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Rapid high-throughput detection of peroxide with an acridinium-9-carboxamide: A homogeneous chemiluminescent assay for plasma choline

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Abstract—Hydrogen peroxide generated from the enzymatic oxidation of choline was detected using a chemiluminescent acridinium-9-carboxamide. The dose–response for choline (0–50 μM) was established in buffer and was applicable to the quantification of choline in human plasma. This homogeneous assay was performed in a 96-well microplate format and required minimal sample volume (1 μL) and analysis time (<5 s per well).

Competitive homogeneous chemiluminescent assays that rely on the signal modulation of acridinium-9-carboxamide-modified ligands upon binding to ligand-specific proteins have been previously reported. Examples have included biotin–avidin,[1] folic acid–folate binding protein,[2] vitamin B_{12}-intrinsic factor,[3] and most recently, boronate–glycoprotein.[4] In each case, the signal recorded from the unbound acridinium-9-carboxamide-labeled ligand is more intense than the bound species when triggered with a large excess of basic hydrogen peroxide.

In this communication, we present a different type of homogeneous chemiluminescent assay, one that is conceptually very simple, namely the acridinium-9-carboxamide mediated detection of hydrogen peroxide generated from an analyte-specific oxidase. This approach was demonstrated using choline/choline oxidase and resulted in an assay for choline in human plasma.

Choline oxidase (ChO, *Alcaligenes* sp.) in the presence of oxygen converts choline **1** to betaine aldehyde **2**, then finally to betaine **3**, with the production of 2 equiv of hydrogen peroxide (Scheme 1).[5] The peroxide thus generated reacts with acridinium-9-carboxamide **4** under basic conditions giving rise to a strained dioxetanone intermediate that decomposes to the acridone **5** with the emission of light.

When light from acridinium-9-carboxamide **4**[6] is triggered with a large excess of basic peroxide, a 'flash' chemiluminescence profile is observed (Fig. 1). The chemiluminescence response from the enzymatic conversion of choline to betaine and hydrogen peroxide produced a different profile that was dependent on the delay time between the addition of the oxidase enzyme and acridinium-9-carboxamide **4** (Fig. 2). Choline bitartrate (1 μL, 50 μM) was distributed in a black round-bottomed polystyrene 96-well microplate, and using a Berthold Mithras microplate reader equipped with three reagent injectors, each well was analyzed separately for 2 s after the addition of choline oxidase (10 μL, 10 U/mL), a delay time of 1–20 s, then addition of acridinium-9-carboxamide **4** (10 μL, 4 μM) and aqueous sodium hydroxide (30 μL, 0.25 N). As the delay time lengthened from 1 to 20 s, the chemiluminescence profiles changed from 'pseudo-flash' to 'glow.' Regardless of the delay time, the chemiluminescent signal required approximately 10 min to decay to background (data not shown). The intensity of the response rose to a maximum at a delay time of 2 s (120,000 RLU) and then decreased by two-thirds (40,000 RLU) after 20 s. The signal intensity was not further diminished for delay times up to 20 min (data not shown).

The chemiluminescence profiles over range of choline concentrations (1 μL, 0–50 μM) using a delay time of

