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Improved method for the estimation of hydroxyl free radical levels in vivo based on liquid chromatography with electrochemical detection

Douglas R. McCabe[a], Timothy J. Maher[b], Ian N. Acworth[a,b,*]

[a]*ESA Inc., 22 Alpha Road, Chelmsford MA 01824-4171, USA*

[b]*Division of Pharmaceutical Sciences, Massachusetts College of Pharmacy and Allied Health Sciences, 179 Longwood Avenue, Boston MA 02115, USA*

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Abstract

Free radical damage to proteins, lipids, DNA and RNA has been thought to play an important role in many diseases as well as the aging process. One free radical, the hydroxyl free radical (HFR), is extremely reactive and is difficult to measure directly. HFRs were quantified by measuring the hydroxylation products 2,3- and 2,5-dihydroxybenzoic acids (DHBAs) formed as a result of the reaction between HFR and systemically administered salicylate (SAL). DHBAs and SAL concentrations were determined using RP-HPLC with dual coulometric electrode detection. The method has limits of detection of 1 pg for the DHBAs and 100 pg for SAL (signal-to-noise ratio 3:1). A detailed interference study as well as analyte stability and linearity studies were performed. This method was used to determine basal ratios of DHBA/SAL in a variety of tissues and to study the effects of glutamatergic and dopaminergic drugs on DHBA/SAL ratios in brain region homogenates.

Keywords: Hydroxyl free radicals; Dihydroxybenzoic acid; Salicylic acid

1. Introduction

A variety of oxygen based free radical species exist in biological systems and include the hydroxyl free radical ($^{\cdot}OH$), the superoxide anion ($^{\cdot}O_2^-$) and the hydroperoxyl ($HO_2^{\cdot}$) free radical, collectively called the reactive oxygen species (ROS) [1]. ROS are actively involved in many normal physiological processes such as the destruction of pathogens by phagocytic polymorphonuclear leucocytes [2] but their rate of production does not normally exceed the capacity of the tissue to catabolize them [3]. However, during periods of oxidative stress, for example, brought about by exposure to UV irradiation or environmental toxins, or in those individuals with a genetic predisposition, levels of ROS can overwhelm the body's control mechanisms with subsequent damage to proteins, lipids, DNA and RNA, which can alter their biological activity and cause cellular damage [3–5]. Consequently, ROS have been implicated in many diseases including cancer, arthritis, reperfusion injury and several neurodegenerative

*Corresponding author.

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diseases including Parkinson's and Alzheimer's diseases [5–8].

Of all the ROS that can occur in vivo the HFR is considered to be the most reactive and hazardous [9]. Several approaches have attempted to accurately quantitate HFR levels in vivo. Although the use of spin traps such as α-phenyl *N-tert.*-butyl nitrone coupled to electron paramagnetic resonance (EPR) is a powerful technique [10,11], this approach has proven to be difficult due to poor sensitivity, toxicity of some spin traps, instability of the spin trap adduct and quenching in vivo (Refs. [12,13] and references therein). A variety of spin trap HFR adducts have also been measured by HPLC–UV [13–16], fluorescence [17], electrochemical detection (ED) [18–20], ED with UV [20–23] and ED with fluorescence [24] detection using phenol [18], phenylalanine [17], 2-deoxyguanosine (2DG) [14], or SAL [15,16,18–24] as the spin trap. However, not all of the above methods measured the precursor spin-trap and the inherent problems associated with this approach are discussed in more detail below. In general SAL is the preferred spin trap as it reacts exceedingly rapidly with the HFR [25]. Unlike phenylalanine and 2DG, SAL is not normally found in tissue and furthermore, at least one of its products, 2,3-DHBA, does not occur endogenously and once formed cannot be further metabolized [26] (Fig. 1). HPLC with ED is approximately 1000 times more sensitive than EPR [13] and HPLC with spectrophotometric detection [27]. Consequently, ED detection permits the use of lower concentrations of SAL than would be possible with UV, fluorescence, ED–UV and ED–fluorescence detection approaches, where the mandatory use of high concentrations of SAL may be producing physiological affects ([28] and references therein).

