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Measurement of hydroxyl radical in rat blood vessel by microbore liquid chromatography and electrochemical detection: an on-line microdialysis study

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Abstract

Salicylic acid (0.5 m*M*) is used as a trapping reagent of hydroxyl radical, and the formed 2,3- and 2,5-dihydroxybenzoic acids were collected via an on-line microdialysis device from the blood vessels. This study revealed the use of a sensitive liquid chromatographic system with electrochemical detection for the determination of 2,3- and 2,5-dihydroxybenzoic acids. Mobile phase consisted of 0.1 *M* monochloroacetic acid, 10 m*M* EDTA, 0.5 m*M* sodium octylsulfate, 20% acetonitrile and 5% tetrahydrofuran in 1 l (pH 3.0 adjusted with 1 *M* NaOH), and the flow-rate of 0.05 ml/min were found to be optimum. Isocratic separation of these adducts on a microbore column (reversed-phase C_{18}, 150×1 mm I.D., 5 μm) was achieved within 10 min. The optimal applied potential of dihydroxybenzoic acids was set at 750 mV based on a hydrodynamic study. This method has the detection limits of 1.3 pmol/ml (or 0.2 ng/ml) for 2,3- and 2,5-dihydroxybenzoic acids in Ringer solution (at signal-to-noise ratio=3).

Keywords: Hydroxyl radical

1. Introduction

Hydroxyl radical, one of the most reactive and toxic oxygen metabolites, is formed via the Fenton reaction in living systems [1]. The half-life of hydroxyl radicals in cells though difficult to measure directly by conventional analytical techniques is estimated around 10^{-9} s. But, these hydroxyl radicals can be easily trapped by aromatic compounds [2,3]. Salicylic acid is widely used for this purpose because of its lack of toxicity, rapid reaction with hydroxyl radical, and ease in analysis of hydroxylation products [4–7].

A variety of hydroxyl radical adducts have been measured by high-performance liquid chromatography (HPLC) with ultraviolet [6], fluorescence [7], and electrochemical detection (ED) [8,9]. The spin trap coupled to electron paramagnetic resonance has not been proved to be a good method for the measurement of hydroxyl radical because of poor sensitivity, instability of the spin trap adducts and

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quenching in vivo [10,11]. In general salicylic acid is preferred for trapping as it reacts very rapidly with the hydroxyl free radical [12,13]. Recently, chemiluminescence [14] and liquid chromatography–electrochemical detection (LC–ED) coupled with on-line microdialysis [15] have been used for the measurement of 2,3- and 2,5-dihydroxybenzoic acids (DHBAs) in cell-free screening and in rat brain, respectively. Baseline levels of DHBAs in brain dialysates have been reported to in the range 0.3 to 30 μmol/ml. The detection limit for DHBAs, using conventional LC–ED is a few μmol/ml. This is sufficient for determinations of DHBAs in biological fluids and brain dialysates. However, DHBA levels in blood and some other compartments may be below the detection limit of conventional LC–ED [16]. In order to accommodate the low levels of DHBAs, continued efforts have been made to improve LC–ED in this laboratory. Microbore LC–ED has resulted in steady, encouraging progress and led to 40–200-fold enhancement in detection limits. In the present study, in vivo on-line microdialysis–microbore LC–ED was evaluated for the determination of DHBA levels in blood vessels.

2. Experimental

2.1. Chemicals and reagents

2,3-, 2,5-Dihydroxybenzoic acids, sodium salicylate and sodium octylsulfate were purchased from Sigma (St. Louis, MO, USA). Monochloroacetic acid, LC-grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Animals

Adult, male Sprague–Dawley rats (280–320 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and allowed to acclimatize to their environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least five days before the experiments. At the start of the experiments, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Supplements of sodium pentobarbital were given as and when needed throughout the experimental period.

