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AN AROMATIC HYDROXYLATION ASSAY FOR HYDROXYL RADICALS UTILIZING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC). USE TO INVESTIGATE THE EFFECT OF EDTA ON THE FENTON REACTION

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A highly sensitive HPLC method for the separation of hydroxylation products derived from the attack of hydroxyl radical upon phenol is described. Catechol and hydroquinone are the major hydroxylation products formed, with little resorcinol. The effect of EDTA upon hydroxyl radical generation from an iron (II)-H_2O_2 system is shown to depend upon the order of addition of chelator and metal ion to the reaction mixture, the ratio [iron salt]/[chelator] and the presence or absence of a phosphate buffer. Reasons for these different effects are discussed.

Key words: phenol; aromatic hydroxylation; Fenton reaction; hydrogen peroxide; iron; cobalt; titanium; EDTA.

INTRODUCTION

Superoxide dismutase, an enzyme specific for the O_2^- radical as substrate, is an important member of the arsenal of antioxidants necessary for life in the presence of oxygen[1,2]. The function of superoxide dismutase implies that the O_2^- radical must be a damaging species *in vivo*, yet O_2^- is poorly reactive in aqueous solution. Many of its effects are probably due to O_2^--dependent formation of more reactive species, of which special attention has been given to the hydroxyl radical[2,3]. Superoxide-dependent formation of hydroxyl radicals requires the presence of metal catalysts and the hydroxyl radical appears to arise mainly by a direct reaction between a metal ion in a lower oxidation state and H_2O_2. Metal-ion catalysts of ·OH production from H_2O_2 include Fe(II), Cu(I), Co(II) and Ti(III)[3,4,5,6].

Formation of hydroxyl radicals in biochemical systems has been studied by a number of techniques, including the ability of ·OH to oxidise methional and related compounds into ethene[7], degrade tryptophan[8], convert dimethylsulphoxide into formaldehyde and methane[9], decarboxylate benzoic acid[10] and oxidise deoxyribose into thiobarbituric-acid-reactive material[11]. However, the two methods with the

greatest potential for *specifically* identifying hydroxyl radical are esr spin-trapping[12] and aromatic hydroxylation[13]. Hydroxyl radicals produce a characteristic pattern of hydroxylation of aromatic ring structures[13].

The principle of the aromatic hydroxylation assay is to include an aromatic compound in the reaction mixture and measure the formation of hydroxylated products[14]. This can be done semiquantitatively, with salicylate as a detector molecule, by using a colorimetric method[15] or quantitatively (with phenol as detector molecule) using gas-liquid chromatography (GLC) to separate derivatized hydroxylation products[15,16]. The use of aromatic hydroxylation as an assay for hydroxyl radical has produced results comparable with those of spin-trapping experiments in the hypoxanthine-xanthine oxidase system[12,15], the NADH-phenazine methosulphate system[17,18,19] and thiol-H_2O_2 systems[20,21,22]. However, the tedious derivatization procedure required before GLC analysis of hydroxylated metabolites[15,16] has probably precluded more general use of this method. High-performance liquid chromatography (HPLC), which requires no product derivatization, is a much superior way of analysing hydroxylation products[5,23]. In the first part of this paper we describe an HPLC technique combined with electrochemical detection for the measurement of hydroxylation products derived from phenol.

Chelation of iron to EDTA affects the reactivity of iron[3,11] in superoxide-dependent or ascorbate-dependent systems producing ·OH, but the precise effect produced depends upon the order in which iron and EDTA are added to the reaction mixture and the nature of the buffer, if any, present in the reaction mixture[11,24,25,26,27]. Chelation of Fe(III) to EDTA facilitates its reduction by O_2^{-}[29] or ascorbate. In the second part of this paper we have used the aromatic hydroxylation technique to investigate the effects of EDTA on the Fenton reaction itself.

MATERIALS AND METHODS

Reagents

Phenol, catechol, resorcinol and hydroquinone were from Aldrich. HPLC solvents (including water) and other reagents were of the highest quality available from BDH Chemicals Ltd. Solutions of H_2O_2 were made up fresh when required. Standard solutions of hydroquinone, resorcinol, catechol and phenol were prepared in 0.10M HCl/KCl buffer pH 2.0 and stored in the refrigerator for a period not exceeding one week.