We have refined the method of Floyd et al. [18] to measure both the DHBAs and SAL electrochemically on one analytical system with one detector. Our approach uses two flow-through graphite coulometric electrodes placed in series. The first electrode completely oxidizes the DHBAs thereby preventing them from reacting at the second, down-stream electrode set at a higher potential for the measurement of SAL. The ability to optimize each electrode for a particular class of analyte has several advantages: lower background noise and, hence, greater sensitivity due to the lower, optimized potential; independent sensitivity settings which allows the simultaneous measurement of analytes occurring at markedly different concentrations (e.g., DHBAs and SAL); less co-elutions at the lower potential upstream electrode(s) due to less compounds oxidizing and responding at these lower potentials; and the ability to screen lower-potential oxidizing compounds from reacting at the higher potential downstream electrode. The advantages of this type of electrode have previously been discussed in detail [29,30]. We have used our method to measure concentrations of DHBAs and SAL in homogenates of different brain regions, liver, kidney and deproteinized serum following systemic administration of SAL. Furthermore, concentrations of DHBAs and SAL were measured in brain region homogenates obtained from animals pre-dosed with SAL and following pharmacological treatments chosen to affect the glutamatergic and dopaminergic pathways which are thought to produce ROS.

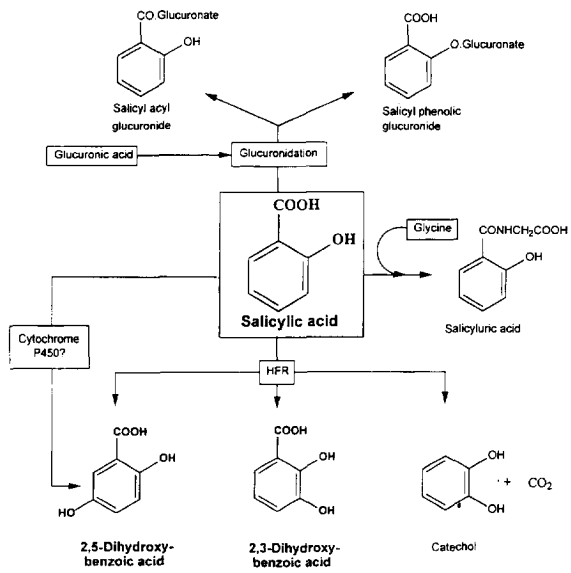

Fig. 1. Metabolism of salicylic acid showing reactions with HFR, enzymatic hydroxylation, conjugation with glycine and glucuronidation.

2. Experimental

2.1. Chemicals and reagents

2,3-DHBA, 2,5-DHBA and SAL were obtained from Sigma (St. Louis, MO, USA). All standards

used in the interference study were from Sigma or Aldrich (Milwaukee, WI, USA). *S*-(+)-MK801 hydrogen malate, *N*-methyl-D-aspartic acid (NMDA) and *S*-(+)-amphetamine sulfate were from RBI (Natick, MA, USA). All other reagents were of the purest grade available. The water used throughout the experiments had a resistivity of 18.2 MΩ cm obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). This water was then further purified by passing through a C_{18} solid-phase extraction cartridge (Waters, Milford, MA, USA) to remove trace organics. This step greatly reduces background currents and noise within the HPLC–ED system. Each 1 cm^3 cartridge can purify approximately 2 l of purified water.

Stock solutions of DHBAs and SAL were prepared at 1 mg/ml in 0.2 *M* PCA containing 100 μ*M* EDTA and 100 μ*M* sodium metabisulfite. Stock solutions were stored at 4°C and were stable for several months. Stock solutions of the standards used for the interference study were prepared at a concentration of 100 ng/ml (except where noted- Table 1) in saline (pH 3.0, acidified with phosphoric acid) containing ascorbic acid (10 μg/ml) and were stable at −80°C for several months.

Table 1
Possible endogenous interferences injected onto HPLC system[a]