2.3. Chromatography

The microbore LC system consisted of a chromatographic pump (BAS PM-80, Bioanalytical System, West Lafayette, IN, USA), an on-line injector (CMA/160, Stockholm, Sweden) equipped with a 10-μl sample loop and a BAS-4C amperometric detector (Bioanalytical System). DHBAs were separated using a microbore column (BAS, reversed-phase C_{18}, 150×1 mm I.D., particle size 5 μm) maintained at ambient temperature. The mobile phase was comprised of 0.1 *M* monochloroacetic acid, 10 m*M* EDTA, 0.5 m*M* sodium octylsulfate, 20% acetonitrile and 5% tetrahydrofuran in 1 l (pH 3.0 adjusted with 1 *M* NaOH), and the flow-rate was 0.05 ml/min. The buffer was filtered through a Millipore 0.22-μm filter and degassed prior to use. Various potentials (from 100 to 1000 mV) were applied to the glassy carbon working electrode and referenced to a Ag/AgCl electrode to determine optimal potentials for the determination of DHBAs. Fig. 1 shows hydrodynamic voltammograms of

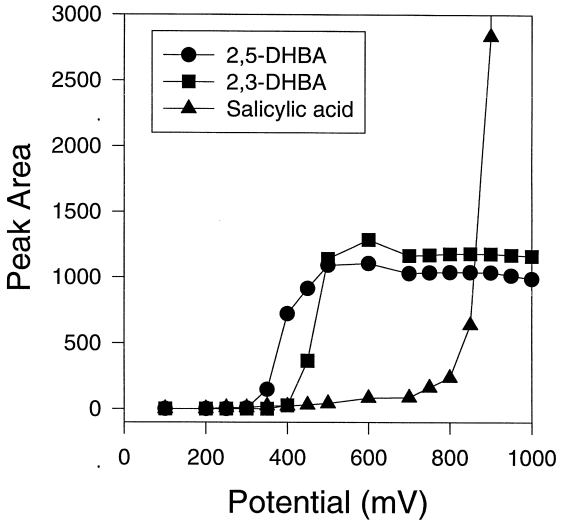

Fig. 1. Hydrodynamic voltammograms of salicylic acid, 2,3- and 2,5-dihydroxybenzoic acids in a standard mixture using microbore LC–ED.

salicylic acid, and 2,3- and 2,5-DHBAs as a function of applied potentials. The optimal potentials of DHBAs were between 700 and 750 mV. In the present study, the potential was set at 750 mV. Output signal from the LC–ED was recorded via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.4. Method validation

All calibration curves of DHBAs were made in standard mixtures (1, 5, 10, 50, 100 ng/ml, $n=3$) prior to the experiments with correlation values of at least 0.995. The intra- and inter-assay variabilities for DHBAs were determined (six replicates) at concentrations of 5, 10, 50 and 100 ng/ml on the same day and for six successive days, respectively. The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: bias (%)= $[(C_{obs}-C_{nom})/(C_{nom})]\times 100$. The precision relative standard deviation (RSD) was calculated from the observed concentrations as follows: % RSD= [standard deviation (SD)/C_{obs}]×100. Accuracy (% bias) and precision (% RSD) values of within ±15% were considered acceptable over this concentration range [17].

2.5. Analysis of hydroxyl radical

The formation of hydroxyl radical was quantified by its ability to convert salicylate (2-hydroxybenzoic acid) to 2,3-DHBA and 2,5-DHBA (Fig. 2). DHBAs were determined by microbore LC–ED as described earlier. The stock solutions of DHBAs (1 mg/ml) were prepared in 0.2 *M* perchloric acid containing 0.1 m*M* EDTA and 0.1 m*M* sodium metabisulfite. Stock solutions were stored at 4°C and are stable for several months [12].

