Lares-Asseff.qxd:Article template 8/5/10 8:37 AM Page 1

HPLC Method for Quantification of Oxidative Stress by Salicilate Hydroxylation in Human Plasma

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Abstract

The aim of the present study was to modify and validate a high-performance liquid chromatographic (HPLC) method for determining 2,3 and 2,5 dihydroxybenzoic acid (2,3-DHBA and 2,5-DHBA) from salicylic acid in human plasma. The mobile phase was a mixture of sodium acetate/citrate (pH 2.5) 30 mM–methanol (93:7, v/v). The injection volume was 10 µL. Retention time for 2,5-DHBA, and 2,3-DHBA was 4.5 ± 0.10 and 5.8 ± 0.15 min, respectively. The detection and quantification limits were 10 and 40 nM for 2,3-DHBA and 8 and 20 nM for 2,5-DHBA. Linearity was evaluated in the range of 40–1600 nM for both metabolites. Inter- and intra-analysis variation coefficient was below 10%. Good recoveries of more than 99% were obtained for both metabolites using this method.

Introduction

Oxidative stress (OS) results from an imbalance in the prooxidant-antioxidant equilibrium favoring prooxidants, which can be due to an excess of prooxidant substances, an antioxidant deficiency, or by both factors simultaneously (1). The main oxidative substances in biological systems are free radicals (FR). The majority of the cell components are susceptible to FR, but nucleic acids, lipids, proteins, and carbohydrates are their main targets (2). Several diseases have been associated with OS, such as inflammatory atherosclerosis, diabetes, Alzheimer's diseases, various types of cancer, and cardiovascular disorders (3,4).

A variety of biological markers have been developed for evaluating OS, which specifically target the damage caused by FR or the generation of FR in vivo, including lipids, proteins, and oxidized DNA (5). Nevertheless, few methods directly determine reactive oxygen species, especially the hydroxyl radical ($OH^{\bullet}$), due to their short average lifespan. Furthermore, although the antioxidant defense system can be increased or decreased in the presence of OS, the determination of FR in fact indicates the oxidative level present in the organism. Determination of $OH^{\bullet}$ is carried out by evaluating the generation of stable derivatives such as salicylic acid (SAL), 2,3-dihydroxybenzoic acid (2,3-DHBA) and comparing with CYP_{450} oxidation route, 2,5-dihydroxybenzoic acid (2,5-DHBA) using high-performance liquid chromatography (HPLC) techniques (6–9). Because these metabolites are stable and freely circulate in the bloodstream, they can easily be detected and quantified in plasma (10,11).

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Experimental

Reagents and chemicals

Analytical-grade 2,3-DHBA, 2,5-DHBA, and 3,4-dihydroxybenzoic acid (3,4-DHBA) from Sigma Aldrich (St. Louis, MO) were used together with analytical-grade granular anhydrous sodium acetate and sodium citrate dihydrated from J.T. Baker (Phillipsburg, NJ). Methanol and ethyl acetate were HPLC grade from J.T. Baker.

Chromatography

Samples were analyzed using an HP Agilent series 1100 chromatographic system consisting of a solvent delivery pump Model G131A (Palo Alto, CA), an electrochemical detector Model 1049A, a Waters Spherisorb 5-µm ODS2 reversed-phase column (25 × 0.46-cm i.d.) (Milford, MA), and a ChemStation for LC3D software, version A.08.03 (847) (Agilent).

Plasma samples preparation

The proposed method is based on the method by Coudray et al. (7). One-hundred microliters of 3,4-DHBA (1000 nM) was added to 400 µL plasma as an internal standard. A total of 100 µL mixture containing 2,3-DHBA and 2,5-DHBA at known concentrations (between 40 and 1600 nM) were added to 100 µL 1 M HCl. The mix was vortexed for 30 s, and 3 mL of ethyl acetate was added and then vortexed for another 1 min. Finally the tubes were centrifuged at 2000 × *g* for 10 min. The resulting organic layer was separated and evaporated by aeration. The sample was reconstituted in 400 µL of mobile phase, and the injection volume was of 10 µL.

