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Short communication

The salicylate trapping method: Is oxidation of salicylic acid solution oxygen and time dependent and metal catalysed?

Michael Gruber [a,*], Gunther Wiesner [b], Ralf Burger [c], Regina Lindner [a]

[a] *Department of Anaesthesiology, University of Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg, 93053, Germany*
[b] *Institute of Anaesthesiology, German Heart Centre Munich, Lazarettstrasse 36, Muenchen 80636, Germany*
[c] *Department of Neurosurgery, University of Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg, 93053, Germany*

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Abstract

For a microdialytic trapping method we systematically investigated changes in concentrations of 2,5-dihydroxy-benzoic acid (2,5-DHBA) and 2,3-dihydroxy-benzoic acid (2,3-DHBA) in freshly prepared solutions of salicylic acid (SA). The solvent was 0.9% saline exposed to different atmospheric concentrations of oxygen (0, 21, and 100%). The solutions were treated by freezing–thawing and an ultrasonic bath in presence and absence of aluminium foil. Without aluminium the concentrations of 2,5-DHBA and 2,3-DHBA kept constant over an observed period of 160 min on different levels from below 20 ng/ml to about 100 ng/ml. In presence of aluminium the concentrations increased to maximum 307 ng/ml after 160 min. Ultrasonic irradiation amplified this effect to maximum 341 ng/ml. HPLC/ECD processing and quantitative analysis of dihydroxy-benzoic acids (DHBAs) in microdialysis may be artificially influenced by varying oxygen environment and metal catalysis.

Keywords: Salicylate trapping method; 2,5-DHBA; 2,3-DHBA; Hyperoxia; Oxygen; Metal catalysis

1. Introduction

Indirect detection of reactive oxygen species (ROS) in experimental research of traumatic brain injury and cerebral ischemia has been focussed on since at least 20 years [1–4]. Therefore, trapping molecules as salicylic acid (SA) [5–9] or 4-hydroxy-benzoic acid [10,11] are commonly used and has been recently reviewed [12]. Quantification of the reaction products of these molecules and ROS by chromatographic methods [5,13] are employed. The reaction mechanism to the main products involving the OH-radicals and the aromatic ring is well-investigated [14,15] (Fig. 1). Possible influences on the reaction by molecules of biological origin particularly lipids [16] or fatty acids [17] are realized.

With our attempt to establish the salicylate trapping method for evaluation of free radical production in a novel traumatic focal mass lesion model [18,19] involving a microdialysis system and HPLC/ECD we detected unexpected changes in concentrations of the reaction products 2,5-dihydroxy-benzoic acid (2,5-DHBA) and 2,3-dihydroxy-benzoic acid (2,3-DHBA) (both: DHBAs) of the trapping SA. In a further course we systematically characterized oxygen concentration, time, freezing–thawing and metal catalysis as potential parameters for the concentration shifts excluding biological activity.

2. Material and methods

2.1. Experimental reagents

A 5 mM standard solution of salicylic acid (Sigma-Aldrich Corp., Steinheim, Germany) was freshly prepared in isotonic sodium chloride every day (Braun Corp., Melsungen, Germany). 2,5-DHBA, 2,3-DHBA and the aluminium foil (weighing boats 4 mm × 12 mm × 4 mm about 25 mg each) as well as 1-octanesulfonic acid sodium salt and EDTA were purchased from Sigma–Aldrich Corporation. Triethylamine and orthophosphoric acid (Fluka Corp., Neu-Ulm, Germany) and

* Corresponding author. Tel.: +49 941 944 7870; fax: +49 941 944 7802.
E-mail address: Michael.Gruber@klinik.uni-regensburg.de (M. Gruber).

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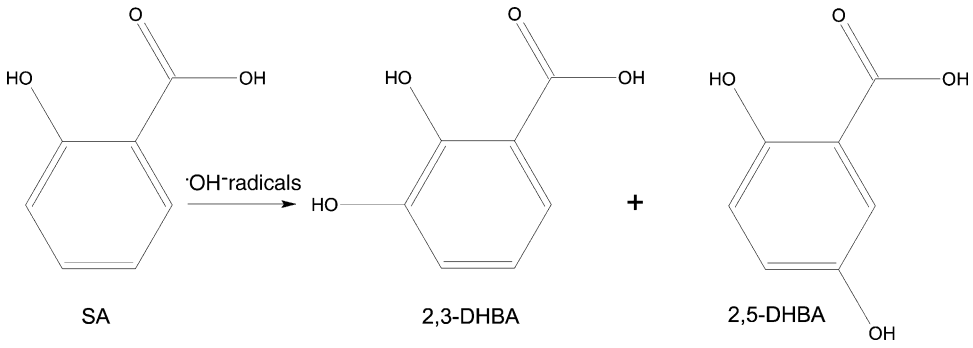

Fig. 1. Nonstoichiometric reaction equation of SA with OH-radicals.

methanol (Mallinckrodt Baker Corp., Deventer, The Netherlands) were also used.