2.6. Microdialysis experiment

The on-line microdialysis system (Fig. 3) consisted of a microinjection pump (CMA/100) and an on-line injector (CMA/160) [18,19]. Microdialysis probes were made of silica capillary and concentrically designed dialysis membrane (10 mm×150 μm O.D., with a cut-off at nominal molecular mass of

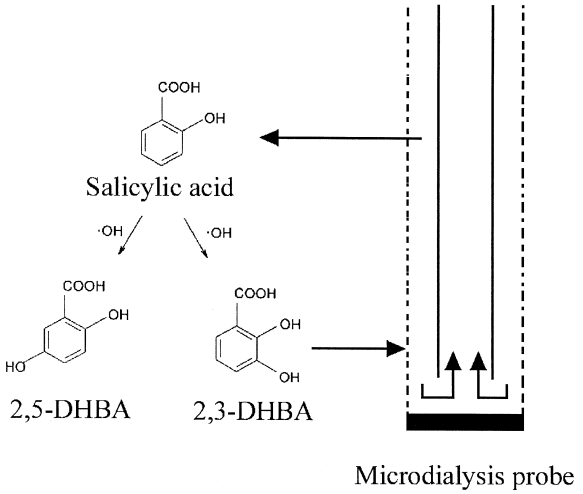

Fig. 2. Reaction of hydroxyl radicals with salicylate through the membrane of microdialysis probe to 2,3- and 2,5-dihyroxybenzoic acids.

13 000; Spectrum, Laguna Hills, CA, USA). Prior to the experiment, perfusate solution (147 m*M* NaCl, 4 m*M* KCl, and 2.3 m*M* $CaCl_2$ of Ringer's solution, pH 7.4) was degassed. Each microdialysis probe was perfused with degassed Ringer's solution for at least 40 min prior to use. A microdialysis probe was inserted into the jugular vein/right atrium (toward the heart) of an anesthetized rat and perfused with salicylate (0.5 m*M*) solution at a flow-rate of 1 μl/min using microinjection pump [20]. The body temperature of rat was maintained at 37°C with the help of a heating pad. Dialysates were collected for every 10-min interval (containing 10 μl of dialysate) into the on-line injector (CMA/160) and assayed with the microbore LC–ED system [21].

2.7. Recovery of DHBAs

For in vitro microdialysis recovery, blood microdialysis probes were calibrated in an Eppendorf vial (1.5 ml) containing DHBAs (C_{in}: 50 or 100 ng/ml) at a temperature of 25°C and 37°C. The in vitro recovery ratio ($R_{in\ vitro}$) of DHBAs across the microdialysis probe was calculated from the concentration in the dialysate (C_{out}) divided by the concentration of the spiked drug in the Eppendorf vial (C_{samp}) [21], that is: $R_{in\ vitro}=C_{out}/C_{samp}$.

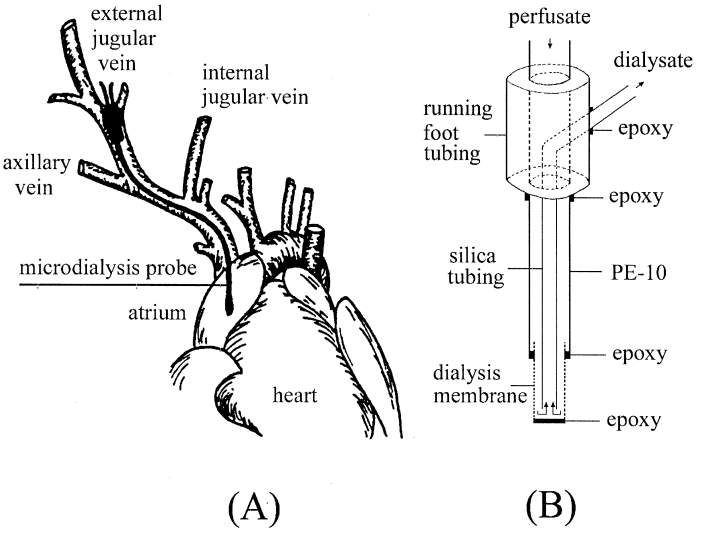

Fig. 3. Use of microdialysis probe in a rat blood vessel: (A) insertion of a microdialysis probe into rat jugular vein/right atrium, (B) the laboratory-made microdialysis probe.

