ μmol L_{-1}). These results clearly show that the dialysis treatment induces a significant reduction of the TAC values and that TAC might be adopted as a clinical marker.

Finally, human cerebrospinal fluid was tested (30 patients, 14 males and 16 females) to investigate the response of the CUPRAC–BCS method in a different biological matrix and in a different range of antioxidant capacity. Preliminary measurements gave TAC responses on cerebrospinal fluids around 300 μmol L^{-1}. These values are of very small extent with respect to those obtained on plasma and the linear range used to the quantification of the TAC on plasma is not appropriate. New linear working range was then investigated to better determine the TAC on cerebrospinal fluid samples, namely 2.5×10^{-3}–0.5 mmol L^{-1} ($r^2 = 0.999$). TAC values show that the method responds to the redox reactivity of the cerebrospinal fluid: $TAC_{mean} = 318 \pm 98$ μmol L^{-1}.

The TAC values have demonstrated that the method response is sensible to the variation of redox reactivity of plasma. Moreover, determination of the TAC on cerebrospinal fluid has shown that the method is suitable for the study in a biological matrix different from plasma, even if dealing with lower capacity values.

To conclude, the photometric method proposed for the TAC determination was found to be simple, rapid, widely linear, reliable for routine analysis and suitable for further study of plasma from patients under severe oxidative stress conditions and on other biological fluids.

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