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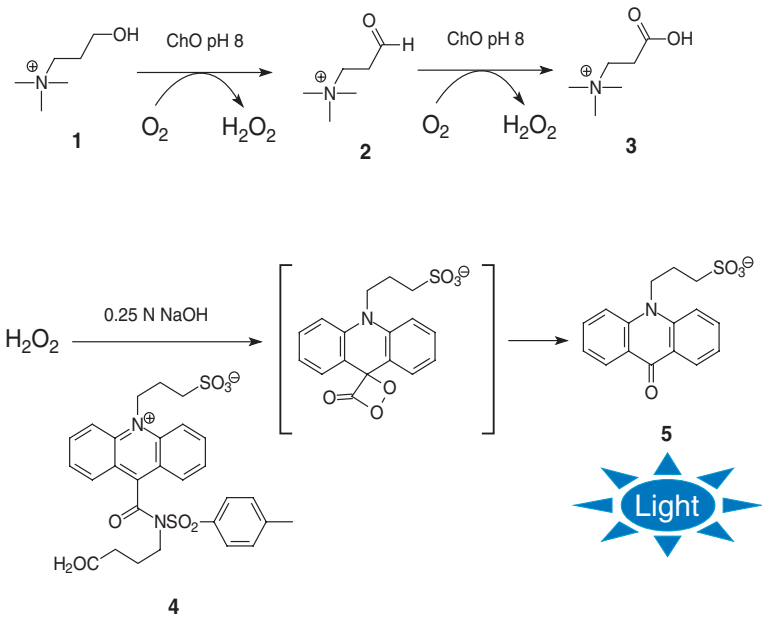

Scheme 1. Conversion of choline to betaine and hydrogen peroxide.

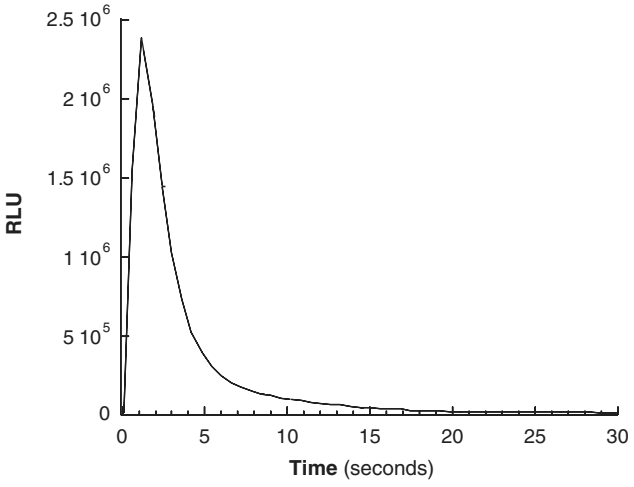

Figure 1. 'Flash' chemiluminescence profile. Conditions: 0.18 N NaOH, 0.7% H_2O_2, 1% Triton X-100, and 0.05% diethylenetriaminepentacetic acid.

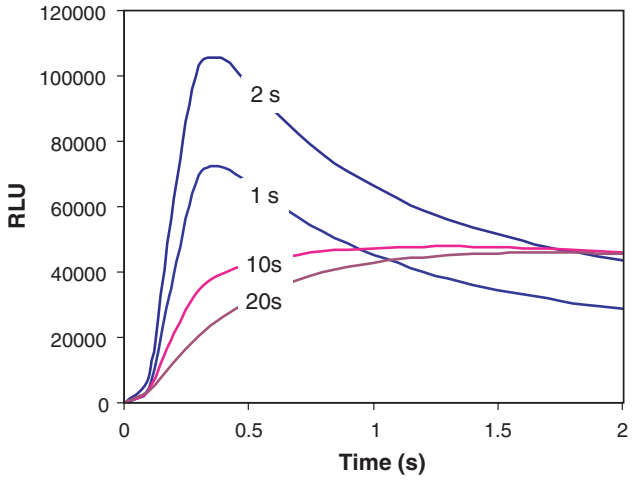

Figure 2. Effect of delay time on chemiluminescence response. Sample: choline (1 μL, 50 μM, pH 8, 0.2 M sodium phosphate). Berthold Mithras microplate luminometer analysis sequence: (a) add choline oxidase solution (10 μL, 10 U/mL, pH 8, 0.2 M sodium phosphate, 0.1% sodium cholate,); (b) 1–20 s delay; (c) add **4** (10 μL, 4 μM in water, 0.1% sodium cholate); (d) add sodium hydroxide (30 μL, 0.25 N); (e) read for 2 s.