Analyte	
2,4-Dihydroxybenzoic acid	Ferulic Acid
2,6-Dihydroxybenzoic acid	Glutathione (oxidized)
2-Hydroxyhippuric acid	Glutathione (reduced)
2-Hydroxyphenylacetic acid	Guanine
3,3,5-Triiodo-DL-thyronine	Guanosine
3,4-Dihydroxybenzoic acid	Homogentisic acid
3,4-Dihydroxymandelic acid	Homovanillic acid
3,5-Dihydroxybenzoic acid	Homovanillyl alcohol
3-*O*-Methyldopa	Homoveratic acid
3-Hydroxy-4-methoxyphenethylamine	Hydroquinone
3-Hydroxyanthranilic acid	Hypoxanthine
3-Hydroxybenzoic acid	Indole-3-acetic acid
3-Hydroxykynurenine	Indole-3-proprionic acid
3-Hydroxymandelic acid	Isatin
3-Hydroxyphenylacetic acid	Kynurenine
3-Methoxytyramine	L(−)-Isoproterenol
4-Hydroxy-3-methoxymandelic acid	L-Dopa
4-Hydroxybenzoic acid	Melatonin
4-Hydroxyphenylacetic acid	Metanephrine
4-Hydroxyphenylactic acid	Methionine
5-Hydroxyindoleacetic acid	Methoxamine
5-Hydroxytryptophan	Methoxyhydroxyphenylglycol
5-Hydroxytryptophol	α-Methyl-L-DOPA
5-Methoxy-DL-tryptophan	N-Methyldopamine
5-Methoxytryptamine	N-Methylserotonin
5-Methoxytryptophol	Norepinephrine
5-Methyl-L-cysteine	Normetanephrine
5-Serotonin	Pyroxidal
6-Hydroxydopamine	Resorcinol
6-Hydroxymelatonin	Tryptamine
7-Methylguanine	Tryptophan
Acetaminophen	Tryptophol
Acetylserotonin(N)	Tyramine
Anthranillic acid	Tyrosine
Ascorbic acid (10 μg/ml)	Uric acid (10 μg/ml)
Catechol	Vanillic acid
Cysteine	Vanillylmandelic acid
Dihydroxyphenylacetic acid	Xanthine (1 μg/ml)
DL-Indole-3-lactic acid	Xanthosine
Dopamine	
Epinephrine	

[a] On-column injection mass of 1 ng except where noted.

2.2. Chromatography

The chromatographic system consisted of a dual piston pump (Model 580), a refrigerated autosampler (Model 540), and a Coulochem II (Model 5200) dual potentiostat electrochemical detector equipped with an RS232 interface. Data collection and system control were performed using a PC-based data station (Model 500). Separation of analytes was achieved on a reversed-phase DHBA-250 column (5 μm, 250×3.0 mm). Analytes were detected on a dual electrode analytical cell (Model 5010) with the first electrode (E1) set to oxidize the DHBAs at +250 mV (vs. Pd) and the second electrode (E2) set to oxidize SAL at +750 mV (vs. Pd). A guard cell (Model 5020) was placed between the pump and the autosampler at a potential of +775 mV (vs. Pd) to oxidize contaminants in the mobile phase. All equipment was from ESA (Chelmsford, MA, USA).

The mobile phase consisted of 50 m*M* sodium acetate, 50 m*M* sodium citrate, 8% methanol, 2% 2-propanol (v/v). The pH of the mobile phase was adjusted to 2.5 with phosphoric acid after the addition of organic modifiers. The mobile phase was passed through the system at 0.5 ml/min. All analyses were performed at 27°C.

2.3. Biological experiments

Male Sprague–Dawley rats (250–300 g) were housed in pairs and were given ad libitum access to food and water prior to experimentation. Animals

were maintained at 20–22°C and were exposed to a 12 h light–dark cycle. All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and had prior approval of the Institutional Animal Care and Use Committee.

In the first experiment the feasibility of this method was examined in animals receiving either vehicle (0.9% w/v sodium chloride) or SAL (100 mg/kg i.p.). After 30 min animals were killed (decapitated) and the brain, liver, kidneys and blood were immediately removed. The liver and kidneys were rapidly frozen on dry ice. The brain was quickly dissected on ice and the striata and cortex were removed and frozen on dry ice and stored at −80°C until analysis. Blood was centrifuged (12 500 *g*, 5 min, 4°C) and the serum frozen as above.

In the second experiment, four groups of animals (three per group) received either vehicle or SAL (30, 100 or 300 mg/kg i.p.). After 30 min animals were killed (decapitated) and the brain was rapidly dissected. Cortex, striata and hippocampi were immediately frozen on dry ice and stored at −80°C until analysis.

In the third experiment eight groups of animals received either vehicle or SAL (100 mg/kg i.p.) 30 min prior to receiving one of the following treatments i.p.: vehicle; NMDA (30 mg/kg); NMDA and MK-801 (30 and 1 mg/kg respectively); (+)-amphetamine (5 mg/kg). After 30 min the animals were killed (decapitated) and the brain rapidly dissected into cortex, hippocampi and striata, and stored as above at −80°C.

