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Measurement of Hydroxyl-Radical Formation in the Rat Striatum by In Vivo Microdialysis and GC–MS

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Abstract

A GC-MS method was developed for measuring hydroxyl-radical capture products of salicylic acid, a common trapping agent for this reactive oxygen species, in samples obtained by in vivo cerebral microdialysis experiments. The assay employed liquid-liquid extraction followed by derivatization of 2,3- and 2,5-dihydroxybenzoic acid, along with 3,5-dihydroxybenzoic acid added as an internal standard. Due to their simple electron ionization mass spectra featuring $[M - 57]^+$ ions through the loss of tertiary alkyl group from the corresponding molecular ions, *tert*-butyldimethylsilyl (TBDMS) derivatives afforded straightforward method development based on selected-ion monitoring. In addition, tandem mass spectrometry probing collision-induced dissociation of $[M - 57]^+$ ions obtained from the isomeric *tert*-butyldimethylsilyl derivatives revealed characteristic differences in the resultant product-ion spectra. Our work has demonstrated the applicability of GC-MS for the assay of microdialysates for 2,3- and 2,5-dihydroxybenzoic acid by confirming that local administration of the excitotoxic glutamate into the rat striatum significantly increased in vivo hydroxyl-radical production in this brain region and that subsequent systemic administration of α -phenyl-*tert*-butylnitron reversed glutamate-induced oxidative stress.

Keywords

Dihydroxybenzoic acid; Derivatization; Gas chromatography-mass spectrometry; In vivo intracranial microdialysis; Oxidative stress

Introduction

Considered the most toxic reactive oxygen species (ROS), the hydroxyl radical ($\cdot\text{OH}$) is an extremely short-lived oxidant that reacts at near-diffusion limited rates with a wide variety of biological macromolecules [1] and has been directly implicated in oxidative stress leading to the development and/or progression of numerous neurodegenerative processes and neurotraumas [2-4]. However, monitoring of its formation in tissue has been a challenge.

In vivo intracranial microdialysis has been found suitable for sampling this ROS in localized brain regions [5-7]. By using a marker or “trapping” compound that is delivered to the origin of its production, monitoring of changes in $\cdot\text{OH}$ concentrations is possible by the analysis of radical-capture end products in samples obtained by microdialysis. An excellent marker is salicylic acid that converts to 2,3- and 2,5- dihydroxybenzoic acid (2,3- and 2,5-DHBA) upon reaction with $\cdot\text{OH}$ (Scheme I). The latter radical-capture products are essentially unreactive

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and, thus, their measurement indicates $\cdot\text{OH}$ formation in vitro and in vivo. While 2,3-DHBA is produced exclusively by reaction with $\cdot\text{OH}$, 2,5-DHBA may also be formed enzymatically [8].

The assay that has been employed to analyze 2,3- and 2,5-DHBA relies on high-performance liquid chromatography with electrochemical detection (HPLC–ECD) [1,5,6]. As a potentially more selective and robust alternative to HPLC–ECD, we aimed at developing a GC–MS-based method. For GC–MS analysis of DHBAs, derivatization is commonly employed to improve separation and detection. Trimethylsilylation has been applied to aromatic carboxylic acids [9]. In this study, we also considered a reagent that afforded *tert*-butyldimethylsilyl (TBDMS) derivatives. TBDMS derivatives exhibit reduced fragmentation compared to the corresponding trimethylsilyl (TMS) esters and ethers, which facilitates easy development of quantitative assays based on selected-ion monitoring (SIM) [10]. TBDMS ethers are also considered about 10,000-times more stable towards hydrolysis than their TMS analogues [10]. However, reduced fragmentation may not afford straightforward identification of positional isomers [9]. Therefore, we also aimed at studying the potential use of tandem mass spectrometry (MS/MS) to distinguish TBDMS-derivatized DHBAs.