3. Results and discussion

In the present study, a microbore liquid chromatographic method was used to determine DHBAs from rat jugular veins. Typical chromatograms of standard mixtures containing DHBAs (1, 10 and 100 ng/ml) are shown in Fig. 4. Separation of DHBAs from some endogenous chemicals in blood dialysates was achieved in an optimal mobile phase containing 0.1 *M* monochloroacetic acid, 10 m*M* EDTA, 0.5 m*M* sodium octylsulfate, 20% acetonitrile and 5% tetrahydrofuran (pH 3.0 adjusted with 1 *M* NaOH). Retention times for 2,5-DHBA and 2,3-DHBA were 5.4 and 6.9 min, respectively. Peak-areas of DHBAs were linear (r^2>0.995) over a concentration range from 1 to 100 ng/ml. Fig. 5A shows a typical chromatogram of a standard mixture containing 2,5-DHBA and 2,3-DHBA (5 ng/ml each). The baseline levels of 2,3- and 2,5-DHBAs in dialysate obtained from rat jugular vein were 1.1 and 8.7 ng/ml, respectively (Fig. 5).

DHBAs are not stable in Ringer's solution or solution with pH values higher than 7.0. However, DHBAs are stable in acidic solution in presence of antioxidants [12]. In order to preserve DHBAs, the stock solutions of DHBAs were prepared with 0.2 *M* perchloric acid, 100 μ*M* EDTA, and 100 μ*M* sodium metabisulfite [12]. Intra-assay (Table 1) and inter-assay (Table 2) precision and accuracy values for DHBAs fell well within predefined limits of acceptability. All % bias and % RSD values were within ±15%. This method has detection limits of 0.2

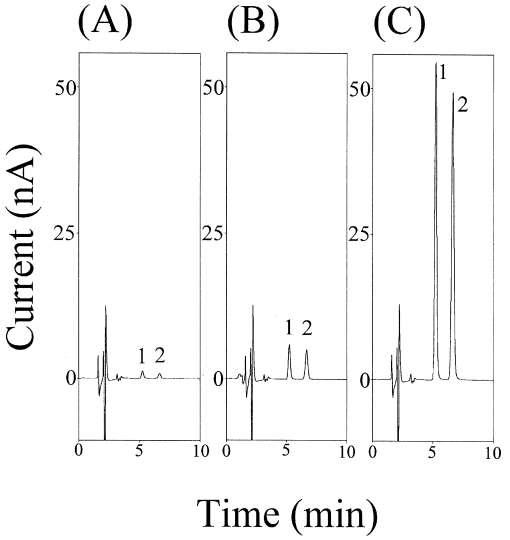

Fig. 4. Typical chromatograms of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid standards: (A) 1 ng/ml, (B) 10 ng/ml, (C) 100 ng/ml. Peaks: 1=2,5-dihydroxybenzoic acid; 2=2,3-dihydroxybenzoic acid.

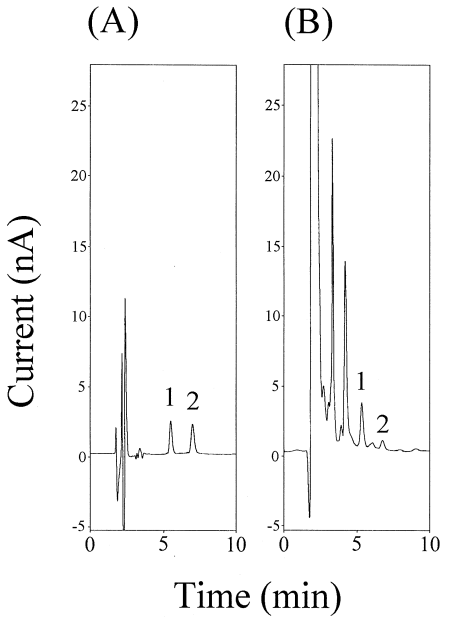

Fig. 5. Chromatograms of (A) 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid standards each 5 ng/ml; (B) the baseline levels of 2,3-dihydroxybenzoic acid (1.1 ng/ml) and 2,5-dihydroxybenzoic acid (8.7 ng/ml) in rat jugular vein/right atrium. Peaks: 1=2,5-dihydroxybenzoic acid; 2=2,3-dihydroxybenzoic acid.

ng/ml (or 1.3 pmol/ml) for 2,3- and 2,5-dihydroxybenzoic acids (at signal-to-noise ratio=3).