2.4. Sample preparation

Tissue was ultrasonicated in 0.2 *M* PCA (containing 100 μ*M* EDTA and 100 μ*M* sodium metabisulfite) 1:5 or 1:10 (w/v) at 4°C using a Heat Systems cell disrupter (Model W-220F, Plainview, NY, USA). Samples were then centrifuged (12 500 *g*, 5 min, 4°C) and the supernatant was passed through a Micro-Spin centrifuge tube containing a 0.2 μm cellulose acetate filter (Alltech, Deerfield, IL, USA) by centrifugation (as above). A sample volume of 10 μl was used throughout this study. Serum samples were deproteinized by the addition of PCA (containing EDTA and sodium metabisulfite) 1:5 v/v and were centrifuged and filtered as above.

3. Results and discussion

3.1. Chromatography

There is still a great debate as to which DHBA isomer better reflects HFR levels. Some researchers have suggested that the 2,3-isomer is a more accurate representation of HFR levels as the 2,5-isomer can be formed in vivo by the action of cytochrome P450 [31,32]. However, others disagree and have suggested that the 2,5-isomer is indeed an accurate reflection of in vivo HFR levels [21,24,33–37]. Unfortunately, as the concentrations of 2,3-DHBA are typically much less than the 2,5-isomer, these lower concentrations of the 2,3-isomer may be below the detection limit of some approaches, thereby hampering the measurement of both isomers [21,28]. Many researchers do not measure concentrations of SAL and interpret changes in tissue DHBA concentrations as changes in HFR levels [16,20,34–43]. Such an approach may make interpretations of data difficult, as concentrations of DHBA may just be the result of differences in SAL concentrations due to inter-animal variability in SAL distribution and metabolism or accuracy and location of SAL administration. Hall et al. [21] showed a linear increase in 2,5-DHBA formation in the gerbil brain as SAL dose was increased. The 2,5-DHBA/SAL ratio remained constant, however, regardless of i.p. dose administered. For these reasons we developed a sensitive method capable of measuring both the DHBA isomers and SAL substrate simultaneously.

The correct choice of applied potentials to the two working electrodes was obtained from the analytes' current–voltage (CV) curves. CV curves were generated by analyzing a constant mass (100 ng on column) in duplicate of DHBAs and SAL with E1 set to 0 mV and E2 increasing in 50 mV increments. The potential to E2 was only changed between each pair of injections. The CV curves are presented in Fig. 2. From these data the major differences in maximal oxidation potentials between the analytes allowed the system to be configured so that the first electrode measured the DHBAs (+250 mV vs. Pd)

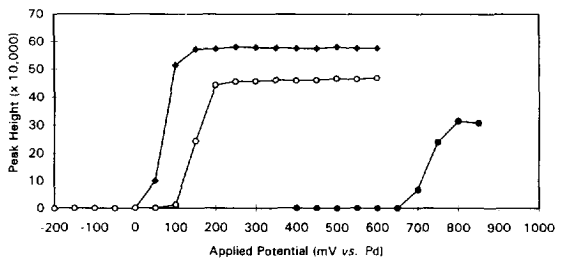

Fig. 2. Current–voltage curves for 2,5-DHBA (♦), 2,3-DHBA (○) and SAL (·). Each point is the average of two injections (100 ng on column) of authentic standard. Mobile phase consisted of 50 m*M* sodium acetate, 50 m*M* citric acid, 8% methanol (v/v), and 2% 2-propanol (v/v), pH 2.5 with phosphoric acid.

and the second measured SAL (+750 mV vs. Pd). Although the analytes could be detected on one electrode set at +750 mV (vs. Pd) the resulting solvent front would increase dramatically due to the increase in the number of analytes responding at this higher potential, thereby increasing the likelihood of undetected co-elutions with the early eluting DHBAs. In addition, the resulting increase in background currents and noise would substantially reduce the sensitivity of the assay. Kaur and Halliwell [44] analyzed DHBAs and SAL in human plasma on a one electrode system with success. However, this involved a complicated sample clean-up procedure and resulted in a complex chromatogram with multiple detector sensitivity changes within the chromatographic run. In addition, it is unknown what the resulting chromatography would be as a result of processing and analyzing other biological sample matrices utilizing this high potential, one electrode approach.

Fig. 3 shows the DHBAs and SAL were all well resolved and the analysis was completed within 18 min. Fig. 3A shows that there is a perturbation on the first electrode at SAL's retention time. This is an artifact, a consequence of the excessive amount of SAL disrupting the double layer of the first electrode and not due to the actual oxidation of SAL per se [SAL does not start to oxidize until >650 mV (vs. Pd), see Fig. 2]. Attempts to quantitate SAL based on this perturbation on E1 would not be expected to be an accurate indicator of SAL's actual concentration.