To test the developed assay, changes in $\cdot\text{OH}$ levels were monitored in the rat striatum by in vivo microdialysis using salicylate as a detector molecule introduced through diffusion across the semipermeable membrane of the probes employed [5]. Local perfusion of glutamate was used to increase oxidative stress in the target brain region, and reversal of this neurotoxic insult was assessed by the systemic administration of α -phenyl-*tert*-butylnitron (PBN).

Experimental

Chemicals

Sodium salicylate (meeting USP testing specifications), 2,3-DHBA (99%), 2,5-DHBA (>99%) and 3,5-DHBA (97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetamide (MTBSTFA) containing 1% (v/v) *tert*-butyldimethylsilylchlorosilane (TBDMS) as a catalyst was obtained from Pierce (Rockford, IL, USA). Deriva-Sil (a mixture of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide, trimethylchlorosilane, trimethylsilylimidazole and pyridine, 3:2:3:10 v/v, to afford TMS-derivatives) was supplied by Regis (Morton Grove, IL, USA). Artificial cerebrospinal fluid (ACSF) was purchased from Harvard Apparatus (Holliston, MA, USA). All other chemicals were of analytical grade and obtained from Thermo Fisher Scientific (Waltham, MA, USA).

In Vivo Microdialysis

Male Sprague-Dawley rats (250–300 g body weight; three animals) were used for the brain microdialysis experiments. They were housed in an environmentally controlled room on a 12-hour light and 12-hour dark cycle, and were maintained ad libitum on water and rodent chow. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center before initiation of the study.

The implantation of a guide cannula (CMA/Microdialysis, Inc., Acton, MA) was done stereotactically [11] under aseptic conditions by using a frame equipped with a micromanipulator after the rats had been completely anesthetized (60 mg/kg ketamine and 10 mg/kg xylazine, administered intraperitoneally). The top of the head was shaved and a midline cut was made through the skin. Fascia over the skull was scraped away, and the cut was protracted. With a hand-held dental drill, one hole was drilled through the skull to place a guide cannula into the brain. Two additional holes were made anterior and posterior to this hole for the anchor screws. The guide cannula was inserted through the hole into the striatum (based

on its location at anterior to bregma, A: 0.5 mm; lateral to midline, L: 3.0 mm; vertical, V: 2.5 mm [11]), and held permanently in place with cranioplastic cement filled into the protracted area. The actual process of insertion of the probe into the striatum started after allowing 5 to 7 days for the wound to heal following the implantation of the guide. After connecting the tubing for the influx and efflux of the perfusion fluid to the microdialysis probe and then inserting the probe into the cerebral guide cannula, the animal was placed into the clear plastic bowl of an animal handling unit (Stand-alone Return, BASi, West Lafayette, IN, USA) for sample collection.

Concentric microdialysis probes (CMA/12, CMA/Microdialysis, Inc., Acton, MA) with a 4-mm long polycarbonate membrane (cutoff molecular weight 20,000 Da) were used. ACSF containing 5 mM salicylic acid was employed as a perfusion medium. Syringe pumps (1-mL BeeStinger), their controller (BeeKeeper) and a refrigerated fraction collector (HoneyComb) used throughout the experiments were purchased from BASi (BASi, West Lafayette, IN, USA). The system was first equilibrated at $2 \mu\text{L min}^{-1}$ with the perfusion medium for an hour and, then, baseline samples were collected for 2 h. A manual valve (CMA/110, CMA/Microdialysis, Inc., Acton, MA) was used for switching perfusion to a solution of 5 mM salicylic acid and 50 mM glutamate in ACSF without the interruption of the flow through the probe. Two hours after switching, PBN (100 mg/kg body weight) was administered intraperitoneally (i.p.) and perfusion of the salicylic acid plus glutamate solution through the probe was carried on for an additional 2 h period. Microdialysis samples were collected continuously in 20-min fractions.