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Lares-Asseff.qxd:Article template 8/5/10 8:37 AM Page 2

The HPLC system was equilibrated with the mobile phase consisting of 30 mM acetate/sodium citrate (93:7, v/v) (pH 2.5)–methanol, varying the proportion of both solvents to find the optimum, at a flow rate of 1.0 mL/min. The electrochemical detection was optimized in order to found the adequate oxidation potential that detected the signal of the analyzed metabolites. The column was maintained at ambient temperature.

Validation procedure

The analytical method was validated in adherence to the FDA Guidance for Industry: Bioanalytical Methods Validation and the Official Mexican Norm NOM-177-SSA1-1998 in the following parameters.

Linearity

Linearity was assessed within the range of 40–1600 nM for solution and plasma, which covered the clinically expected trough plasma levels.

Precision and accuracy

Intra-day validation was carried out using the runs of one day (n = 5), while inter-day validation was assessed using data collected in three days (n = 2). Three different concentrations were analyzed within the linearity interval for 2,3-DHBA and 2,5-DHBA in plasma. These concentrations were different from those present in the calibration chart, although they were representative of the linearity interval. Precision was characterized by the coefficients of variation (CV%), whereas accuracy was expressed as a percentage error (PE%) of nominal versus measured concentration.

Table I. Retention Times of the Acid 2,5-DHBA, 3,4-DHBA, and 2,3-DHBA*

Metabolite	Retention time (min)
2,5-DHBA	4.5 ± 0.10
3,4-DHBA	5.2 ± 0.09
2,3-DHBA	5.8 ± 0.15

* The retention time is expressed as mean ± standard deviation; n = 10. Room temperature of 20 ± 3°C.

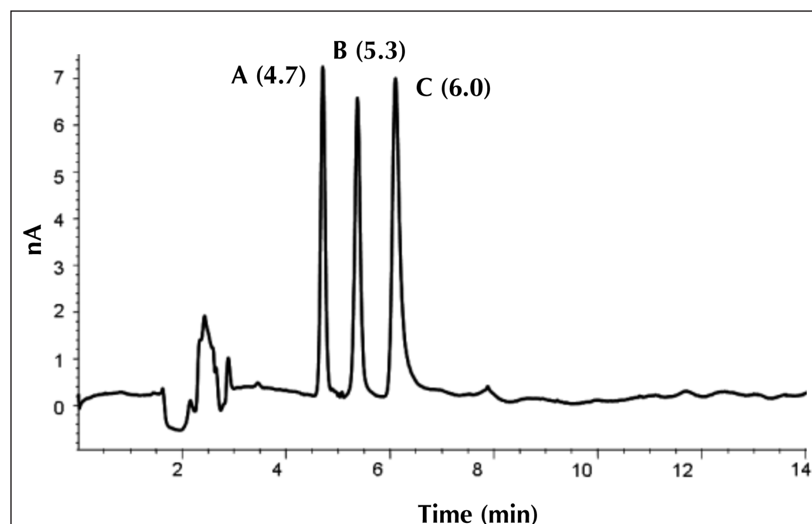

Figure 1. Standard chromatogram of plasma with 1000 nM: (A) 2,5-DHBA acid, (B) 3,4-DHBA, and (C) 2,3-DHBA and their corresponding retention times.

Recovery

Recovery was assessed by determining three concentration levels in plasma and solution (700, 1400, and 2000 nM). The response of plasma samples, to which 2,3-DHBA and 2,5-DHBA had been added at the aforementioned concentrations, were compared to the response of the standard 2,3-DHBA and 2,5-DHBA solutions at the same concentrations.

Quantification limit

The lowest working concentration level was determined in quintuplicate, and it was determined to be valid if the values obtained were within 20% and if the CV% was no greater than 20%.The limit of quantification of 2,5-DHBA was 20 nM with a CV% of 5.57% and for 2.3-DHBA was 49 nM with a CV% of 0.3%.