2.2. Sample preparation

Before solving the salicylic acid, a stream (7 l/min) of 0, 21 or 100% oxygen in nitrogen was passed through the saline for 30 min using a degasing frit (Agilent Corp., Waldbronn, Germany) and an anaesthesia apparatus (Trajan 808, Draeger Corp., Lübeck, Germany). The oxygen concentration in a glove box containing the saline bottle was monitored (Capnomac Ultima, Datex-Ohmeda Corp., Duisburg, Germany) to guarantee the desired oxygen content over the conditioning period. The final partial oxygen pressures in the saline were measured by a blood gas analyser (Bayer 248, Fernwald Corp., Germany). The oxygen preconditioned salines were transferred to a 25 ml polypropylene narrow-necked graduated flask (Brand Corp., Wertheim, Germany) already containing 17.3 mg SA and a stopper. For optimizing the dissolving process the flask was put in an ultrasonic bath (Sonorex super RK 510H, Bandelin Corp., Berlin, Germany) at room temperature (RT) for 1 min. The further processing of the SA/saline solution was separated into the four illustrated modes (Table 1).

In every group 1 ml oxygenated SA/saline solution was placed into a 1.5 ml glass vial and closed airtight. Vials from groups 2 to 4 were immediately frozen on dry ice (−78 °C). Vials from groups 3 and 4 already contained an aluminium foil before the SA/saline solutions were added. Vials from group 1 were immediately chromatographed and kept at room temperature (20 °C) until the following two injections. Frozen vials were rewarmed to room temperature just before injection (groups 2 and 3) or before ultrasonic treatment for 10 min (group 4). Room temperature was maintained until the second and third injection (80 min/160 min) out of the same vial. All series were done in duplicate.

Table 1
Pre treatment of SA stock solution

Group	Aluminium foil	Freezing–thawing	Ultrasound	Injection time after (min) at RT
1	No	No	No	0; 80; 160
2	No	Yes	No	0; 80; 160
3	Yes	Yes	No	0; 80; 160
4	Yes	Yes	10 min	0; 80; 160

2.3. Chromatography

The chromatographic system consisted of an Agilent 1090 with an Agilent 1050 UV detector connected to an electrochemical detector Coulochem II (Esa Corp., Chelmsford, MA, USA) with a 5011 analytical cell. A volume of 5 μl was injected. Conditions were isocratic with a flow rate of 0.3 ml/min and a Luna 5 μ C18 (2) 250 mm × 2 mm column and a C18 4 mm × 2 mm pre-column (Phenomenex Corp., Aschaffenburg, Germany). The solvent composition was a mixture of 25% solvent A and 75% solvent B. Both solvents were basically composed of 0.1 M $NaH_2PO_4 \cdot H_2O$, 2.6 mM 1-octansulfonic acid sodium salt, 0.1 mM EDTA and 250 μM triethylamine and adjusted to pH 3.5 with *ortho*-phosphoric acid. Methanol was added to solvents A and B to yield a final concentration of 8% (A) and 16% (B). The solvents were filtered (0.45 μm filter pores; RC 55, Schleicher & Schuell Corp., Dassel, Germany) and kept oxygen free by degasing with helium (99.999% pure, Linde Corp., Höllriegelskreuth, Germany) in the HPLC bottle holder.

The limit of detection was about 5 ng/ml (33 nM, 160 fmol on column) and the LOQ at about 20 ng/ml. Both were ascertained by the software B.E.N. 2.0 (Institute for Forensic and Traffic Medicine, University of Heidelberg, Heidelberg, Germany) according to DIN 32645 for both dihydroxybenzoic acids in 0.9% NaCl using an external standard quantification and 400 mV (500 nA range) in the analytical cell. Linearity of the electrochemical signal was given at least up to 800 ng/ml.

The outside of the stainless steel injection needle was flushed after every run with methanol/water 50/50 vol.% to remove residues. The retention times for the target substances were 4.2, 5.6 and 13.4 min for 2,5 DHBA, 2,3-DHBA and SA, respectively. SA was detected at 215 nm and the DHBAs electrochemically with the ECD. An exemplary chromatogram of a SA/saline solution is shown in Fig. 2.