The influx of $\cdot\text{OH}$ -trapping agent into the brain was estimated by an in vitro method [5]. ACSF containing 5 mM salicylic acid was perfused at $2 \mu\text{L min}^{-1}$ through the CMA/12 probes placed into 0.5-mL microcentrifuge tubes containing a small volume of ACSF (100 μL) and immersed into a temperature-controlled water bath set to maintain 37°C . Microdialysates were collected continuously by the refrigerated fraction collector as described in the previous paragraph. The influx of salicylic acid (in nmol min^{-1}) into the acceptor compartment was estimated from the difference of its entry (c_{in} , 5 $\text{nmol } \mu\text{L}^{-1}$) and exit concentration (c_{out} , in $\text{nmol } \mu\text{L}^{-1}$) and from the perfusion flow rate (V , $2 \mu\text{L min}^{-1}$) as $(c_{\text{in}} - c_{\text{out}}) \cdot V$ [12]. We also calculated $(c_{\text{in}} - c_{\text{out}}) / c_{\text{in}} \cdot 100$ that expressed the percentage of trapping agent delivered from into the acceptor compartment. Reversed-phase HPLC with UV detection was employed for the determination of c_{out} . Three separate perfusion experiments were done. Measurements were performed on an Agilent (Palo Alto, CA, USA) 1050 system equipped with a diode-array detector and autosampler. Data acquisition was done by the manufacturer's ChemStation system. An Alltech (Deerfield, IL, USA) 150×3.2 mm octadecylsilica column (Econosil, 5 μm) was used for the analyses. The mobile phase was water–acetonitrile (75:25, v/v) adjusted to pH 2.5 with phosphoric acid, and the flow rate was 0.5 mL min^{-1} . The injection volume was 20 μL , and the analyte was detected at 254 nm. Three 20- μL microdialysis fractions collected after 1 h of initial probe perfusion were combined, mixed with an aliquot of 2.5 mM mobile-phase solution of 4-hydroxybenzoic acid used as an internal standard, and the resultant solution was injected without any further dilution. Assay calibration was performed by 2.5, 5, 7.5 and 10 mM salicylic acid solutions in ACSF.

Samples and Sample Preparation

Calibration and quality control (QC) solutions (0.05, 0.26, 1.28, 6.4, 32 and 96 nM) of 2,3-DHBA and 2,5-DHBA were prepared by serial dilutions in ACSF containing 4.5 mM salicylic acid. Ten μL of sample was mixed to 500 μL deionized water containing a fixed quantity (400 pg) of 3,5-DHBA as an internal standard. The solution was acidified by the addition of 5 μL of aqueous 2 M HCl. The sample was then extracted with ethyl acetate ($2 \times 8 \text{ mL}$). After evaporation of the solvent under nitrogen stream, the residue was derivatized with 50 μL of MTBSTFA + 1% TBDMS for 30 min at 60°C . The derivatized samples were transferred into

a limited-volume glass insert in an auto-sampler vial (Alltech Associates, Deerfield, IL, USA) and were analyzed without further dilution.

GC-MS

Analyses were performed on a TRACE GC interfaced to a Polaris Q ion-trap mass spectrometer (Thermo Fisher Scientific). Separations were performed using a 30 m $\times$ 0.25 mm i.d., 0.25- μ m d $_f$ Rxi-5MS fused silica column (Restek, Bellefonte, PA, USA). Helium (1.0 mL/min) was used as a carrier gas. Column temperature was held at 175 °C for 2 min, and then raised to 300 °C at 10 °C/min. One μ L of the derivatized sample was injected in splitless mode with injector temperature set to 250 °C. Transfer line and ion source temperatures were 250 and 300 °C, respectively. Electron ionization (EI, 70 eV) was employed to obtain mass spectra. Collision-induced dissociation (CID) product-ion tandem mass spectra (MS/MS) were collected using 1.0 Th parent ion isolation width and 16 ms isolation time, followed by 2.5 V applied to the endcap electrodes for 30 ms (excitation time) with 0.3 selected as resonance parameter (q). Data acquisition and processing were performed by the manufacturer's XCalibur (version 1.4) software. Linear regression was employed to calibrate analyte over internal standard peak area ratios of the SIM chromatograms to the 2,3-DHBA and 2,5-DHBA concentrations, respectively.