Detection limits

The concentration at which the 2,3-DHB and 2,5-DHB signal could be discerned from background noise in samples without these compounds added was determined. The detection limits were 10 nM for 2,3-DHBA and 8 nM for 2,5-DHBA.

Specificity

The specificity of the method was established by analyzing samples from six random human volunteers that had no additives in them.

The stability in time of 2,3-DHBA, 2,5-DHBA, and 3,4-DHBA in mobile phase was tested at different storage temperatures (refrigeration and frozen).

Moreover, the HPLC method was preliminarily applied to determine 2,3-DHBA and 2,5-DHBA concentrations from healthy subjects treated with therapeutic single dose of aspirin (500 mg).

When the aim of the study and the possible side effects of drug administration had been explained to the subjects, a written informed consent was requested and received. The present work was approved by the Ethics and Research Committee of the Durango General Hospital of the Mexican Health Ministry.

Results and Discussion

We developed and validated a quick and sensitive method for determining 2,3-DHBA, 2,5-DHBA, and 3,4-DHBA as an internal standard in human plasma samples. Chromatographic conditions were investigated, and the maximum resolution and sensitivity of the method were obtained at 0.55 V oxidation potential, 1.0 mL/min flow rate, and retention times of 2,5-DHBA, 3,4-DHBA, and 2,3-DHBA were 4.5 ± 0.10, 5.2 ± 0.09, and 5.8 ± 0.15 min, respectively (Table I). The injection volume was 10 µL.

The total run time of the method was 10 min, and

Lares-Asseff.qxd:Article template 8/5/10 8:37 AM Page 3

standard chromatograms were obtained after injecting plasma samples with 2,3-DHBA, 2,5-DHBA, and 3,4-DHBA added at a 1000 nM concentration (Figure 1).

The resolution was obtained using a Waters reversed-phase column Spherisorb Phenyl (4.6 × 250 mm, 5 µm). We found the optimum chromatographic conditions for its detection and obtained higher sensitivity and resolution to an oxidation potential of 0.55 V, a mobile phase flow rate of 1 mL/min. The mobile phase consisted of a mixture of acetate/30 mM sodium citrate–methanol (93:7, v/v) at pH 2.5 plus metabolites in the dissolved phase with a running time of 15 min. The chromatogram shows the retention times of the metabolites as shown in Figure 1.

Linearity

Linearity of the method was established at the 40–1600 nM range (Figure 2), with the following linear regressions: for 2,5-DHBA acid: $y = 1.9411 + 0.1032x$, $r = 0.9985$ ($p = 0.001$) and for 2,3-DHBA acid: $y = 8.4641 + 0.0916x$; $r = 0.9925$ ($p = 0.001$).

Precision and accuracy

Inter- and intra-assay precision was determined by analyzing three known concentrations (80, 200, and 1000 nM). Inter- and intra-analysis CV% for 2,3-DHBA and 2,5-DHBA analytes were less than 10% (Table II), which is below the acceptable 15% level (12). The relative error did not exceed the ± 15% acceptance limit in the three different concentrations levels that were tested (Table II).