3.2. Stability

It is known that many catechol-based compounds, several of whose structures are similar to those of the DHBAs, are unstable at neutral pH and room temperature and rapidly form quinones [45,46]. Therefore, the effects of pH and a variety of antioxidants on the stability of the DHBAs were tested. The DHBAs were prepared at a concentration of 100 ng/ml and were placed in capped vials on the autosampler at 4°C. Two 10 μl samples of each standard solution were analyzed over the course of 4 days – see Fig. 4. Both DHBAs were found to be unstable in saline or artificial CSF (aCSF) at pH 7.0, decomposing within 4 h even though they were maintained at 4°C. This may be an issue for the measurement of DHBAs in tissue extracellular fluids obtained using in vivo microdialysis perfusion where the DHBAs come in contact with aCSF (pH 7.0, 37°C) during the course of an experiment [19,34,35,38–40,47]. Furthermore, we have recently found that when prepared in aCSF, SAL spontaneously forms DHBAs. This may pose a problem when administering SAL through the microdialysis probe or if SAL enters the probe after its peripheral administration. The addition of typical SAL concentrations (5 m*M*) to aCSF caused the formation of 2,3-DHBA (195 pg/10 μl) which remained at this concentration for 4 days and was not affected by light. There was less formation of 2,5-DHBA (9 pg/10 μl) which slowly increased over 4 days and its formation was inhibited by dark. 2,3-DHBA spontaneously formed when SAL was added to each of the individual components of the aCSF. Neither DHBA isomer could be measured in aCSF devoid of SAL.

In general, 2,5-DHBA was less stable than 2,3-DHBA which is in agreement with their observed electrochemical behavior where the 2,5-isomer's maximal oxidation potential is 100 mV less than the 2,3-isomer (Fig. 2). The greater stability of 2,3-DHBA may also be due to intramolecular hydrogen bonding which occurs between the phenol and carboxylic acid group [15]. Both DHBAs were more stable in acidic media but showed the greatest stability in either the unacidified EDTA solution or in PCA containing EDTA and sodium metabisulfite. Although either of the solutions could have been