The in vitro recoveries of 2,3- and 2,5-DHBAs were measured at temperature of 25°C and 37°C (Table 3). Two different concentrations (50 and 100 ng/ml) of analytes dissolved in Ringer's solution were tested at each temperature. There were no significant differences between the same temperature but the recovery at 37°C was found to be higher than recovery at 25°C. Telting-Diaz et al. [22] studied the in vitro recovery of microdialysate using Ringer's solution and plasma, and found no significant difference in the tested solutions. Hence, calibration in the present study was only performed with Ringer's solution.

Sampling by microdialysis is based on the theory of passive diffusion that the microdialysis probe acts as a blood vessel with dialytic exchange of mainly small molecular substances with the surrounding tissues, but this method may be limited by its dialytic efficiency of microdialysis probe. For the determination of free radicals in biological samples, microdialysis offers many advantages over another in vivo method which uses iron-induced luminol chemiluminescence [14] such as continuous monitoring of analytes concentrations in the extracellular compartment in the same animals, less biological fluid loss and, therefore, minimal stress on hemodynamics [23]. The measurement of DHBAs using microdialysis and LC–ED was previously performed under cardiac nerve stimulation [23] and cerebral ischemic conditions in rats [24,25].

The present on-line microdialysis–microbore LC–ED method requires no pretreatment, provides high sensitivity, and enhances detection limits. As compared to the conventional LC–ED system, the microbore LC–ED has a number of practical advantages: (1) in general, the microbore system enhances the

Table 1
Intra- and inter-assay of accuracy and precision values for 2,3-DHBA

Nominal concentration (ng/ml)	Observed concentration[a] (ng/ml)	RSD (%)	Accuracy (% bias)
Intra-assay (n=6)			
5	5.47±0.53	9.7	9.4
10	10.84±0.28	2.6	8.4
50	47.28±0.32	0.7	−5.4
100	101.55±0.83	0.8	1.6
Inter-assay (n=6)			
5	4.96±0.56	11.3	−0.8
10	10.08±0.49	4.9	0.8
50	47.77±0.15	0.3	−4.6
100	102.57±1.62	1.9	2.6

[a] Observed concentration data are expressed as rounded means±SD.

Table 2
Intra- and inter-assay of accuracy and precision values for 2,5-DHBA

Nominal concentration (ng/ml)	Observed concentration[a] (ng/ml)	RSD (%)	Accuracy (% bias)
Intra-assay (n=6)			
5	4.97±0.2	4.0	−0.6
10	10.35±0.41	4.0	3.5
50	49.72±0.91	1.8	−0.6
100	100.53±0.86	0.9	0.5
Inter-assay (n=6)			
5	5.11±0.4	7.8	2.2
10	10.3±0.43	4.2	3
50	48.99±1.48	3.0	−2.0
100	100.44±0.68	0.7	0.4

[a] Observed concentration data are expressed as rounded means±SD.

detection ability over conventional packed columns LC–ED by at least 40–200-fold. (2) By decreasing the flow-rates from 1 ml/min down to 0.05 ml/min a dramatic reduction in waste of organic solvents and other chemicals can be achieved. (3) The total analytical time is within 10 min (Figs. 4 and 5) which allows dialysate load into the loop (10 μl) of the on-line injector. The sample injection volume (5 or 10 μl) has been tested and does not cause peak broadening. Several baseline values between 110 n*M* to 160 m*M* in microdialysates were reported by many other groups. These variations can be attributed to the concentrations of sodium salicylate (0.5–5.0 m*M*) used, the time delayed during injection of dialysates after collection (at once, 10–30 min, or on-line injection), contamination of interfering peaks, or LC column resolution. The detection limit of conventional LC–ED is often sufficient for determination of DHBAs in biological fluids and is probably difficult in brain dialysates at basal conditions. It might not be able to detect very low DHBAs levels in blood vessels or some other circumstances, which only generate very low levels of DHBAs. To the best of our knowledge, the present work provides the lowest detection ability to measure hydroxyl radical adducts. In summary, we have established a determination of DHBAs in the rat blood vessel using in vivo on-line microdialysis with microbore LC–ED. This present assay has versatile applications in vivo.