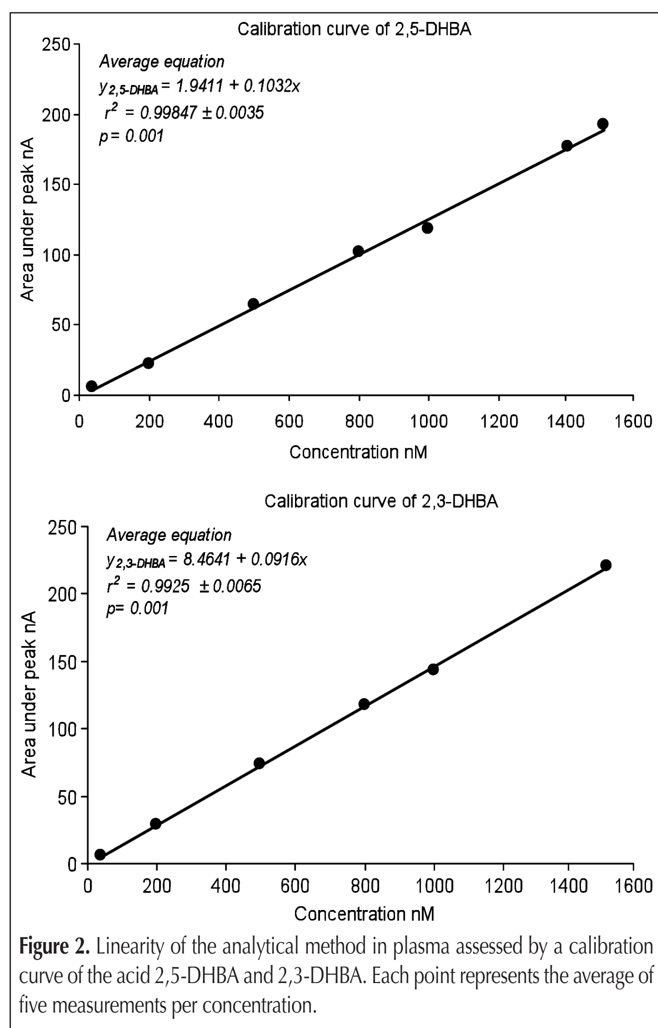

Figure 2. Linearity of the analytical method in plasma assessed by a calibration curve of the acid 2,5-DHBA and 2,3-DHBA. Each point represents the average of five measurements per concentration.

Recovery

The recovery of each metabolite was analyzed in quadruplicate at the three concentration levels (80, 200, and 800 nM). Table III shows that analyte recoveries were close to 100%, and the extraction efficiency ranged between 99.27–101.68% for 2,5-DHBA and 99.62–104.67% for 2,3-DHBA (CV% < 6%).

Sensitivity

The sensitivity was determined by evaluating the change in response per unit concentration, whereas sensitivity is the slope of the concentration-response curve, or change in response per unit concentration.

The quantification limit for 2,5-DHBA acid was 20 nM with a CV% of 5.57%, whereas for 2,3-DHBA it was 40 nM with a CV% of 0.30%. The detection limits were 10 nM and 8 nM for 2,3-DHBA and 2,5-DHBA, respectively.

Specificity

No endogenous interference was observed in the retention times for the analytes, as noted in six chromatograms obtained from the samples of healthy individuals without any added substances (not shown).

Stability

The stability of 2,3-DHBA, 2,5-DHBA, and 3,4-DHBA was assessed in mobile phase. Results indicated the three analytes were stable at 4 ± 2°C for at least 24 h and at –24 ± 1°C for a month. After three months, the three analytes degraded in plasma, in contrast the compounds in the solution, were stable for six months at –24 ± 1°C.

In addition, in vivo detection of 2,3-DHBA produced was tested. Typical chromatograms found from plasma blank, and plasma added with metabolites and patient's plasma samples are presented in Figure 3.

The purpose of this work was to develop a quick and sensitive chromatographic method for determining 2,3-DHBA, 2,5-

Table II. Inter-Assay and Intra-Assay Precision and Accuracy of Plasma Samples for Validation of the Method

	2,5-DHBA			2,3-DHBA		
Conc. added (nM)	Conc. found (nM)	PE (%)*	CV (%)*	Conc. found (nM)	PE (%)	CV (%)
Inter-day (n = 5)						
80	81.18	1.47	3.67	78.20	–2.25	1.19
200	201.89	0.94	4.82	208.21	4.10	5.64
800	786.89	–1.63	5.70	797.92	–0.26	3.39
Intra-day (n = 6)						
80	81.43	1.79	3.86	79.55	–0.56	9.80
200	191.30	–4.35	5.93	201.89	0.94	7.33
800	776.52	–2.93	3.73	773.14	–3.35	2.31

* CV = coefficient of variation, calculated as (standard deviation/mean concentration) × 100; PE = percentage error, calculated as [(mean concentration – nominal concentration)/nominal concentration] × 100.