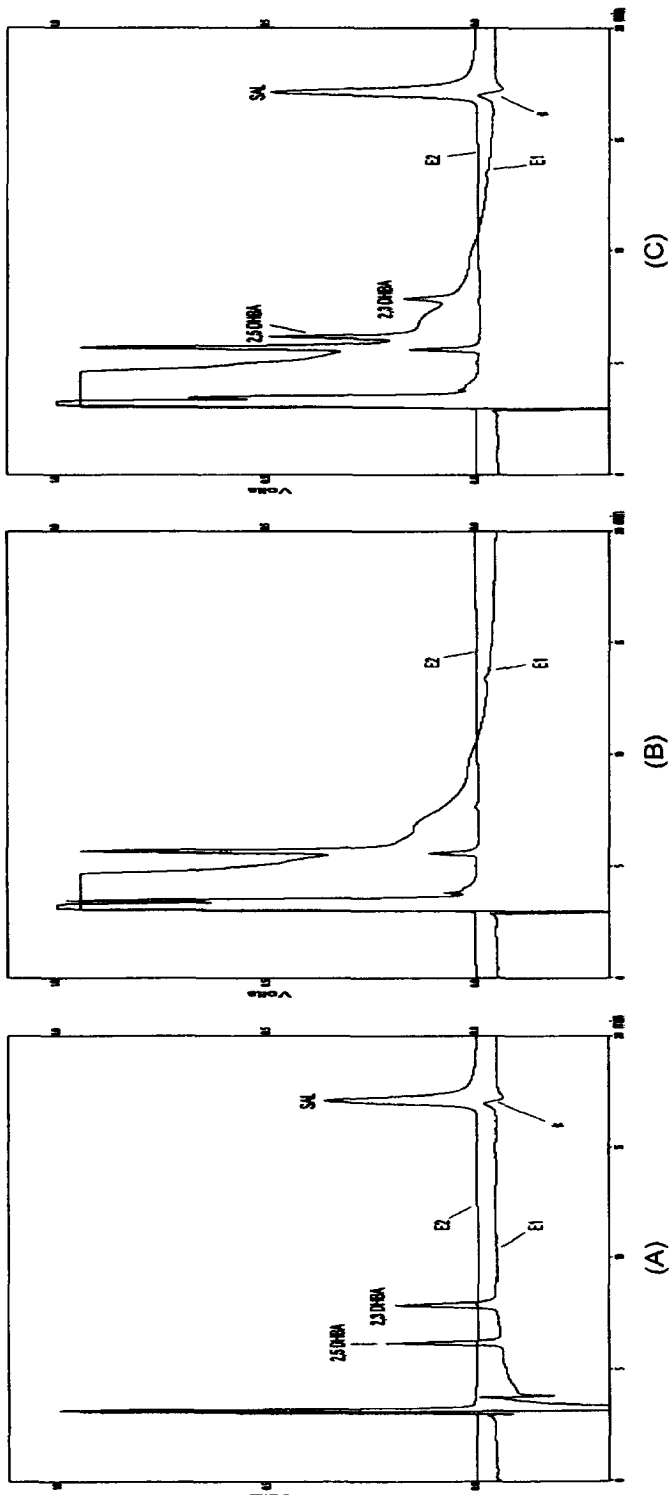

Fig. 3. Chromatograms of (A) 250 pg DHBA and 125 ng SAL standard, (B) rat hippocampus homogenate injected with vehicle, and (C) rat hippocampus homogenate injected with SAL (100 mg/kg) followed by amphetamine (5 mg/kg). DHBAs detected on E1 at +250 mV (vs. Pd, 100 nAFS) and SAL detected on E2 at +750 mV (vs. Pd, 10 μAFS). (*) denotes SAL baseline perturbation on E1 caused by the excessive amount of SAL disrupting the double layer of the first electrode. Mobile phase composition is given in Fig. 2.

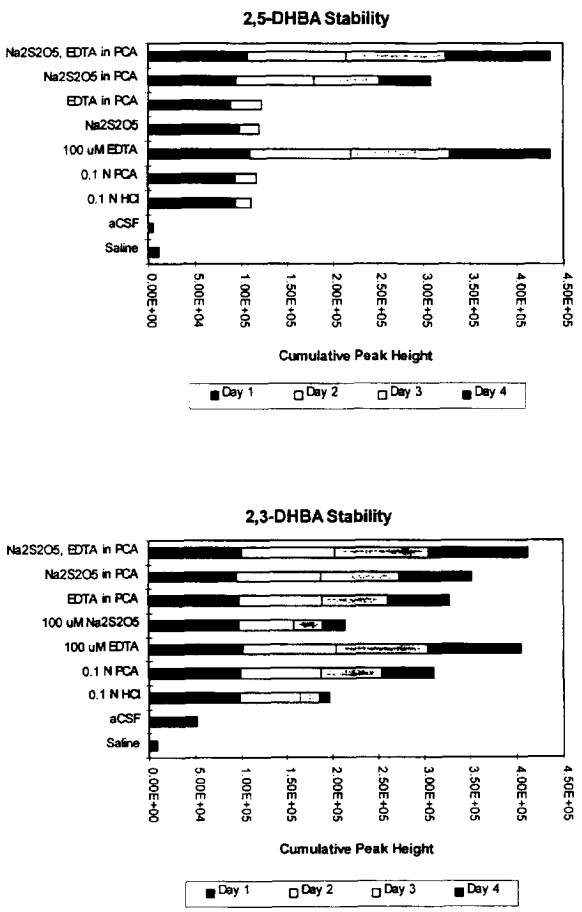

Fig. 4. Stability of DHBA standard as a function of storage solution at 4°C. Two 10 μl samples containing 1 ng of authentic standard were injected onto the HPLC system with detector settings of $E=+250$ mV (vs. Pd, 10 μAFS). Mobile phase composition is given in Fig. 2.

chosen, the latter was used to prepare all DHBAs and SAL standard solutions and sample extracts because PCA was used in the sample preparation procedures.

3.3. Interference

Table 1 lists a variety of endogenous compounds and metabolites of SAL that were tested and found not to interfere with the measurement of the DHBAs and SAL. Standards were prepared at concentrations of 100 ng/ml (except where noted).

3.4. Linearity, limits of detection, retention time and peak height precision

Standards containing 5–800 pg/10 μl of 2,3- and 2,5-DHBA were analyzed in duplicate at a potential of +250 mV (vs. Pd) and a current range of 100 nA. Results showed the response to be linear over this concentration range with correlation coefficients (r^2) of 0.9999 and 0.9997 for 2,3-DHBA and 2,5-DHBA, respectively. The results for SAL (5–250 ng/ml) were also linear, with an r^2 of 0.9977. The limits of detection were found to be 1 pg on column for DHBAs and 100 pg on column for SAL.

