AAPS PharmSciTech, Vol. 11, No. 3, September 2010 (© 2010)
DOI: 10.1208/s12249-010-9484-y

Research Article

Autographic Assay for the Rapid Detection of Antioxidant Capacity of Liquid and Semi-solid Pharmaceutical Formulations Using ABTS$^{\bullet+}$ Immobilized by Gel Entrapment

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Received 15 November 2009; accepted 30 June 2010; published online 24 July 2010

Abstract. An autographic assay suitable for the detection of antioxidant compounds in a complex matrix (liquid and semi-solid pharmaceutical formulations) or in isolated compounds was described. The preformed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) was generated by oxidation of ABTS with potassium persulfate and reduced in the presence of hydrogen-donating antioxidants. For a further comparative estimation of its applicability and sensitivity, different medicinal plant extracts, hydrogels and antioxidant compounds were dot seeded or chromatographed on silica gel (TLC) and revealed with ABTS$^{\bullet+}$ solution (System I) or ABTS$^{\bullet+}$ immobilized by gel entrapment (System II). Both systems were effective and able to detect antioxidant activity in a micromolar range in seconds. System II was more sensitive and reproducible than System I. This micromethod is quick, inexpensive, and particularly helpful whether it works with numerous samples or on a small scale.

KEY WORDS: ABTS$^{\bullet+}$; antioxidant activity; pharmaceutical formulations; plant extract; TLC-autographic method.

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are produced constantly in cells with normal metabolism. Oxidative stress occurs when free radical production exceeds the tissue antioxidant capacity. In this condition, free radicals induce cellular membrane damage, DNA base oxidation, DNA strand break, chromosomal aberrations, and protein alterations. Oxidative stress in humans is thought to be closely associated with many chronic diseases, such as arteriosclerosis, ischemic–reperfusion, cancer, aging, and other degenerative diseases (1). Several epidemiological studies suggest the importance of vegetable product consumption in reducing the incidence of degenerative diseases like cancer and arteriosclerosis. The role of plants in human health and nutrition has been attributed, in part, to the antioxidant properties of their constituents (2).

Several colorimetric assays have been used to determine the antioxidant activity of biological samples, beverages, plants and fruits. Trolox equivalent antioxidant capacity (TEAC) using ABTS (2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid) as an oxidant, ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging potential, oxygen radical absorption capacity (ORAC), total radical absorption potential (TRAP), and photochemiluminiscence are some of the most commonly used assays (1,3–10). Depending upon the reaction involved they can be classified into two types: assays based on hydrogen atom transfer reactions and assays based on electron transfer (ET). ET-based tests measure the capacity of an antioxidant in the reduction of an oxidant which changes color when reduced. ABTS, FRAP, and DPPH are methods that measure the former, and ORAC and TRAP represent the latter. ABTS and DPPH are frequently chosen to compare antioxidant capacity in foodstuffs, medicinal plant extracts and to guide the isolation of bioactive compounds. Both spectrophotometric methods are simple and fast. In addition to the spectrophotometric methods, there are known autographic methods that not only detect biological activity in total complex mixtures but also combine chromatographic separation and *in situ* biological activity determination. Autographic methods have been used to detect antibacterial and antifungal compounds (11–13) as well as xanthine oxidase inhibitors (14), and DPPH and ABTS$^{\bullet+}$ scavenging activities (15,16). A modification of the ABTS$^{\bullet+}$ autographic method to increase its stability and sensibility and its application on liquid and semi-solid pharmaceutical formulations was described in this work.

MATERIALS AND METHODS

Reagents

The reagents employed are listed as follows: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

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(ABTS), (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Trolox), and Folin–Ciocalteau reagent. Standard antioxidants (ascorbic acid, quercetin, rutin, β-carotene, naringenin). All solvents used were of analytical grade and obtained from Merck and Sigma-Aldrich Canada Ltd.

Medicinal Plants

The aerial parts of *Sechium edule* Swartz, *Baccharis incarum* (Wedd.) Perkins, *Baccharis boliviensis* (Wedd.) Cabrera, *Chuquiraga atacamensis* Kuntze, *Parastrephia lucida* (Meyen) Cabrera were used.