Assay Validation

Limit of detection (LOD) and limit of quantitation (LOQ) was defined as $3\sigma/S$ and $10\sigma/S$, respectively, where σ was the standard deviation of the response (estimated by the standard deviation of y-intercept of the linear regression in the 50 pM to 6.4 nM concentration range) and S was the slope of the calibration line [13]. Assay reproducibility was determined by analyzing QC samples containing 6.4 nM of 2,3- and 2,5-DHBA in five replicates on five consecutive days.

Results and Discussion

Trapping assays employ reactions of $\bullet$ OH with a foreign "detector" molecule to form specific products that allow for the monitoring of this extremely short-lived ROS in vitro and in vivo [1]. Salicylic acid has been one of the most common trapping agents. A typical application of the method is an assay for the estimation of $\bullet$ OH levels in the brain of experimental animals (usually rats) via sampling by in vivo microdialysis [5-7]. About 13.1% ($\pm$ 2.2%) of the trapping agent, an acceptable agreement with 16% reported by others [5], was shown to leave the CMA/12 probes at 2 μ L/min perfusion flow rate according to our HPLC analysis. Therefore, the estimated influx of salicylic acid into the tissue in the employed experimental paradigm was merely 2.62 ± 0.44 nmol/min. Obviously, the concentration of the trapping agent in the microdialysates also decreases proportionally with the amount delivered into the tissue and, thus, we approximated this loss by making calibration/QC solutions in ACSF containing 4.5 mM salicylic acid.

As exemplified in Fig. 1 with 2,3-DHBA, TBDMS derivatives of DHBA's indeed yield intense $[M - tBu]^+$ (m/z 439, Fig. 1b) ions and exhibit reduced fragmentation compared to the corresponding TMS derivatives (Fig. 1a), which facilitates easy development of quantitative assays based on selected-ion monitoring (SIM). Fig. 1c, 2c and 2d summarize results of CID-MS/MS experiments for the isomeric $[M - tBu]^+$ ions from the TBDMS-derivatized DHBA's revealing characteristic differences in their fragmentation. Tentative origins of the observed fragment ions are indicated in the spectra; however, more detailed interpretation of fragmentation pathways probably strongly influenced by the interactions of the neighboring *tert*-butyldimethylsiloxy groups on the aromatic core of the derivatives requires additional studies. Nevertheless, the use of MS/MS is recommended for identification or development of

isomer-selective quantification in complex mixtures. In vivo microdialysates are moderately complex due to the nature of the sampling process [14] and GC–MS–SIM is a straightforward method to quantify 2,3-DHBA and 2,5-DHBA. By screening isomeric DHBAs for retention on the GC stationary phase employed, we selected 3,5-DHBA as an appropriate internal standard (clearly separated from the analytes, but with retention time closest to 2,3-DHBA and 2,5-DHBA).

Assay calibration correlating 2,3- and 2,5-DHBA concentrations with SIM (m/z 439) ion–current ratios afforded linear fits ($R^2 \geq 0.99$) in the target concentration range (5 to 100 nM [5–7]). LODs were 0.15 and 0.32 nM, while LOQs were 0.5 and 1.06 nM for 2,3- and 2,5-DHBA, respectively. In comparison, LOD for 2,3-DHBA by HPLC–ECD was ~ 0.1 nM [5]. Therefore, GC–MS–SIM has provided a true alternative to the previously employed assay method in this regard. No assay validation data to compare the two methods were available for HPLC–ECD. Because sample preparation involving extraction, solvent evaporation, derivatization and GC–MS analysis required several hours to complete, intra-day assay reproducibilities were not addressed. Coefficients of variation (CV) for accuracy and inter-day reproducibility of the assay tested with the 6.4 nM QC sample were $\leq 1.3\%$ and $\leq 6.1\%$, respectively. GC–MS analyses of microdialysates were also very consistent; CV of analyte to internal standard peak area ratios of the SIM chromatograms (m/z 439) was $<10\%$ based on five injections. A drawback of the method we developed is the need for a simple yet time-consuming sample preparation. On the other hand, assay calibration involving the use of an internal standard very similar to the analyte could result in improved accuracy over the previously employed HPLC–ECD assay, where external calibration was used [5].