An homogenized and filtered rat cortex solution was spiked with DHBA and SAL standards yielding an on-column injection mass of 250 pg for the DHBAs and 100 ng for SAL. The intra-day variation in retention time over an 8 h period was 7.67±0.03, 0.37; 5.47±0.02, 0.30; and 16.93±0.11, 0.46 min (mean±S.D., R.S.D.; $n=8$) for 2,3-DHBA, 2,5-DHBA and SAL respectively. The inter-day variation in retention time over a 5 day period was 7.69±0.03, 0.36; 5.48±0.01, 0.22 min (mean±S.D., R.S.D.) for 2,3-DHBA and 2,5-DHBA, respectively. The intra-day peak height precision over an 8 h period was 25.71±3.11, 12.08; 34.36±3.99, 11.62; and 58.29±0.38, 0.65 (mean±S.D., R.S.D.) for 2,3-DHBA, 2,5-DHBA and SAL respectively. The inter-day peak height precision over a 5 day period was 26.40±3.29, 12.47 and 35.12±4.34, 12.37 (mean±S.D., R.S.D.) for 2,3-DHBA and 2,5-DHBA, respectively.

3.5. Biological data

The first biological experiment measured the concentrations of the DHBAs and SAL in a variety of tissues when animals received either saline or SAL (100 mg/kg i.p.). No endogenous interferences were found in any of the tissues. Table 2 shows that basal concentrations of DHBAs could only be measured in kidney, liver and serum (they were below the detection limit in the brain). The concentrations of DHBAs and SAL in tissue following SAL administration were similar to those previously reported [19,22,48,49]. The ratio of 2,3-DHBA/SAL was much lower in the periphery than the ratio of 2,5-DHBA/SAL supporting the fact that 2,5-DHBA

Table 2
Concentrations of DHBAs and SAL, and ratios of DHBA/SAL in the brain and periphery[a]

Sample matrix	2,5-DHBA (ng/g)	2,3-DHBA (ng/g)	SAL (μg/g)	2,5-DHBA/SAL ($\times 10^{-3}$)	2,3-DHBA/SAL ($\times 10^{-3}$)
Striatum	7.4	14.3	3.9	1.9	3.6
Cortex	20.9	17.6	6.6	3.2	2.7
Kidney	950 (29.3)	120 (8.4)	41.2	23.1	2.9
Liver	515	41.2 (7.5)	28.4	18.2	4.9
Serum	1560 (33.0)[b] ng/ml	116 (5.6)[b] ng/ml	129 μg/ml	12.1($\times 10^{-3}$)	0.9($\times 10^{-3}$)

[a] Animals were killed 30 min post-SAL administration (100 mg/kg i.p.).
[b] Basal (control) concentrations found in kidney, liver and serum are given in parenthesis.

may better reflect cytochrome P450 activity than HFR levels [31,32]. Furthermore, concentrations of 2,5-DHBA were much lower in the brain, an organ with limited P450 activity [50].

The second biological experiment examined the effects of different doses of SAL on DHBA formation in different brain regions- Table 3. Concentrations of DHBAs were below the analytical detection limits with the 0 and 30 mg/kg doses. However, central concentrations of DHBAs could be measured in rats receiving SAL at 100 mg/kg doses or greater. The normal ranges of SAL used in the literature varied from 100–300 mg/kg. It is important to keep the concentrations of SAL as low as possible to avoid possible toxicological effects such as acid–base, hemostatic and neurological disturbances commonly observed with high doses of SAL ([28] and references therein, [51]). It is interesting to note that, in general, the ratios of 2,3-DHBA/SAL and 2,5-DHBA/SAL tended to decrease in all brain regions as the dose of SAL increased and is in agreement to previously published data [19,21]. This suggests that under basal conditions the reactions for the formation of DHBAs are already proceeding maximally at the 100 mg/kg dose.