Lares-Asseff.qxd:Article template 8/5/10 8:37 AM Page 4

DHBA, and 3,4-DHBA analytes. Reliability of the method was determined by validating the method taking into account previous reports (6,7,10,13). The oxidation potential signal detected from the metabolites that were analyzed (0.55 V) was lower than that of Coudray et al., (7), which was 0.7 V, and others (6,10,13), which ranged from 0.60 to 0.96 V (Table IV). The mobile phase was similar to that used by Coudray et al. (7), varying only in the amount of each compound used. Whereas Coudray et al. used 85:15 (v/v) 30 mM sodium acetate/citrate–methanol in their method, the present work used a 93:7 (v/v) amount. The advantages offered by this method is that the mobile phase flow rate was higher than that used by these authors (1 mL/min versus 0.2 mL/min), which made the retention and run times shorter than those of Coudray et al. (7) and others authors (6,10,13) (Table IV). This in turn helped us to obtain better peak resolution.

Table III. Recovery and Coefficient of Variation for 2,3-DHBA and 2,5-DHBA Metabolites

	Recovery (n = 3)			
	2,5-DHBA		2,3-DHBA	
Conc. (nM)	Mean (%)	CV (%)	Mean (%)	CV (%)
80	101.68	5.79	101.83	4.12
200	99.27	4.50	104.67	5.69
800	101.10	3.90	99.62	1.84

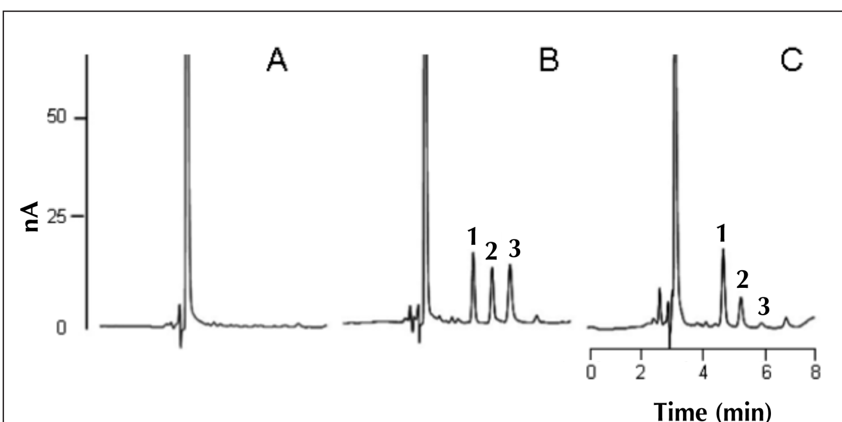

Figure 3. Characteristic chromatograms: (A) blank human plasma, (B) blank plasma spiked with 1000 nM of 2,5-DHBA, 1; the internal standard 3,4-DHBA, 2; and 2,3-DHBA, 3; and (C) plasma sample from a healthy subject administered with a single dose of aspirin (500 mg, oral route) and monitored for the detection of the three metabolites.

Table IV. Comparison of Analytical Methods for Quantifying Metabolites 2,3 and 2,5-DHBA

Oxidation potential	Flow rate	Mobile phase	Retention time	References
0.70 V	0.2 mL/min	30 mM acetate/sodium citrate–methanol (85:15, v/v) pH 3.9	2,3-DHBA = 22 min 2,5-DHBA = 19.6 min 3,4-DHBA = 27 min	7
0.77 V	0.5 mL/min	50 mM acetate/sodium citrate–methanol–2-propanol (90:8:2, v/v) pH 2.5		13
0.60 V	1.0 mL/min	30 mM sodium citrate/27.7 mM acetate pH 4.75	2,3-DHBA = 10 2,5-DHBA = 12	6
0.96 V	0.9 mL/min	30 mM sodium citrate/27.7mM acetate pH 4.75–methanol (92.2:12.8, v/v) pH 2.5	2,3-DHBA = 7 2,5-DHBA = 8	10
0.55 V	1.0 mL/min	30 mM acetate/ sodium citrate–methanol (93:7, v/v) pH 2.5	2,3-DHBA = 5.8 min 2,5-DHBA = 4.5 min 3,4-DHBA = 5.2 min	This study

Correlation of both metabolites indicates that more than 99% of the peak area is explained by analyte concentration (12). Inter-assay and intra-assay CV% for 2,3-DHBA and 2,5-DHBA were below the 15% acceptance level established for this type of method (Official Mexican Norm, 1998; Guidance for industry, 2001), indicating that the method is highly precise. Furthermore, this method is accurate because the percentage error was below 4.3% and did not exceed ± 15% acceptance levels.