2 s are shown in Figure 3. A chemiluminescence dose–response curve was constructed by plotting the maximum relative light units (RLU_{max}) versus choline concentration (Fig. 4).

Having demonstrated a dose–response, we next investigated the detection of hydrogen peroxide generated from the action of choline oxidase on choline in human plasma.

The importance of choline **1** as an analyte has been emphasized by its identification as an essential human nutrient with physiological activity in phospholipid, cholinergic, and methionine metabolism.[7] It has been investigated as a biomarker during pregnancy and in neonates,[8–11] end stage renal disease,[12] neurological disorders,[13–15] and ischemia.[16–21] The normal mean concentration of choline in plasma is 10 μM (range 7–20 μM)[7] (28–35 μM, newborns)[8,10] and rises 3- to 4-fold under pathological conditions.

While chemiluminescent assays for choline from plasma samples are known, they have most often been reported using choline oxidase (ChO) coupled with luminol and a second enzyme, horseradish peroxidase (HPRO)[22,23,13] or a metal catalyst.[24,23,25] With the exception of Das et al.,[13] all employed immobilized enzyme(s). Recently, de Silva et al.[26] demonstrated that an acridan could replace luminol as the HRPO substrate. Others have approached the analysis using a flow injection method

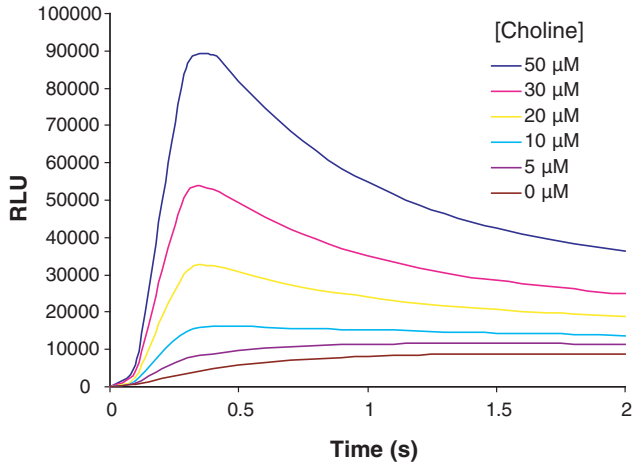

Figure 3. Choline-dependent chemiluminescence response. Sample: choline bitartrate (1 μL, 0–50 μM, pH 8, 0.2 M sodium phosphate). Berthold Mithras microplate luminometer analysis sequence: (a) add choline oxidase solution (10 μL, 10 U/mL, pH 8, 0.2 M sodium phosphate, 0.1% sodium cholate,); (b) 2 s delay; (c) add **4** (10 μL, 4 μM in water, 0.1% sodium cholate); (d) add sodium hydroxide (30 μL, 0.25 N); (e) read for 2 s.

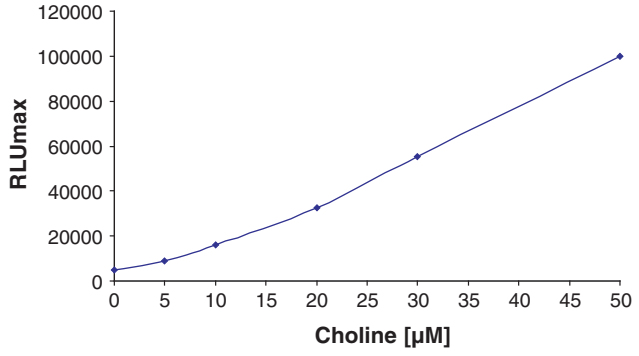

Figure 4. Choline dose–response.

based on immobilized enzymes and peroxyoxalate chemiluminescence.[27]

Akin to assays for choline, 9-acridinecarbonylimidazole,[28] and phenoxy-substituted acridinium esters[29] were used to detect hydrogen peroxide generated from glucose oxidase, while 10-methyl-9-(*p*-formylphenyl)-acridinium carboxylate trifluoromethanesulfonate[30] was used to quantitate peroxide in natural waters.