The third biological experiment was designed to measure the effects of pharmacologically induced stress on HFR production. Oxidative stress can damage neuronal tissue by several interacting mechanisms. Ischemia and trauma produces free radicals which promote the release of the excitatory amino acids (EAA) glutamate and aspartate. EAA receptor stimulation then elevates the intracellular free Ca^{2+} concentration which in turn activates a variety of intracellular enzymes [9,52,53]. These then cause more neuronal damage and also the formation of more free radicals which culminates in stimulated EAA release, thereby completing this positive feedback loop [54]. In our paradigm the actions of the EAA agonist NMDA on HFR production was studied alone or after being blocked with MK-801, a potent and selective non-competitive NMDA receptor antagonist. Neither administration of the NMDA alone nor coadministration of NMDA and MK-801

Table 3
Effects of varying SAL injection doses on DHBA formation in different brain regions[a]

Ratio of DHBA to SAL	Brain region	SAL injection doses (mg/kg i.p.)			
		0	30	100	300
2,5-DHBA/SAL ($\times 10^{-3}$)	ST	n.d.[b]	n.d.	1.37±0.86	0.57±0.28
	H	n.d.	n.d.	1.56±0.81	1.36±0.19
	CX	n.d.	n.d.	2.88±0.34	1.51±0.56
2,3-DHBA/SAL ($\times 10^{-3}$)	ST	n.d.	n.d.	13.55±9.25	4.11±0.40
	H	n.d.	n.d.	7.75±1.48	6.16±1.88
	CX	n.d.	n.d.	6.55±0.69	5.15±0.97

[a] $n=3$, mean value±SEM.
[b] n.d.=none detected (LOD=1.0 pg on column).
Animals killed 30 min post-SAL administration. CX=Cortex; ST=striata; H=hippocampi.

Table 4
Ratios of DHBA/SAL in various brain regions after i.p. administration of SAL followed by vehicle, NMDA, NMDA and MK-801, or D-amphetamine[a]

Ratio of DHBA to SAL	Brain region	Post-SAL administered drug (i.p.)[b]			
		Vehicle	NMDA	NMDA and MK-801	(+)-Amph.[c]
2,5-DHBA/SAL ($\times 10^{-3}$)	ST	1.92±0.30	1.62±0.24	1.52±0.16	2.53±0.14
	H	1.82±0.31	1.89±0.26	1.69±0.14	2.25±0.38
	CX	1.49±0.21	1.76±0.24	1.39±0.10	2.37±0.53
2,3-DHBA/SAL ($\times 10^{-3}$)	ST	0.63±0.11	0.56±0.05	0.61±0.05	0.68±0.07
	H	0.56±0.04	0.70±0.08	0.59±0.02	0.72±0.10
	CX	0.14±0.07	0.28±0.03	0.19±0.03	0.28±0.08

[a] $n=3$, mean value±SEM.
[b] SAL administered at time=0 min, followed by drug or vehicle at time=30 min, and sacrifice at time=60 min.
Parallel experiment run with vehicle administered instead of SAL. No endogenous DHBAs or SAL were detected.
[c] (+)-Amph.=(+)-amphetamine.
CX=Cortex; ST=striata; H=hippocampi.

had any affect on the regional production of the DHBAs in brain tissue (Table 4). Although equivalent doses are known to stimulate glutamate release in vivo [55], it is not clear why NMDA failed to affect HFR production. However, these data also suggest that a major insult such as ischemia and reperfusion may be a prerequisite to activation of the free radical–glutamate–Ca^{2+} cycle and that stimulation of the EAA receptor by NMDA is not a powerful enough stimulant.

Several compounds such as the catecholamines can spontaneously auto-oxidize and generate free radicals [9,56]. A second approach attempted to increase HFR levels by stimulating the extracellular concentration of the catecholamine dopamine by the mixed-acting sympathomimetic agent (+)-amphetamine. Animals receiving SAL following vehicle showed no change in the DHBA/SAL ratios compared to controls. Animals receiving SAL followed by amphetamine did show a slight increase in the 2,5-DHBA/SAL ratio (Table 4) but all animals died prematurely. At present it is unclear whether these data are the direct result of the stimulation of dopamine release and free radical generation or the consequence of ischemia following death. Current experiments have shown that the combination of high doses of SAL and (+)-amphetamine causes an enhanced hyperthermic response to (+)-amphetamine resulting in death of the animal. In addition to the elevation in body temperature, the coadministration of these two agents resulted in significant tachycardic response, without significantly affecting mean arterial blood pressure [57].

4. Conclusions

A sensitive LC method using a high efficiency electrochemical detector was developed for HFR levels as reflected by the ratio of DHBA-isomers to SAL. The new dual electrode method permits the use of lower concentrations of SAL and offers great selectivity. This method is routine, exhibits no interferences from endogenous metabolites and possesses more than sufficient sensitivity for the measurement of both DHBA-isomers in a variety of tissue homogenates following low doses of SAL. Although this approach is sufficiently sensitive for microdialysis studies the spontaneous formation of DHBAs when SAL is prepared in aCSF is a great cause for concern and warrants further investigation.

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