Aromatic hydroxylation assay

An HPLC Technology Ltd Spherisorb 5 ODS column (25 cm × 4.6 mm) was used to separate the hydroxylation products obtained from phenol. The mobile phase was 82% (v/v) 20 mM sodium citrate buffer pH 5.0 and 18% (v/v) methanol at a flow rate of 1.1 ml/min, continuously sparged with helium during elution of hydroxylation products. These were detected with an EDT model LCAI5 electrochemical detector equipped with a glassy carbon working electrode. An oxidation potential of +1.1 volts against an Ag/AgCl reference electrode was selected to obtain maximum current response and minimum background noise for the hydroxylation products of phenol.

RESULTS

Separation of hydroxylation products

Figure 1 shows that the HPLC technique achieved a clear separation of the three hydroxylation products

catechol (1,2 dihydroxybenzene)

resorcinol (1,3 dihydroxybenzene)

and hydroquinone (1,4 dihydroxybenzene)

from phenol. The electrochemical detection method was extremely sensitive. A solution containing less than 10^{-9} mol/dm^3 of hydroxylation product could be analysed, i.e. less than 2×10^{-14} moles of product per 20 μl sample injected into the column. For example, it was possible to measure the concentration of aromatic compounds in Central London tap water: one sample contained 20 μM phenol, 2 μM catechol and 1–2 μM hydroquinone, although these values varied widely. Even greater sensitivity could be achieved by acidifying reaction mixtures and extracting hydroxylated products into HPLC-grade ether to concentrate them, gently evaporating the ether in a water bath at 40°C and dissolving the residue in a small amount of the mobile phase.

The response of the electrochemical detector was linear over a wide concentration range, up to at least 10^{-4} moles/dm^3 (Figure 2), for all the hydroxylation products. The detector was calibrated at the beginning of each day's experiments. The usual retention times were hydroquinone 4.5 min, resorcinol 6.4 min, catechol 7.6 min and phenol 14.4 min under the conditions given and these times varied by less than 5% on different days.

Phenol hydroxylation by an Fe(II)-H_2O_2 system: the effect of phosphate and EDTA

The use of a phosphate buffer introduces complexities into studies of iron-dependent radical reactions[24], but it can be justified because intracellular metal ions will exist in the presence of phosphate ion at concentrations in the high millimolar range. It should also be pointed out that buffers such as Tris[30] and Hepes[31] are powerful scavengers of ·OH radicals.

Table I shows the effect of varying concentrations of phosphate buffer, pH 7.4, on the generation of ·OH from Fe(II) and H_2O_2, both in the presence and in the absence of EDTA. In the absence of EDTA, addition of phosphate decreased the formation of hydroxylated products to an extent increasing with phosphate concentration. In the presence of EDTA in two-fold excess over the iron, low concentrations of phosphate increased the formation of hydroxylated products but higher concentrations caused a decrease. It may also be seen from Table I (last column) that the presence of EDTA always increased the formation of hydroxylated products, but to a much greater extent in the presence of phosphate buffer than in the unbuffered system.

In these experiments iron(II) was added to the reaction mixture last. If the EDTA was pre-mixed with the iron(II) salt and then added to the reaction mixture, the amounts of hydroxylated product obtained were reduced by 90% or more (data not shown).

Table II shows the effect of varying iron(II)-EDTA ratios on the formation of hydroxylated products at a fixed concentration of phosphate buffer (20 mM). The

