Microchemical Journal 124 (2016) 261–266

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

High-sensitivity programmable flow method for assessment of total antioxidant capacity in biological samples

Sara S. Marques, Ildikó V. Tóth *, Luís M. Magalhães, Salette Reis, Marcela A. Segundo

UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

ARTICLE INFO

Article history:
Received 19 June 2015
Accepted 1 September 2015
Available online 6 September 2015

Keywords:
Total antioxidant capacity
Copper reducing assay
Automatic programmable flow
Tears
Saliva
Serum

ABSTRACT

A programmable flow system is proposed for assessment of total antioxidant capacity of biological fluids based on Cu(II) reduction. A high-sensitivity procedure was implemented by using a 1 m pathlength liquid core waveguide capillary cell (LWCC), fostering high oxidant to antioxidant ratio. This feature promoted a faster reaction development and the response of the developed method towards several model antioxidant compounds, including ascorbic acid, Trolox (soluble analogue of vitamin E), uric acid, cysteine, glutathione and serum albumin, was studied. Application to less studied biological samples available at low volumes (tear, saliva), and also to serum samples, was performed. Analytical features include the low limit of detection (200 nM) and application range up to 10 μM Trolox with intraday repeatability of <3%. Trolox equivalent antioxidant capacity (TEAC) values between 26 ± 1 and 155 ± 7 μM were found for tears and saliva samples, while values between 421 ± 3 and 759 ± 14 μM were found for serum samples. The mean recovery was 101.9 ± 7.3% when uric acid was added to the different biological sample matrices, showing the suitability of the proposed method towards this application at low μM levels.

1. Introduction

The term oxidative stress is applied when the pro-oxidant/antioxidant balance is shifted towards the pro-oxidant agents due to an overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) or when the levels of antioxidant protection are diminished [1]. The excess of ROS/RNS can oxidize cellular lipids, proteins and nucleic acids modifying their physiological function and fostering the pathogenesis of several human diseases, including atherosclerosis, cancer, cardiovascular diseases, diabetes mellitus and neurodegenerative disorders [2,3]. On the other hand, antioxidants are substances that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate [4]. This biological role can be based on different mechanisms of action such as the ability to reduce oxidant species, the scavenging capacity of free radicals, the inhibition of activity of enzymes involved in the formation of ROS/RNS and/or the formation of complexes with metallic species that elicit the formation of oxidant species [5].

In this context, the oxidative status of biological fluids can be determined following three perspectives: (i) measurement of the ROS/RNS formed *in vivo* such as nitric oxide ($NO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$); (ii) assessment of circulating biomarkers of ROS/RNS oxidative damage like serum lipid hydroperoxides, plasma malondialdehyde or urine F2-isoprostanes; and (iii) determination of total antioxidant capacity (TAC) of biological fluids [5,6]. The measurement of ROS/RNS is difficult to perform due to the low lifetime of reactive species and high reactivity towards biomolecules, requiring *in vivo* procedures that are expensive and not practical [7]. The measurement of oxidative biomarkers needs time-consuming sample treatment, sensitive and selective analytical instruments (typically LC-MS), which are not available in routine laboratories [8,9]. The assessment of TAC represent a suitable approach to predict the redox status of biological samples since the analytical assays are simple involving spectrophotometric, fluorimetric or chemiluminometric detection [5,6,10,11]. These methodologies are based on the ability of biological sample to reduce or scavenge oxidative species, such as metallic species (Cu(II) or Fe(III)), synthetic radicals ($DPPH^{\bullet}$ or $ABTS^{\bullet+}$) or different ROS/RNS formed in vitro. The TAC of plasma samples have been determined for treatment monitoring especially during supplementation trials for boosting plasmatic antioxidant levels [12] and for diagnostic purposes of diabetes, cardiovascular disorders and metabolic syndrome [13]. Saliva have been used to measure TAC in periodontal diseases [14], while TAC of tears has been used for patients with ophthalmologic pathologies such as cataracts, glaucoma and diabetic retinopathy [15].

TAC assays have been adapted to microplate format aiming to attain high-throughput results suitable for bioanalysis purposes [5,16,17]. TAC assays are extremely affected by reaction conditions, fostering non-comparable results between studies [18]. Automation of TAC assay is beneficial in providing strict control of reaction conditions, including reaction time. The literature describes strategies that include microplate and flow injection assays with matrix matching approach [16] and flow

* Corresponding author. Tel.: +351 220 428 664; fax: +351 226 093 483.
E-mail address: tothildiporto@gmail.com (I.V. Tóth).

http://dx.doi.org/10.1016/j.microc.2015.09.001
0026-265X/

through spectrophotometric method with kinetic assessment [19]. The general downscaling of the assay also provides reduced sample consumption, as illustrated by the semi-micro FIA microdialysis probe hyphenated system that was applied for evaluation of the *ex vivo* antioxidant effect of the microdialysate after ascorbic acid administration in rats for preclinical assays [20]. The strictly controlled timing in flow assays also provides the valuable possibility for in-line *in vitro* production of highly reactive radical species [21–23], additionally to testing of biological samples against various reactive species is also possible resorting to a single flow set up [24]. However, the analytical assays described do not measure the TAC of biological samples in concentrations similar to those found in cellular environment. For this reason, the development of rapid and sensitive analytical approaches that measure TAC of biological fluids in low μM level with minimal sample treatment is of great interest for routine and screening analysis.