We found that the homogeneous choline oxidase/acridinium-9-carboxamide system rapidly detects choline in plasma directly without any sample extraction, additional enzymes or catalysts. Frozen normal donor plasmas ($N = 50$) from the Abbott Specimen Collection Bank that had been collected on lithium heparin were analyzed in triplicate and the results calculated from a point-to-point fit of the dose–response curve using MikroWin 2000 on the Berthold Mithras. The distribution of results is shown in Figure 5. The mean concentration of this normal population was 11.97 ± 2.83 μM, which is in agreement with the expected values from the literature.[7]

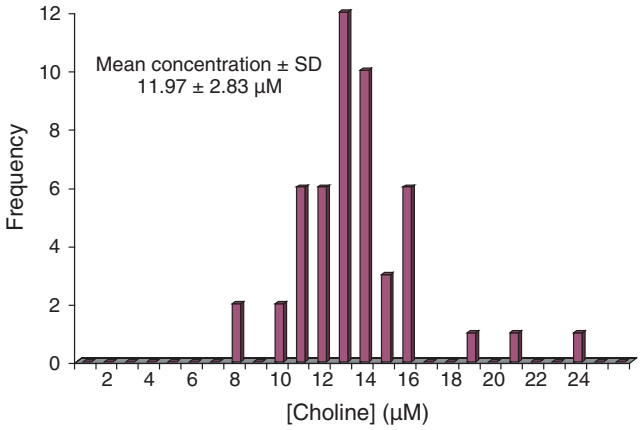

Figure 5. Analysis of normal donor plasma. Standards: choline bitartrate (1 μL, 0–50 μM, pH 8, 0.2 M sodium phosphate). Samples: human plasma collected in lithium heparin tubes (1 μL). Berthold Mithras microplate luminometer analysis sequence: (a) add choline oxidase solution (10 μL, 10 U/mL, pH 8, 0.2 M sodium phosphate, 0.1% sodium cholate,); (b) 2 s delay; (c) add **4** (10 μL, 4 μM in water, 0.1% sodium cholate); (d) add sodium hydroxide (30 μL, 0.25 N); (e) read for 2 s.

The application of the homogeneous chemiluminescent detection of oxidase-generated peroxide to other sample matrices and analytes is underway.

References and notes

1. Adamczyk, M.; Mattingly, P. G.; Moore, J. A.; Pan, Y. *Org. Lett.* **2003**, *5*, 3779.
2. Adamczyk, M.; Fino, J. R.; Mattingly, P. G.; Moore, J. A.; Pan, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2313.
3. Adamczyk, M.; Johnson, D. D.; Mattingly, P. G.; Moore, J. A.; Pan, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3917.
4. Adamczyk, M.; Chen, Y.-Y.; Johnson, D. D.; Mattingly, P. G.; Moore, J. A.; Pan, Y.; Reddy, R. E. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1324.
5. Ohta-Fukuyama, M.; Miyake, Y.; Emi, S.; Yamano, T. *J. Biochem.* **1980**, *88*, 197.
6. Adamczyk, M.; Chen, Y.-Y.; Mattingly, P. G.; Pan, Y. *J. Org. Chem.* **1998**, *63*, 5636.
7. Zeisel, S. H. *Nutrition* **2000**, *16*, 669.
8. Ilcol, Y. O.; Ozbek, R.; Hamurtekin, E.; Ulus, I. H. *J. Nutr. Biochem.* **2005**, *16*, 489.
9. Velzing-Aarts, F. V.; Holm, P. I.; Fokkema, M. R.; van der Dijs, F. P.; Ueland, P. M.; Muskiet, F. A. *Am. J. Clin. Nutr.* **2005**, *81*, 1383.
10. Buchman, A. L.; Sohel, M.; Moukarzel, A.; Bryant, D.; Schanler, R.; Awal, M.; Burns, P.; Dorman, K.; Belfort, M.; Jenden, D. J.; Killip, D.; Roch, M. *Nutrition* **2001**, *17*, 18.
11. Ilcol, Y. O.; Uncu, G.; Ulus, I. H. *Arch. Physiol. Biochem.* **2002**, *110*, 393.
12. Ilcol, Y. O.; Dilek, K.; Yurtkuran, M.; Ulus, I. H. *Clin. Biochem.* **2002**, *35*, 233.
13. Das, I.; de Belleroche, J.; Moore, C. J.; Rose, F. C. *Anal. Biochem.* **1986**, *152*, 178.
14. Miller, B. L.; Read, S.; Tang, C.; Jenden, D. *Neurobiol. Aging* **1991**, *12*, 61.
15. Jope, R. S.; Wright, S. M.; Jenden, D. J. *Psychopharmacol. Bull.* **1984**, *20*, 674.