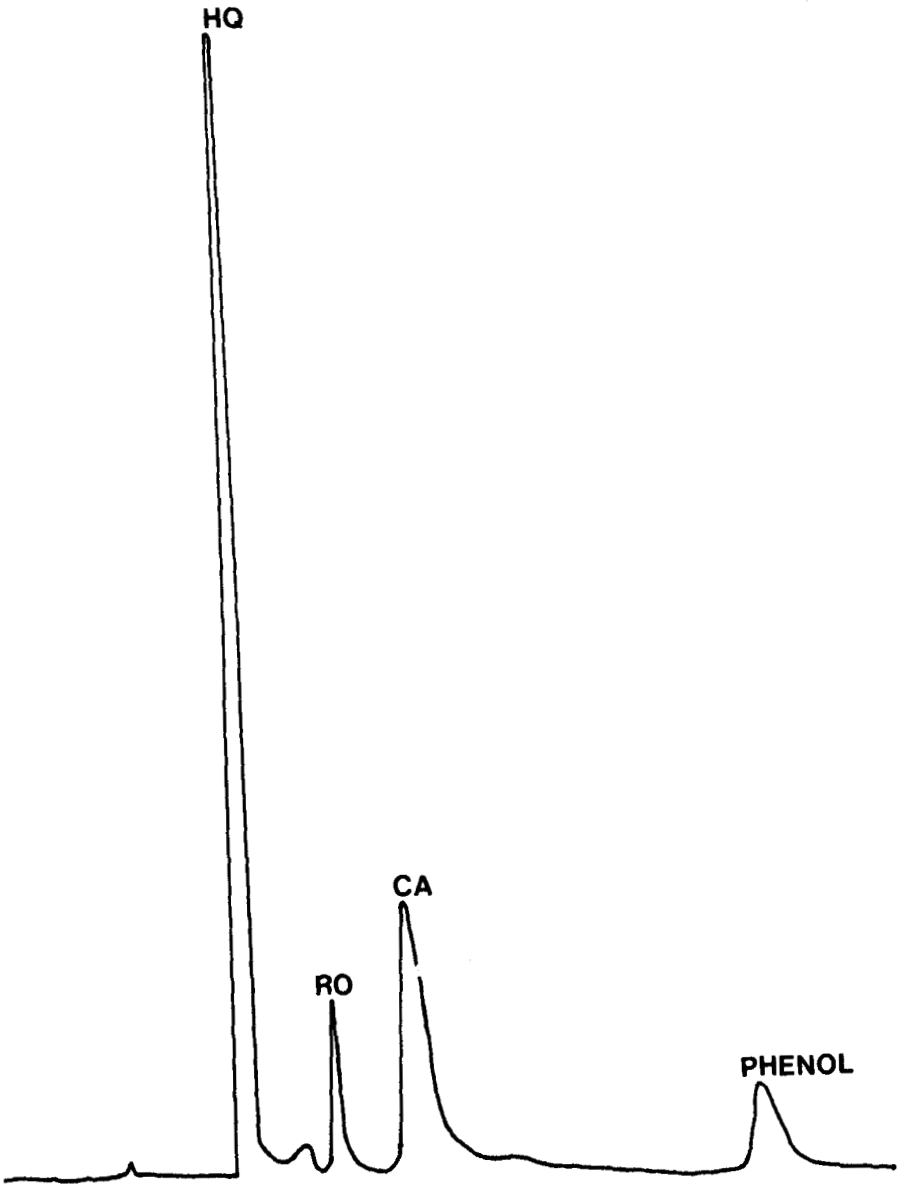

FIGURE 1 HPLC separation and electrochemical detection of dihydroxyphenols.

A 20 μl sample of a standard mixture of phenols was injected into the HPLC system and the figure shows the clear separation achieved. The retention times were (in min) hydroquinone 4.5, resorcinol 6.4, catechol 7.6 and phenol 14.4 under the conditions described. Abbreviations: catechol CA; resorcinol RO; hydroquinone HQ.