Hence, in the present work, a flow system exploiting the principles of flow programming [25] was developed for sensitive determination of TAC in several biological fluids. This flow approach allows the assessment of different kinetic responses of antioxidant compounds or biological samples under controlled conditions with enhanced sensitivity provided by long path spectrophotometry using a liquid core waveguide capillary cell (LWCC). The selected chemistry was based on the reduction of the copper(II)-bathocuproine complex [26] because the reduced form of this complex have high intrinsic molecular absorptivity when compared to other copper chelates [27]. Hence, TAC of biological fluids was targeted for low sample volumes at low μM level, with application to human plasma, saliva and tears.

2. Materials and methods

2.1. Reagents

All chemicals used were of analytical reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO) if not otherwise stated. Ultrapure water from Sartorius AriumPro system (resistivity > 18 MΩ cm; Sartorius, Goettingen, Germany) was used in the preparation of all solutions.

For the developed method, 10 mM copper stock solution was prepared by dissolving the solid $CuCl_2 \cdot 2H_2O$ in water. The complexing agent solution (7.5 mM) was prepared from bathocuproinedisulfonic acid disodium salt (BCS) by dissolution in water. Ammonium acetate buffer (1.0 M) pH 7.0 was prepared by dissolving 3.85 g of ammonium acetate in 50.0 mL of water. For the daily preparation of the reagent mixture 3.6 mL of copper stock solution and 3.6 mL of BCS solution were mixed with 10 mL of the buffer solution followed by dilution of the resulting mixture to 100 mL with water. The resulting reagent mixture (R) contained 0.36 mM Cu, 0.27 mM BCS and 0.1 M ammonium acetate at pH 7.0.

Stock solutions of 2.00 mM ascorbic acid (AA), 1.00 mM of reduced glutathione (GSH), 1.00 mM cysteine (CSH) and 10 g L^{-1} (≈150 μM) of human serum albumin (HSA) were prepared by dissolving the respective solids in water. Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) solution in 1.00 mM concentration was prepared by dissolving 25.0 mg of the solid in 5 mL of ethanol followed by completing the volume to 100 mL with water. The stock solution of 1.00 mM uric acid (UA) was prepared by suspending the corresponding solid in 20 mL water, the pH of the resulting solution was raised by dropwise addition of 0.1 M NaOH solution until complete dissolution of the solid (pH 11–12), followed by the elimination of the excess of base by addition of 0.1 M HCl solution until reaching pH 7.0. Afterwards, the total volume of the solution was completed to 100 mL with water [16]. Working standard solutions of antioxidants in the range of 1 to 10 μM were prepared by adequate dilution in water. All antioxidant solutions, including the stocks, were prepared daily.

2.2. Equipment

The programmable FIA manifold developed for TAC assessment in biological fluids comprised a Cheminert six-port rotary injection valve (model E80-CE, VICI AG International, Schenkon, Switzerland) equipped with a 30 μL injection loop, a peristaltic pump (Minipuls 3, Gilson, Villiers-le-Bel, France) with Tygon pumping tubes. The connection of the different components of the flow system was made with PTFE tubes with 0.8 mm i.d. (Omnifit, Cambridge, UK). End-fitting and connectors (Gilson), including a knitted reaction coil with 100 cm length were also used.

As detection system, an Ocean Optics (Dunedin, FL, USA) S2000-UV-VIS CCD-spectrometer with ADC1000USB converter, one 600 μm fibre-optic illuminating cable (QP600-2-UVBx, Ocean Optics), one 200 μm (QP200-2-UVBx, Ocean Optics) fibre-optic cable connecting the flow cell to the CCD detector, a halogen light source (F-O-Liteh; World Precision Instruments, Sarasota, FL, USA) and a WPI (Sarasota, FL, USA) liquid waveguide capillary cell (LWCC 2100, with 1.0 m pathlength, 250 μL inner volume and 550 μm inner diameter) was used. Programmable flow injection mode was implemented with computer control of the injection valve position and the peristaltic pump velocity using a purpose-built Quick Basic software. Data acquisition was performed with the OOIBase32 version 2.0.2.2. (Ocean Optics) software, while data analysis was performed using Origin 6.1 software.

2.3. Analysis protocol

First, the absorbance baseline is established by filling the flow cell with a reagent–carrier mixture. Next, the sample is injected into a water carrier stream and mixed further with the reagent stream containing $Cu(II)BCS_2^{2+}$ complex. The flow is then stopped when the peak maximum reaches the flow cell (45 s) for a pre-defined period of time (up to 300 s). Antioxidants present in the sample or in the standard solutions reduce the Cu(II) to Cu(I). The resulting orange-coloured complex ($Cu(I)\text{-}BCS_2^{+}$) is monitored at 485 nm. The summarized protocol sequence is given in Table 1.

2.4. Samples

Samples of different biological origin were studied. A reference serum sample (SRM 909c from the National Institute of Standards and Technology (USA)), containing 0.278 ± 0.006 mM uric acid, was analysed. Other serum samples from healthy adult volunteers were obtained after clotting and centrifugation. Basal tear film samples (50 μL) from healthy volunteers were obtained using the capillary sampling method according to Zakaria et al. [28]. Other tear samples (emotional, 100 μL) were collected in Eppendorf tubes, after natural drainage. Unstimulated whole expectorated saliva (5 mL) was collected from healthy volunteers in disposable tubes [14]. Synthetic saliva samples (OraFlx, Dyna-Tek Industries, TX, USA) were used in recovery assays. All serum and tear samples were stored immediately after collection at −18 °C until analysis within one week. Saliva samples were processed immediately after collection.

Table 1
Operation sequence of the programmable FIA method.

Operation	Time (s)	Flow rate (mL min^{-1})
1. Sample injection	0	1.6
2. Transport of sample/reagent zone to detector	0–44	1.6
3. Signal acquisition, flow stop (90 s)	44–134	0
4. Washout of flow cell	134–200	1.6