16. Kawada, T.; Yamazaki, T.; Akiyama, T.; Shishido, T.; Mori, H.; Sugimachi, M. *Acta Physiol. Scand.* **2005**, *184*, 187.
17. Apple, F. S.; Wu, A. H.; Mair, J.; Ravkilde, J.; Panteghini, M.; Tate, J.; Pagani, F.; Christenson, R. H.; Mockel, M.; Danne, O.; Jaffe, A. S. *Clin. Chem.* **2005**, *51*, 810.
18. Bruhl, A.; Hafner, G.; Loffelholz, K. *Life Sci.* **2004**, *75*, 1609.
19. Adams, J. *Curr. Cardiol. Rep.* **2004**, *6*, 253.
20. Korth, U.; Krieter, H.; Denz, C.; Janke, C.; Ellinger, K.; Bertsch, T.; Henn, C.; Klein, J. *Resuscitation* **2003**, *58*, 209.
21. Danne, O.; Mockel, M.; Lueders, C.; Mugge, C.; Zschunke, G. A.; Lufft, H.; Muller, C.; Frei, U. *Am. J. Cardiol.* **2003**, *91*, 1060.
22. Kiba, N.; Ito, S.; Tachibana, M.; Tani, K.; Koizumi, H. *Anal. Sci.* **2003**, *19*, 1647.
23. Rauch, P.; Ferri, E. N.; Girotti, S.; Rauchova, H.; Carrea, G.; Bovara, R.; Fini, F.; Roda, A. *Anal. Biochem.* **1997**, *245*, 133.
24. Yao, D.; Vlessidis, A. G.; Evmiridis, N. P. *Anal. Chim. Acta* **2002**, *462*, 199.
25. Yaqoob, M.; Nabi, A.; Masoom-Yasinzai, M. *J. Biolumin. Chemilumin.* **1997**, *12*, 135.
26. de Silva, R.; Handley, R. S.; Schaap, A. P.; Akhavan-Tafti, H. Use of new chemiluminescent reagent in detection of oxidase enzymes and their substrates by a coupled enzyme reaction, 13th Int. Symposium on Bioluminescence and Chemiluminescence, August 2–6, 2004, Yokohama, Japan, 2004.;
27. Wada, M.; Nakashima, K.; Kuroda, N.; Akiyama, S.; Imai, K. *J. Chromatogr., B* **1996**, *678*, 129.
28. Waldrop, A. A.; Fellers, J.; Vary, C. P. H. *Luminescence* **2000**, *15*, 169.
29. Brown, R. C.; Weeks, I.; Fisher, M.; Harbron, S.; Taylorson, C. J.; Woodhead, J. S. *Anal. Biochem.* **1998**, *259*, 142.
30. Cooper, W. J.; Moegling, J. K.; Kieber, R. J.; Kiddle, J. J. *Marine Chem.* **2000**, *70*, 191.