Liquid Pharmaceutical Formulation: Tincture

Ground air-dried plant material was macerated in ethanol (5 g of dry tissue per 100 mL of 80º ethanol) for 7 days shaking (40 cycles/min) at room temperature. In all cases, the extracts were filtered through Whatman No. 4 filter paper. The tinctures were named *B. incarum* (MI-1-3800), *B. boliviensis* (MI-2-3800), *C. atacamensis* (MI-3-3800) and *P. lucida* (MI-4-3800).

Semi-Solid Pharmaceutical Formulation: Hydrogels

Ground air-dried leaves of *S. edule* were extracted with 80° ethanol (*v*/*v*) solvent, using a percolator at room temperature until achieving total extraction (fluid extract). The drug was filtered through Whatman No. 1 filter paper. The prepared extract was stored at 4ºC in the dark.

The hydrogel containing the fluid extract of *S. edule* leaves (0.24%) was prepared and stability controlled as reported in the literature (17).

Phenolic Compound Determinations

Total phenolic compound content was determined using the Folin–Ciocalteau reagent (18) and the results were expressed as gallic acid equivalents.

Thin Layer Chromatography

The components of the different extracts (10 μg of total phenolic compound) were separated by TLC (Kieselgel 60 F254 0.2 mm, Merck). Chloroform:methanol (9.5:0.5, *v*/*v*), or benzene:dioxane:acetic acid (9:2.5:0.4 *v*/*v*/*v*) were used as development solvents. The separated components were visualized under ultraviolet light (254 and 365 nm, UV Lamp Model UV 5L-58 Mineralight Lamp) and sprayed with NP/PEG (19).

ABTS Radical Cation Generation

$ABTS^{\bullet+}$ is a stable radical, a blue/green chromophore with maximal absorption at 734 nm, which loses its color against antioxidant agents.

ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting an ABTS stock solution with 2.45 mM potassium persulfate, $K_2O_8S_2$ (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before using (4).

Autographic Assay in Dot Blot

Tinctures of each plant species (5 μl of different dilutions), *Sechium* hydrogel (1×10^{-4} to 1×10^{-2} g), antioxidant compounds, lipophilic (β-carotene), and hydrophilic (ascorbic acid, rutin, luteolin, quercetin, and naringenin) compounds were placed on 4×4 cm TLC plates (Kieselgel 60 F254 0.2 mm, Merck). Dot blots were prepared in triplicate. Once the samples were dry, antioxidant capacity was visualized with ABTS radical cation systems.

Autographic Assay on TLC

An aliquot (0.1; 1 and 10 μg of total phenolic compounds) of each medicinal plant extract was placed on Silica gel F254 plates (4×4 cm). The plates were developed with chloroform:methanol (9.5:0.5 *v*/*v*) or benzene:dioxane:acetic acid (9:2.5:0.4 *v*/*v*/*v*) as solvent system. After the TLC plates were dry, the separated components were vizualized with $ABTS^{\bullet+}$ systems (I and II).

Staining Procedures

(A). System I: 1 mL of $ABTS^{\bullet+}$ solution was sprayed on the 4×4 cm TLC plate. The plate was incubated at room temperature for 0, 0.5, 1 min in the dark.

(B). System II: 3 mL of soft medium (agar 0.9%) containing 1 mL $ABTS^{\bullet+}$ solution was distributed on a 4×4 cm TLC plate. After solidification, the plate was incubated at room temperature for 0, 0.5, 1 min in the dark.

The antioxidant activity in both systems appeared as clear spots against a dark green-blue background.

Total Antioxidant Capacity by Spectrophotometric Assay

$ABTS^{\bullet+}$ solution (1 mL; absorbance of 0.7±0.02 at 734 nm) was added to different amounts of the phenolic compound of each tested sample (standard or plant extracts) and mixed thoroughly. The reactive mixture was allowed to stand at room temperature and absorbance was recorded at 734 nm, 0, 0.5, and 1 min after initial mixing and up to 6 min. A trolox aliquot was thawed and used to develop a 10–100 μmol/L standard curve. Results were expressed as trolox equivalents (TEAC, μmol trolox per 100 g dry weight of sample). SC_{50} values (sample concentration required to scavenge 50% of $ABTS^{\bullet+}$ free radicals) were calculated