3. Results

Stepwise elevation of atmospheric oxygen concentration increased both DHBA compounds from below 20 ng/ml to about 100 ng/ml 2,5-DHBA and 2,3-DHBA directly after preparation. The corresponding partial oxygen pressures were about 20 mmHg (0% O_2); 180 mmHg (21% O_2). In 100% oxygen enriched atmosphere, the partial oxygen pressure of samples

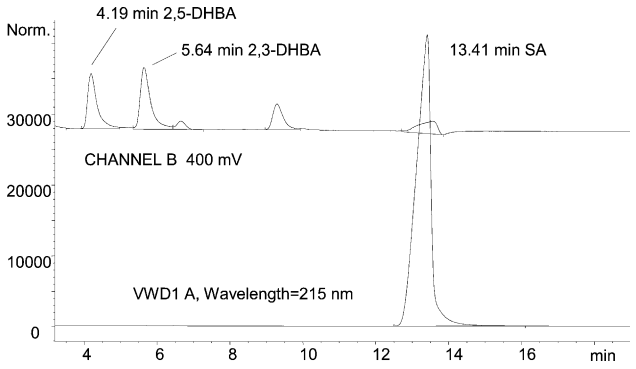

Fig. 2. Chromatogram of SA dissolved in NaCl saturated with 100% oxygen directly after preparation. The areas correspond to 101 ng/ml 2,5-DHBA, 105 ng/ml 2,3-DHBA and 5.02 μM SA (group 1).

was out of the measuring range for the blood gas analyser. Freezing–thawing had almost no effect.

In the absence of aluminium foil in SA/saline solutions no concentration–time dependency was detected at room temperature for the oxidation products 2,5-DHBA and 2,3 DHBA during a time period of 160 min (Fig. 3a and b data of 2,3-DHBA and 2,5-DHBA).

The stability of the SA/saline solution was disturbed by the aluminium foil as shown for 2,3-DHBA (Fig. 4a) and 2,5-DHBA (Fig. 4b). Additional ultrasonic treatment further increased the time depending rise in concentrations of 2,3-DHBA and 2,5-DHBA. (Relating to 100% without irradiation: at 0% oxygen to 293 ± 9.7% (mean ± coefficient of variation), at 21% oxygen to 149 ± 18.9% and at 100% oxygen to 128 ± 9.5%.)

4. Discussion

A large number of experimental studies using brain microdialysis investigated the possible influence on DHBA production from salicylic acid. The effect of an increased [7] or pressure modified [20] inspired oxygen fraction (FiO_2), as well as the action of organic substrates as *N*-methyl-D-aspartate/glutamate [6], 1-methyl-4-phenylpyridinium/histidine [21] or carbon monoxide [8] or inorganic ions as iron (II) [22] (Fenton reaction) or aluminium [5] were studied. CYP-450 activity [23], as a liver dependent organic source for the 2,5-DHBA could be identified aside the local and systemic enzymatic conversion properties from xanthine oxidase [24] or substance P [25]. All of the possible sources of OH radicals [3,4,13,14,5,15,2,16,19] have to be taken into account whenever

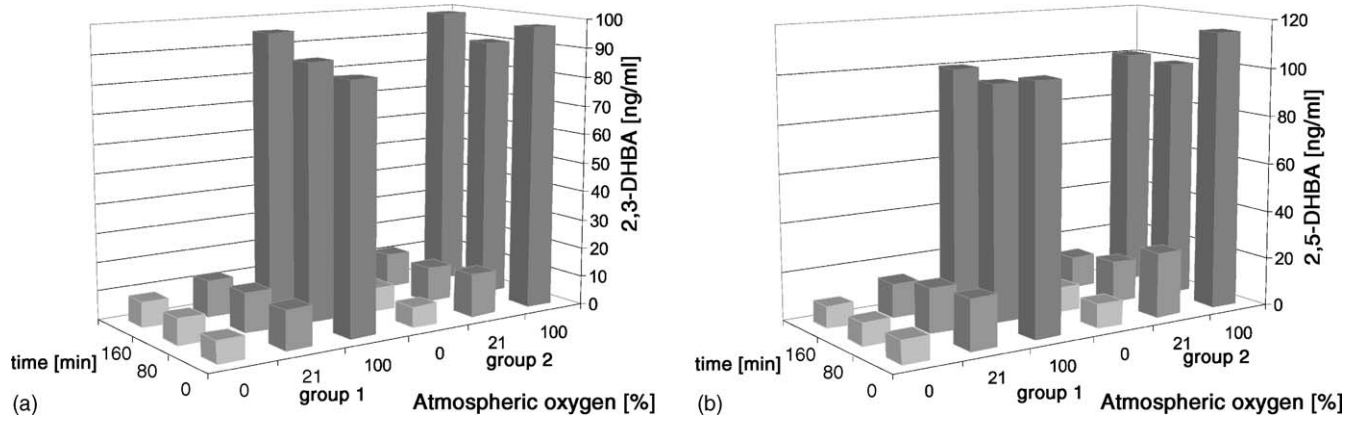

Fig. 3. (a) 2,3-DHBA concentrations in correlation to the oxygen exposure and experimetal duration without metal catalysis and (b) 2,5-DHBA concentrations in correlation to the oxygen exposure and experimental duration without metal catalysis.