Analyte recoveries were close to 100% with ranges of 97.27–103.68% for 2,5-DHBA and 101.83–105.41% for 2,3-DHBA. The quantification limit for 2,5-DHBA was 20 nM and 40 nM for 2,3-DHBA with a CV% ≤ 5.57%, which is well below the acceptance limit of 20% (Official Mexican Norm, 1998). No endogenous interference was seen when analyzing six randomly selected samples from healthy individuals, which provides evidence that this method is highly specific (Official Mexican Norm, 1998).

When healthy individuals were tested for production of 2,3-DHBA, the metabolite produced by an oxidation route by scavenging the $OH^{\bullet}$ free radical, our preliminary results showed a small amount of it (6.0 ± 2.8 nM). Besides, in a few monitored patients with controlled type 2 diabetes, the production of 2,3-DHBA reached in plasma 7.6 ± 1.32 nM. Our results suggest OS presence because of the increased 2,3-DHBA production. Further investigations are needed to assess the diagnostic value of quantifying this metabolite as a marker in several diseases with the presence of OS.

Future application

The method has been optimized successfully to be implemented as a diagnosis test to evaluate oxidative stress in patients by increasing production of the hydroxylated metabolite of salicilates 2,3-DHBA in vivo. This was studied because there are an increasing number of diseases that are associated with OS, such as diabetes, atherosclerosis, cancer, inflammatory process, Alzheimer's, and cardiovascular diseases (3,4).

The results of studies by Ghiselli et al. (14) and Coudray and Favier (15) suggest that the method of salicylate hydroxylation is useful to reveal in vivo OS independently from the peroxidation of lipids, and they support the hypothesis that oxygen radicals are involved in the pathogenesis of chronic complications of diabetes.

Lares-Asseff.qxd:Article template 8/5/10 8:37 AM Page 5

To the authors' knowledge, the study of Strolin-Benedetti et al. (16) showed for the first time that induction of some but not all P450s is associated with the production of hydroxyl radicals in vivo.

Quigley et al. (17) determined the role of hypertension, OS, and inflammation on kidney damage in a rodent model of obesity and diabetes. Their results suggest that obese deoxycorticosterone acetate-salt (DOCA-salt) hypertensive mice exhibited a greater renal injury than lean DOCA-salt hypertensive mice in a manner independent of blood pressure, and this renal injury was associated with obesity-related pre-existing renal OS.

Finally, although many clinical investigations have found a relationship between hearing loss and diabetes mellitus, the pathophysiology of this effect remains controversial. In this sense the outcome of the study by Aladaq et al. (18) suggest that OS may play an important role in hearing impairment in diabetic patients. In this process, increased protein oxidation appears to be more important than lipid peroxidation. Nitric oxide may have a protective effect on hearing, as may some nonenzymatic antioxidants such as vitamin C and E.

Conclusion

A precise chromatography method was developed and validated for determining 2,3-DHBA and 2,5-DHBA. This method provides reliable results and is an additional detection method for determining oxidative damage in pathologies that have been associated with OS, such as diabetes. Furthermore, with this method it will be possible to detect at a very early stage the degree of organic damage produced by an increase in free radicals, as well as to monitor the evolution of the disease. The method is also inexpensive, easy to run, simple, and quick with excellent reproducibility.

On the other hand, the association of OS in vivo with salicylate hydroxylation products in patients with diabetes mellitus is a biochemical marker for monitoring disease progression, complications, and a response to treatment with antioxidant substances and pharmacological management indicated.

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