Table 3
Comparison of in vitro recovery rate at temperature 25°C and 37°C

	Recovery (%)[a]	
	25°C	37°C
2,3-DHBA		
50 ng/ml	32.3±0.6	48.0±3.7
100 ng/ml	35.6±0.7	50.6±5.0
2,5-DHBA		
50 ng/ml	35.6±0.7	50.0±3.0
100 ng/ml	37.1±0.7	50.5±4.3

[a] Data are expressed as mean±SD (n=6).

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References

[1] P.M. Reilly, J. Schiller, G.B. Bulkey, Am. J. Surg. 161 (1991) 488.
[2] S.R. Powell, D. Hall, Free Radic. Biol. Med. 9 (1990) 133.
[3] B. Halliwell, H. Kaur, M. Ingelman-Sundberg, Free Radic. Biol. Med. 10 (1991) 439.
[4] A.C. Blackburn, W.F. Doe, G.D. Buffinton, Free Radic. Biol. Med. 25 (1998) 305.
[5] S.R. Powell, Free Radic. Res. 21 (1994) 355.

[6] Z. Maskos, J.D. Rush, W.H. Koppenol, Free Radic. Biol. Med. 8 (1990) 153.
[7] D. Liu, J. Biochem. Biophys. Methods 27 (1993) 281.
[8] R.A. Floyd, J.J. Watson, P.K. Wong, J. Biochem. Biophys. Methods 10 (1984) 221.
[9] A. Giovanni, L.P. Liang, T.G. Hastings, M.J. Zigmond, J. Neurochem. 64 (1995) 1819.
[10] R.A. Floyd, Biochim. Biophys. Acta 756 (1983) 201.
[11] G. Takemura, T. Onodera, M. Ashraf, Circ. Res. 71 (1992) 96.
[12] D.R. McCabe, T.J. Maher, I.N. Acworth, J. Chromatogr. B 691 (1997) 23.
[13] J.M. Duine, F. Floch, C. Cann-Moisan, P. Mialon, J. Caroff, J. Chromatogr. B 716 (1998) 350.
[14] G. Yildiz, A.T. Demiryurek, J. Pharmacol. Toxicol. Methods 39 (1998) 179.
[15] C.S. Yang, P.J. Tsai, N.N. Lin, L. Liu, J.S. Kuo, Free Radic. Biol. Med. 19 (1995) 453.
[16] T. Obata, H. Hosokawa, T. Soeda, K. Karashima, Y. Uchida, Y. Yamanaka, Comp. Biochem. Physiol. 110 (1995) 277.
[17] R. Causon, J. Chromatogr. B 689 (1997) 175.
[18] T.H. Tsai, C.F. Chen, Neurosci. Lett. 166 (1994) 175.
[19] T.H. Tsai, C.F. Chen, J. Chromatogr. A 762 (1997) 269.
[20] T.H. Tsai, C.F. Chen, J. Chromatogr. A 730 (1997) 121.
[21] T.H. Tsai, F.C. Cheng, L.C. Hung, C.F. Chen, J. Chromatogr. B 720 (1998) 165.
[22] M. Telting-Diaz, D.O. Scott, C.E. Lunte, Anal. Chem. 64 (1992) 806.
[23] T. Obata, Y. Yamanaka, Neurosci. Lett. 211 (1996) 216.
[24] C.S. Yang, N.N. Lin, P.J. Tsai, L. Liu, J.S. Kuo, Free Radic. Biol. Med. 20 (1996) 245.
[25] A.A. Timoshin, O.V. Tskitishvili, L.I. Serebryakova, A.I. Kuzmin, O.S. Medvedev, E.K. Ruuge, Experientia 50 (1994) 677.