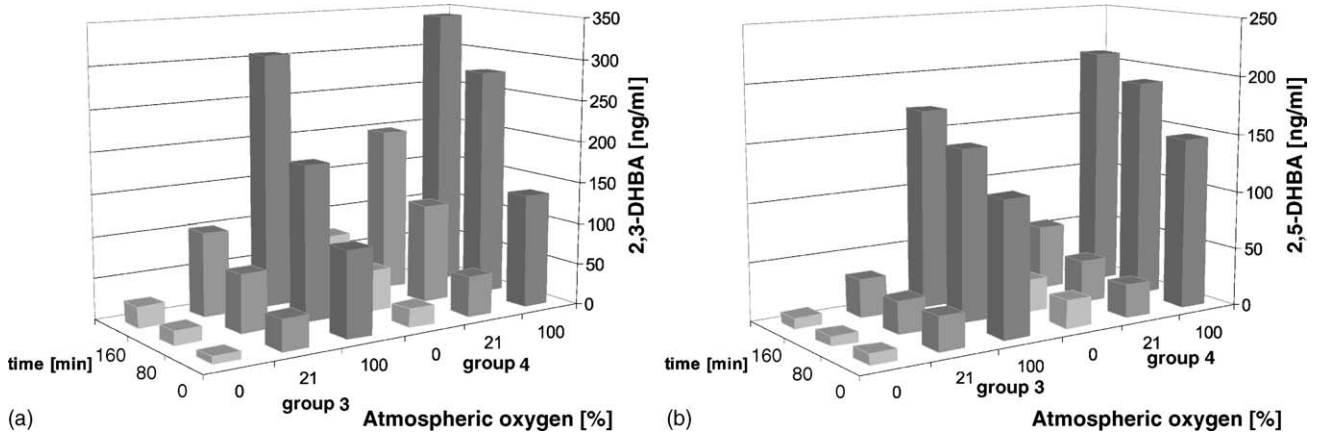

Fig. 4. (a) 2,3-DHBA concentrations in correlation to the oxygen exposure and experimetal duration in presence of aluminium and (b) 2,5-DHBA concentrations in correlation to the oxygen exposure and experimental duration in presence of aluminium.

microdialysis using SA should guarantee robust results. Thereby the steady state of perfusion is a fundamental condition for identification of changes in the radical status after traumatic brain injury or cerebral ischemia. Different baseline levels of DHBA in the perfusion solution before dialysis (Fig. 3a and b) do not automatically confuse the results, as long as the content is stable during the assay period. However, results become unpredictable if simple maneuvers as contact between a residual sample volume and the outer surface of a stainless steel injection needle for 15 min causes variable changes in DHBA concentrations in the next injection. Additionally, our results give rise to the question whether an oxidation reaction from SA to DHBA may be possible without involving a radical mechanism at all. In our solutions existed neither enzymatic activity nor H_2O_2 which are postulated radical sources in an in vivo-situation. Nevertheless the more obvious mechanism of OH radical formation seems to be dependent on the presence of oxygen and a metallic promoter (e.g. Fe(II), Al(0)). A recently filed patent confirms the simple formation of OH radicals in aqueous solution combined with Al_2O_3, SiO_2, Ni–Cr-steel or glass under ultrasonic irradiation [26].

Assuming that the presence of oxygen and metallic promoters are sufficient to induce the formation of OH radicals all microdialysis methods must be aware of the fact that OH radical scavenging might be artificially deluded by biological side effects. Radical scavengers may simply act as sensors for oxygen disturbed by variable concentrations of promoters like enzymes, reperfusion phenomena or inorganic substances. In this connection, it is interesting that elevated levels of inspired oxygen increased radical formation in sham operated animals while traumatized animals showed no effect [27]. Consequently, it is impossible to clarify the origin of the OH radicals. However, OH radicals may be provoked by pathological reasons as disruption of the blood–brain-barrier and acute phase reactions or formed just in the moment of microdialysis because the essential ingredients for their development meet each other by chance. It was shown that an increasing FiO_2 alone does not significantly elevate OH radical levels in ischemic rat brain tissue [7] but miscellaneous sources of iron as transferrin, ferritin or haemoglobin [28] might do. Therefore, the open discussion [20] whether increasing FiO_2 is beneficial for brain metabolism might only be answered by means of microdialysis systems if boundary conditions are perfectly standardized.

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