We essentially reproduced results obtained by similar in vivo microdialysis experiments reported in the literature [5], where HPLC–ECD was used to assay 2,3-DHBA for relative changes in the concentration of this radical-capture product. We introduced the excitotoxic glutamate (Glu) known to increase $\bullet\text{OH}$ -formation [15,16] locally into the rat striatum to induce oxidative stress. As shown by GC–MS–SIM chromatograms of representative samples in Fig. 3a, our measured basal 2,3-DHBA levels were within the range of reported values (17.70 ± 3.30 nM) [5]. In vivo exposure of the rat striatum to Glu indeed resulted in an approximately two-fold increase in the concentration of this $\bullet\text{OH}$ -capture product of salicylic acid (Fig. 3b), whose level was also decreased significantly [17] after the subsequent intraperitoneal injection of PBN (Fig. 3c) shown to attenuate Glu-induced formation of this harmful ROS [5,18]. In Fig. 4, we summarized test data for 2,3-DHBA produced exclusively by reaction with $\bullet\text{OH}$, because 2,5-DHBA might in part be formed enzymatically [8]. Absolute concentrations of DHBAs reflecting $\bullet\text{OH}$ concentrations vary considerably among animal subjects; hence, each animal is used as its own control, when the impact of potential agents that induce changes in extracellular concentrations of this ROS is tested. Therefore, assay results are commonly expressed as percentages of basal mean levels [5].

In conclusion, our study has demonstrated the applicability of GC–MS for the assay of 2,3-DHBA and 2,5-DHBA in conjunction with in vivo intracranial microdialysis experiments to survey changes in $\bullet\text{OH}$ levels by using salicylic acid as a detector molecule. We plan to exploit the developed method to test experimental treatment of animals by various antioxidants with promise to reduce oxidative stress in the brain [19]. The assay can be adapted for measuring 2,3- and 2,5-DHBA in other biological media (e.g., human plasma) to indicate systemic oxidative stress relying on salicylic acid formed from oral aspirin ingestion [1].

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Fig. 1. EI mass spectra of (a) TMS derivative and (b) TBDMS derivative of 2,3-DHBA, and (c) CID product-ion (MS/MS) spectrum of the m/z 439 ion formed upon EI from the TBDMS-derivative of 2,3-DHBA.

Fig. 2. EI mass spectra of TBDMS-derivative of 2,3-DHBA and 3,5-DHBA (**a** and **b**), respectively, and CID product-ion (MS/MS) spectra of the m/z 439 ions formed upon EI of the TBDMS derivatives of 2,3-DHBA and 3,5-DHBA, respectively (**c** and **d**).

Fig. 3. Representative GC-MS-SIM chromatograms (m/z 439) indicating (a) basal levels of 2,3- and 2,5-DHBA in the rat striatum, (b) oxidative stress upon local exposure to glutamate in this brain region, and (c) attenuation of glutamate-induced oxidative stress by systemic (intraperitoneal) administration of PBN. From assay calibrations, concentrations in these samples were as follows: (a) Basal—16 nM 2,3-DHBA and 16 nM 2,5-DHBA; (b) Glu—36 nM 2,3-DHBA and 48 nM 2,5-DHBA; (c) Glu+PBN—27 nM 2,3-DHBA and 41 nM 2,5-DHBA.

Fig. 4. Relative changes in 2,3-DHBA content of in vivo microdialysis samples from rat striatum measured by GC–MS–SIM analyses with assay results were expressed as percentages of basal mean levels. Asterisk and dagger indicate statistically significant differences from basal and glutamate-induced oxidative stress levels of 2,3-DHBA in the microdialysates, respectively, according to analysis of variance (ANOVA) followed by post hoc Tukey test [17].

Scheme I.
Reaction of salicylic acid with hydroxyl radicals.