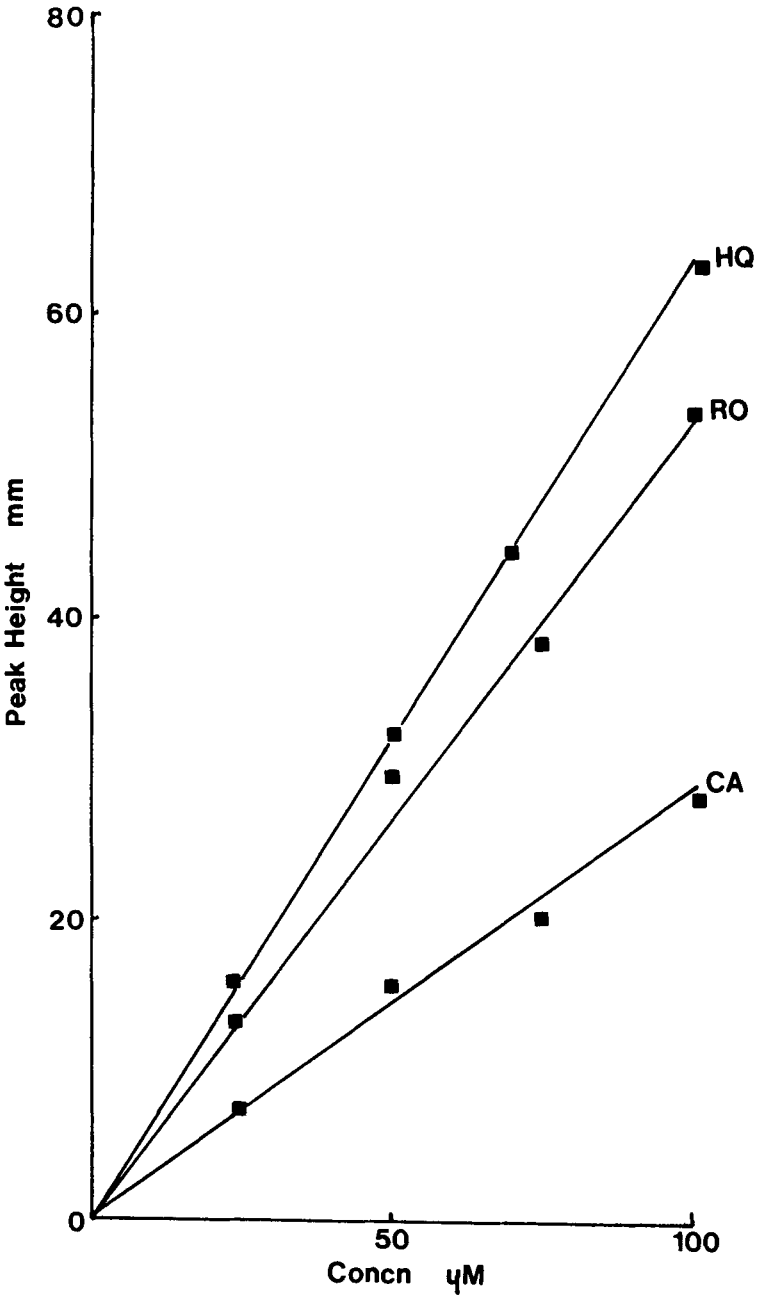

FIGURE 2 Quantitative detection of hydroxylated phenols.
The response of the electrochemical detector (measured as peak height on the chart recorder) was linear up to at least 100 μM for catechol (CA), resorcinol (RO) and hydroquinone (HQ).

TABLE I
Hydroxylation of phenol by Fenton's reagent. The effect of phosphate buffer and EDTA.

Concn. of phosphate buffer (mM)	Hydroxylated products: EDTA present			EDTA absent			% stimulation by EDTA
	C	H	Total	C	H	Total	
0	123	57	180	79	60	139	29
4	170	170	340	48	46	94	262
8	189	166	355	36	44	80	343
12	150	75	225	26	37	63	257
16	90	24	114	21	26	47	143
20	73	16	89	8	6	14	536

Reaction mixtures contained, in a final volume of 300 μl, the following reagents added in the order stated to give the final concentrations in brackets; KH_2PO_4-KOH buffer pH 7.4 (if present, concentration variable); EDTA (2 mM); phenol (1 mM); H_2O_2 (3.3 mM); iron(II) salt as $FeSO_4$ made up immediately before use in deaerated water (1 mM). Reaction mixtures were incubated at 37°C for 5 min and a 20 μl sample injected into the HPLC system. Results are presented as the concentrations of catechol (C) and hydroquinone (H) present in the reaction mixtures after incubation, expressed in μM. No significant amount of resorcinol was produced.

TABLE II
Hydroxylation of phenol by Fenton's reagent under physiological conditions.