RESULTS

In the present work, we used the TEAC III autographic methods in two Systems and compared the results using different pharmaceutical formulations. Antioxidant activity was determined in liquid phytotherapic preparations obtained from aerial parts of medicinal plants (*P. lucida*, *B. boliviensis*, *B. incarum*, and *C. atacamensis*) and semi-solid preparation

(*S. edule* hydrogel). The spectrophotometric assays have shown that *P. lucida* and *B. boliviensis* liquid preparations contained the highest antioxidant concentration (15,588 and 15,000 μmol Trolox/100 g dry weight, respectively) followed by *B. incarum* and *C. atacamensis* with TEAC values of 7,833 and 4,100 μmol Trolox/100 g, respectively. Experimental results demonstrated the ABTS scavenging activity within 1 min without further changes in the subsequent 5 min. SC_{50} values of liquid preparations were 1.5 to 3 μg/mL while semi-solid preparations showed SC_{50} values of 10 μg/mL (Table I).

When different dilutions of tinctures or hydrogel were dot-blotted on silica plates and stained with ABTS, the same reaction occurred (Figs. 1 and 2). With System I, the antioxidant activity was observed on a silica gel plate as clear spots (reduction $ABTS^{\bullet+}$ zones) against a dark green-blue background after 1 min of contact between antioxidant and $ABTS^{\bullet+}$. With System II, the clear spots were observed immediately (0.1 min) in liquid and semi-solid pharmaceutical formulations. The color in the silica gel plate was stable for about 4 h at room temperature in the dark and for 6 days at −20ºC when the plate was revealed with the System II while with System I the intensity of the active bands was increased during the first hour until the color became stable and remained like that for 1 h. Although Systems I and II were appropriate for the assay, antioxidants were detected more clearly and quickly with System II, with the $ABTS^{\bullet+}$ immobilized by gel entrapment. In order to evaluate the sensitivity of the assay, lipophilic and hydrophilic antioxidants were applied in decreasing amounts on the TLC plates; a detection limit was 0.1 μg for naringenin, luteolin, and quercetin, 1 μg for rutin and β-carotene and 2.5 μg for ascorbic acid using both Systems, but the antioxidant activity was observed after 0.1 or 1 min of contact between the antioxidant and the $ABTS^{\bullet+}$ with Systems II and I, respectively (Table I). The minimal amount of hydrogel preparation and medicinal plant extract (tincture) necessary to detect antioxidant activity with System II was 10 μg (fresh weight) and 0.10 μg (in equivalent of gallic acid), respectively (Figs. 1 and 2).

In order to test the applicability of the assays for the localization of active compounds in a complex matrix, the plant extracts were chromatographed and the plates were revealed with both ABTS systems. The minimum amount of total extract necessary to visualize antioxidant components separated by TLC was 10 μg of phenolic compounds in equivalents of gallic acid in both Systems. The autographic test indicated that medicinal plant extracts contained some bioactive bands with ABTS radical cation reduction activity (Fig. 3a and b).

DISCUSSION AND CONCLUSIONS

There are two general types of assays widely used for different antioxidant studies. One is an assay associated with lipid peroxidations and the other assays are associated with electron or radical scavenging, including the DPPH assay, ABTS assay, and FRAP assay. The measured antioxidant capacity of samples varied with the assay method used, pH, and reaction time. In general, these assays are used in aqueous systems. The ABTS assay, which is also called the TEAC assay, has been widely used to evaluate the antioxidant activity of components in foods and beverages due to its applicability in aqueous and lipid phase (20–24) and it can determine antioxidant activity at different pHs (22,25,26). The ABTS assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of the antioxidants under investigation, TEAC I Method (23). More recently, Re *et al.* (4) have described a modified ABTS assay based on the decoloration of the pre-formed radical cation. This method involved formation of the radical cation through oxidation by MnO_2 (TEAC II Method) or $K_2O_8S_2$ (TEAC III Method) exposure of the antioxidant under investigation to the radical cation for a defined time period, and spectrophotometric measurement of the extent of the radical quenched. TEAC I only measures hydrophilic antioxidants. TEAC II usually is an assay for lipophilic antioxidants like carotenoids and tocopherols. TEAC III enables measurement of both kinds of antioxidants by changing the solvent (24). The autographic method described herein is a modification of DPPH and ABTS autographic method described by Soler *et*