Concentration of EDTA (mM) \ Concentration of iron (mM)	0.5	0.75	1.0	1.25	1.50
0.5	C 38 H 11 a	C 113 H 20	C 134 H 26	C 124 H 40	C 110 H 29
0.75	C 60 H 13	C 59 H 11 b	C 45 H 9	C 100 H 15	C 60 H 12
1.00	C 90 H 12	C 73 H 10	C 105 H 25 c	C 116 H 25	C 79 H 24
1.25	C 44 H 8	C 54 H 7	C 77 H 19	C 92 H 35 d	C 68 H 16
1.50	C 66 H 10	C 72 H 17	C 72 H 13	C 65 H 17	C 119 H 24 e

Reaction mixtures contained, in a final volume of 300 μl, the following reagents added in the order stated to give the final concentrations in brackets; KH_2PO_4-KOH buffer pH 7.4 (20 mM); EDTA (concentration as above); phenol (1 mM); H_2O_2 (3.3 mM); iron(II) salt, as $FeSO_4$ made up immediately before use in deaerated water (variable). Reaction mixtures were incubated at 37°C for 5 min and a 20 μl sample injected into the HPLC system. Results are presented as the concentrations of catechol (C) and hydroquinone (H) present in the reaction mixtures after incubation, expressed in μM. No significant amount of resorcinol was produced.

diagonal column (marked a,b,c,d,e) shows that, with equimolar EDTA and iron salt present, the amount of hydroxylation reached a maximum at about 1.00 mM. The variation of hydroxylation with iron salt: EDTA ratio was complex and no clear conclusions could be drawn (Table II).

DISCUSSION

In this paper we have described the further development of our "aromatic hydroxylation" technique[14,15,16] for ·OH by employment of HPLC separation combined with highly-sensitive electrochemical detection. While this work was in progress, two other groups described broadly-similar methods.[23,28] In these studies we have employed phenol as a detector molecule, but work is continuing to determine the pattern of hydroxylation products obtained from other aromatic detector molecules, such as salicylate[14,16] and 4-nitrophenol.[32] Formation of catechol and hydroquinone, with little resorcinol, by the action of a Fenton system on phenol suggests that the hydroxylating species is indeed ·OH[33], since pulse radiolysis studies[13] give a similar pattern.

There has been much debate in the literature over the way in which metal chelating agents affect the Fenton reaction[24,25,26,27] and/or the ability of metal ions to be reduced by O_2^-, ascorbate and NADPH cytochrome P_{450} reductase. The presence of phosphate buffer complicates these studies[24], but is necessary to simulate physiological conditions. Studies performed using Tris[24] are irrelevant physiologically and confusing in view of the radical-scavenging ability of this buffer.[30]

In the present paper we have examined the ways in which EDTA can affect the Fenton reaction. Firstly, pre-mixing of Fe(II) and H_2O_2 greatly decreases production of ·OH. This is not surprising, since the Fe(II)-EDTA complex oxidizes extremely quickly and by the time it is added to the reaction mixture, most will have formed Fe(III)-EDTA.[34,11]

In the absence of phosphate buffer, EDTA can have two opposing effects in the Fenton system. Firstly, it alters the redox potential of the iron to facilitate reaction with H_2O_2. Secondly, it itself scavenges[33] ·OH with k_2 of 2.76×10^9 $l.mol^{-1}$ s^{-1}. Table I shows that the former effect appeared to predominate under the reaction conditions used.

Addition of phosphate buffer to an iron(II)-H_2O_2 system decreases aromatic hydroxylation, presumably by forming unreactive iron-phosphate complexes.[25] In the presence of phosphate buffer, the percentage stimulatory effects of EDTA (Table I) on ·OH generation are greatly increased, probably because the ability of EDTA to maintain iron salts in solution is now an important part of its overall effect. These results, combined with those in Table 2 and reference 25, show that no single explanation[24] of the effect of chelating agents on metal-dependent formation of ·OH in biological systems is adequate.

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References

1. J. Di Guiseppi and I. Fridovich, *CRC Crit. Rev. Toxicol.*, **12**, 315-342, (1984).
2. B. Halliwell and J.M.C. Gutteridge, (1985) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford.
3. B. Halliwell and J.M.C. Gutteridge, *Biochem. J.*, **219**, 1-14, (1984).
4. D.A. Rowley and B. Halliwell, *Arch. Biochem. Biophys.*, **225**, 279-284, (1983).
5. C.P. Moorhouse, B. Halliwell, M. Grootveld and J.M.C. Gutteridge, *Biochim. Biophys. Acta* in press, (1985).

6. C.R.E. Jefcoate and R.O.C. Norman, *J. Chem. Soc.*, (B) 48–53, (1968).
7. C. Beauchamp and I. Fridovich, *J. Biol. Chem.*, **245**, 4641–4646, (1970).
8. J.M. McCord and E.D. Day, *FEBS Lett.*, **86**, 139–142, (1978).
9. S.M. Klein, G. Cohen and A.I. Cederbaum, *Biochemistry*, **20**, 6006–6012, (1981).
10. A.L. Sagone, M.A. Decker, R.M. Wells and C. De Mocko, *Biochim. Biophys. Acta*, **628**, 90–102, (1980).
11. B. Halliwell and J.M.C. Gutteridge, *FEBS Lett.*, **128**, 347–352, (1981).
12. E. Finkelstein, G.M. Rosen and E.J. Rauckman, *Mol. Pharmacol.*, **16**, 676–685, (1979).
13. N.V. Raghavan and S. Steenken, *J. Am. Chem. Soc.*, **102**, 3495–3499, (1980).
14. B. Halliwell, *FEBS Lett.*, **92**, 321–326, (1978).
15. R. Richmond, B. Halliwell, J. Chauhan and A. Darbre, *Anal. Biochem.*, **118**, 328–335, (1981).
16. R. Richmond and B. Halliwell, *J. Inorg. Biochem.*, **17**, 95–107, (1982).
17. B. Halliwell, *Biochem. J.*, **167**, 317–320, (1977).
18. H.W. Richter, M.A. Fetrow, R.E. Lewis and W.H. Waddell, *J. Am. Chem. Soc.*, **104**, 1666–1671, (1982).
19. G. Davis and P.J. Thornalley, *Biochim. Biophys. Acta*, **724**, 456–465, (1983).
20. D.A. Rowley and B. Halliwell, *FEBS Lett.*, **138**, 33–36, (1982).
21. A.J.F. Searle and A. Tomasi, *J. Inorg. Biochem.*, **17**, 161–166, (1982).
22. G. Saez, P.J. Thornalley, H.A.O. Hill, R. Hems and J.V. Bannister, *Biochim. Biophys. Acta*, **719**, 24–31, (1982).
23. D.M. Radzik, D.A. Roston and P.T. Kissinger, *Anal. Biochem.*, **131**, 458–464, (1983).
24. E. Graf, J.R. Mahoney, R.G. Bryant and J.W. Eaton, *J. Biol. Chem.*, **259**, 3620–3624, (1984).
25. W. Flitter, D.A. Rowley and B. Halliwell, *FEBS Lett.*, **158**, 310–312, (1983).
26. H. Rosen and S.J. Klebanoff, *Arch. Biochem. Biophys.*, **208**, 512–519, (1981).
27. G.W. Winston, D.E. Feierman and A.I. Cederbaum, *Arch. Biochem. Biophys.*, **232**, 378–390, (1984).
28. R.A. Floyd, J.J. Watson and P.K. Wong, *J. Biochem. Biophys. Meth.*, **10**, 221–235, (1984).
29. J. Butler and B. Halliwell, *Arch. Biochem. Biophys.*, **218**, 174–178, (1982).
30. W. Paschen and U. Weser, *Hoppe-Seylers Z. Physiol. Chem.*, **356**, 727–737, (1975).
31. B. Halliwell, Unpublished results.
32. T.M. Florence, *J. Inorg. Biochem.*, **22**, 221–230, (1984).
33. C. Walling, *Acc. Chem. Res.*, **8**, 125–131, (1975).
34. D.C. Harris and P. Aisen, *Biochim. Biophys. Acta*, **329**, 156–158, (1973).

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