Table I. Comparison of the Autographic Method Using $ABTS^{\bullet+}$ in Spray (System I) or Immobilized by Gel Entrapment (System II)

	SC_{50}	Dot blot in sílica gel		TLC	
		System I	System II	System I	System II
Samples	(μg/ml)	Detection limit (μg)/detection time (min)/stability(h)		Detection limit (μg)/detection time (min)/stability (h)	
Naringenin (flavanone)	2	0.1/1/1	0.1/0.1/4		
Luteolin (flavone)	2	0.1/1/1	0.1/0.1/4		
Rutin (quercetin-3-rutinoside)	5	1/1/1	1/0.1/4		
Quercetin (flavonol)	1	0.1/1/1	0.1/0.1/4		
Ascorbic acid	3	2.5/1/1	2.5/0.1/4		
β-carotene	3	1/1/1	1/0.1/4		
B. incarum	2	1/1/1	0.1/0.1/4	10/1/1	10/0.1/4
B. boliviensis.	2.5	1/1/1	0.1/0.1/4	10/1/1	10/0.1/4
P. lucida	3	1/1/1	0.1/0.1/4	10/1/1	10/0.1/4
C. atacamensis	1.5	1/1/1	0.1/0.1/4	10/1/1	10/0.1/4
S. edule Hydrogel	10	10/1/2	10/1/4		

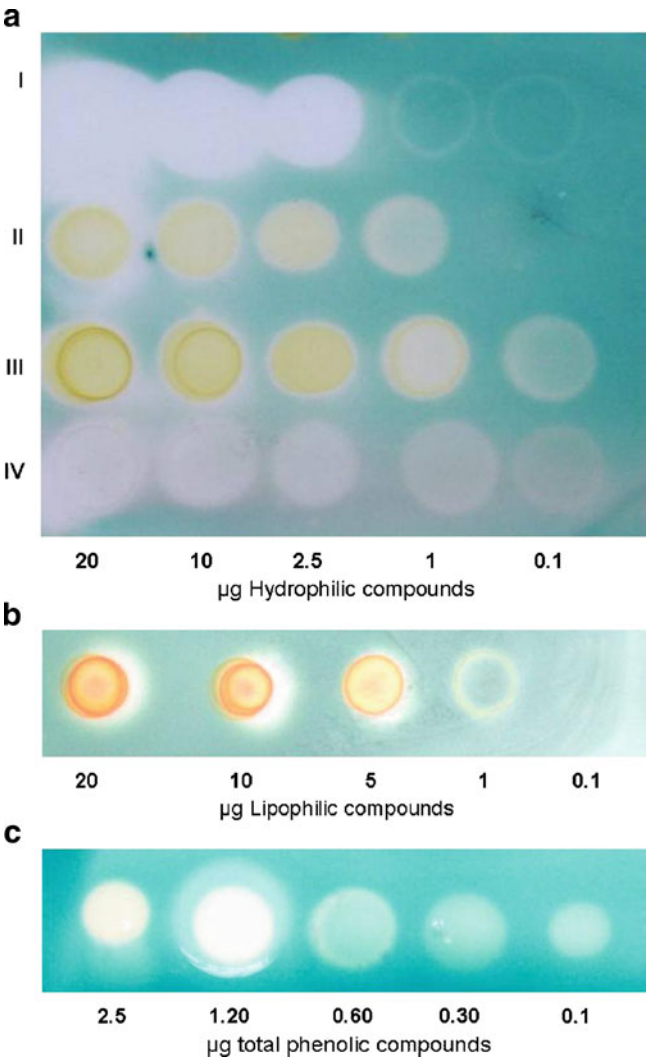

Fig. 1. TLC plate spotted with decreasing amounts of antioxidant compounds (lipophilic and hydrophilic compounds) and liquid pharmaceutical preparation. **a** Hydrophilic compounds: *I* ascorbic acid, *II* rutin, *III* quercetin, *IV* naringenin, **b** Lipophilic compounds: β-carotene. **c** Liquid pharmaceutical preparation. The antioxidant compounds was visualized by System II

al. (16). In this work, TEAC III was used to obtain the ABTS radical cation. ABTS solution sprayed on silica gel plates (System I) was compared with ABTS entrapment in agar (System II). The autographic method using System II is an easy and fast system to detect antioxidant compounds. System II was more reproducible and stable than System I. This assay is a rapid means of total antioxidant activity screening for numerous samples at the same time (chemical compounds and biological mixture), with very limited handling of the sample and with high repeatability, it is simple because it is colorimetric, and does not require analytical equipment. In addition, it is sensitive, since a small amount of antioxidant compounds, in the range from 0.1 to 10 µg, is sufficient to detect bioactive compounds and cheap as its reagents are relatively inexpensive and can be applied to liquid and semi-solid pharmaceutical formulations in dot blot assay or one-dimensional TLC experiments.. The autographic assay using

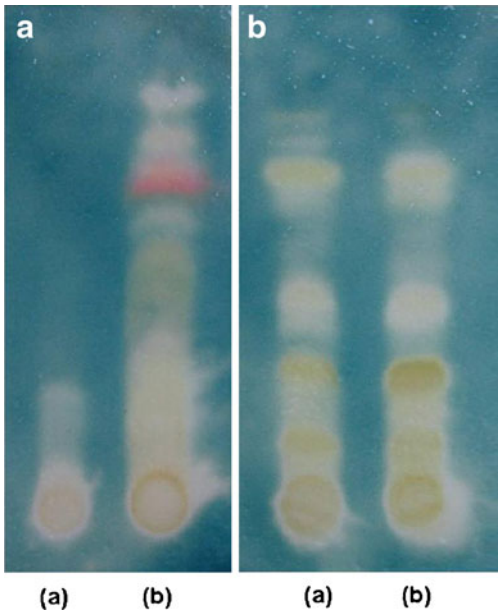

Fig. 2. TLC (Merck Kieselgel 60 F254) of liquid pharmaceutical preparation was realized, **a** 10 µg of phenolic compounds of *C. atacamensis* (*line a*) and *P. lucida* (*line b*) extract/plate was separated using benzene:dioxane:acetic acid (9:2.5:0.4, *v*/*v*/*v*) **b** 10 µg of phenolic compounds of *B. incarum* (*line a*) and *B. boliviensis* (*line b*) extract/plate was separated using chloroform:methanol (9.5:0.5, *v*/*v*) as eluant. The plate was covered with 3 ml of soft medium (0.9% BHI agar) containing $ABTS^{\bullet+}$

$ABTS^{\bullet+}$ immobilized by gel entrapment was more sensitive and fast than DPPH and ABTS autographic method described by Soler *et al.* (16) and was adequate both aqueous and lipophilic preparations.

These data demonstrate that the assay using the System II can be used for the fast screening of diverse pharmaceutical formulations, providing a preliminary indicator of differential antioxidant compositions.

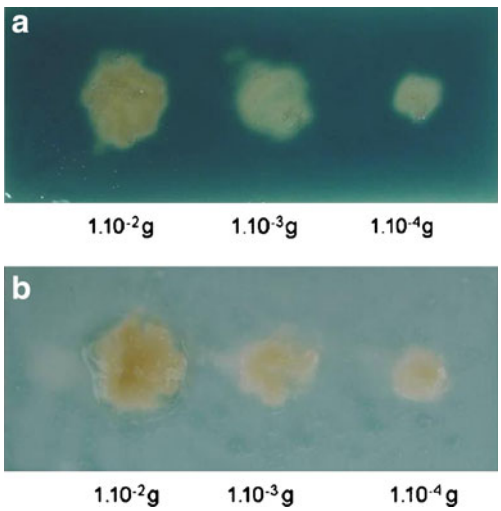

Fig. 3. TLC spotted with decreasing amounts of hydrogel preparation containing fluid extract of *S. edule* (1×10^{-4} to 1×10^{-2} g). The antioxidant compounds was visualized by **a** System II **b** System I

ACKNOWLEDGMENTS

This research was supported by grants from Consejo de Investigación de la Universidad Nacional de Tucumán, Argentina (CIUNT), Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT).

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