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Journal of Chromatography B, 781 (2002) 481–496

JOURNAL OF CHROMATOGRAPHY B

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Review

Hydroxyl radical in living systems and its separation methods

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Abstract

It has recently been shown that hydroxyl radicals are generated under physiological and pathological conditions and that they seem to be closely linked to various models of pathology putatively implying oxidative stress. It is now recognized that the hydroxyl radical is well-regulated to help maintain homeostasis on the cellular level in normal, healthy tissues. Conversely, it is also known that virtually every disease state involves free radicals, particularly the most reactive hydroxyl radical. However, when hydroxyl radicals are generated in excess or the cellular antioxidant defense is deficient, they can stimulate free radical chain reactions by interacting with proteins, lipids, and nucleic acids causing cellular damage and even diseases. Therefore, a confident analytical approach is needed to ascertain the importance of hydroxyl radicals in biological systems. In this paper, we provide information on hydroxyl radical trapping and detection methods, including liquid chromatography with electrochemical detection and mass spectrometry, gas chromatography with mass spectrometry, capillary electrophoresis, electron spin resonance and chemiluminescence. In addition, the relationships between diseases and the hydroxyl radical in living systems, as well as novel separation methods for the hydroxyl radical are discussed in this paper.

Keywords: Reviews; Hydroxyl radicals

Contents

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00620-7

1. Introduction

There has been a tremendous increase in research related to free radicals, and hundreds of laboratories world-wide are now actively investigating many of their aspects. Due to the presence of an unpaired electron, free radicals are highly unstable and tend to react with cellular constitutive elements, particularly lipids and nucleic acids. Among these radicals, reactive oxygen species (ROS) are formed continuously in the body as a result of various biochemical processes [1,2]. The reaction is oxidative in nature and accounts for the potential cytotoxicity of ROS. Of all the ROS, the hydroxyl radical is the most reactive oxygen radical [3,4], which is formed via the Fenton reaction in living systems [5]. The half-life of the hydroxyl radicals in cells, though difficult to measure directly by conventional analytical techniques, is estimated to be around 10^{-9} s [5,6]. These radicals have been postulated to play a direct or indirect role in several pathological conditions, such as brain ischemia, Parkinson's disease, rheumatoid arthritis, respiratory distress syndrome, cardiovascular disease and carcinogenesis [7]. In general, the hydroxyl radical is generally considered to be a harmful byproduct of oxidative metabolism, causing molecular damage in living systems. It is also implicated in various processes such as mutagenesis, aging, and a series of pathological events. Although all of these roles may be justified, evidence is accumulating that free radicals, indeed, are an inherent part of living systems [8].

Hydroxyl radicals cause cell injury when they are generated in excess or the cellular antioxidant defense is impaired. Furthermore, it is well established that levels of free iron play a critical role in initiating and catalyzing a variety of radical reactions in the presence of oxygen [9,10]. At a physiological pH, iron is bound to transferrin or lactoferrin, which prevents it from generating hydroxyl radicals from hydrogen peroxide. However, if these compounds are incorrectly loaded or exposed to an acidic pH, iron is released and can participate in the generation of hydroxyl radicals when in the presence of certain oxidizable molecules, such as superoxide or vitamin C [11]. Living systems have a complex antioxidant defense system, which includes the antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase [12,13]. These enzymes may block the initiation of hydroxyl radicals and their chain reactions. There are some important non-enzymatic antioxidants, such as ascorbic acid, α-tocopherol, and β-carotene, which break free radical chain reactions [14,15]. In general, cellular levels of antioxidant enzymes and non-enzymatic antioxidants respond to levels of oxygen and ROS. This defense mechanism enables cells to defend against increased oxyradical production [16].

Although the spin trap coupled with electron spin resonance (ESR) has been proved to be a good

method for the measurement of hydroxyl radicals, it is restricted in sensitivity and quenching in vivo [17]. Because formation of the highly reactive hydroxyl radical in vivo is difficult to detect, indirect methods have been used to monitor the process. Numerous hydroxyl radical adducts have been measured by high-performance liquid chromatography (HPLC) with ultraviolet (UV), electrochemical detection (ED) and mass spectrometry (MS) [18]. In addition, chemiluminescence [19], gas chromatography MS (GC–MS) [20] and capillary electrophoresis (CE) [21] have been developed for the determination of hydroxyl radical adducts.

Methods used in these measurements can be relatively crude and dependent on single chemical derivatization, or on the separation of different products by means of the above techniques. In this review, these analytical techniques will be discussed in detail. We can now assume that the living state of cells and organisms implicitly requires the production of oxyradicals, including the hydroxyl radical. There are many methods of studying the hydroxyl radical, but all have to deal with the major problems associated with its reactivity, making the measurement of the hydroxyl radical very difficult. Due to the impressive developments in analytical tools, many important aspects of hydroxyl radical research are open for investigation. It is important to evaluate the appropriate usage of these analytical methods. Thus, the overall mechanism involved in hydroxyl radical-mediated physiological and pathological conditions in the living systems can be investigated. This accumulated knowledge will undoubtedly lead to the development of new therapeutic approaches to prevent or control diseases related to hydroxyl radicals.

2. Hydroxyl radical generation

2.1. In vitro

The hydroxyl radical is an oxygen-containing chemical species that has the capacity to abstract an electron from a variety of organic or inorganic molecules and atoms. It can be produced when water is exposed to ionizing radiation, leaving a single electron on hydrogen and one on oxygen [22].

$$H{-}O{-}H \rightarrow {}^{\cdot}H + {}^{\cdot}OH$$

In addition, many of the hydroxyl radicals are generated from the metal (M) ion-dependent breakdown of hydrogen peroxide. In the presence of ferrous ions, hydrogen peroxide is converted into the hydroxyl radical by Fenton's reaction or hydrogen peroxide, and the superoxide radical can assist the hydroxyl radical formation via the Haber–Weiss reaction [23,24].

$$M^{(n)+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\cdot}OH + OH^-$$

(Fenton's reaction)

$$H_2O_2 + {}^{\cdot}O_2^- \rightarrow O_2 + OH^- + {}^{\cdot}OH$$

(Haber–Weiss reaction)

The formation of the hydroxyl radical has been postulated to occur via interaction between the superoxide radical and nitric oxide, a vasodilator radical produced by several cell types [25].

$${}^{\cdot}NO + {}^{\cdot}O_2^- \rightarrow ONOO^-$$ (peroxynitrite)

$$ONOO^- + H^+ \rightarrow ONOOH$$

$$ONOOH \rightarrow {}^{\cdot}OH + {}^{\cdot}NO_2$$

2.2. In vivo

In vivo, hydroxyl radicals are generated under physiological conditions during aerobic metabolism or the metal ion catalytic reaction known as the Fenton reaction [26–28]. Several studies have reported that metals generating the hydroxyl radical, such as copper or ferrous iron, are classical transition metals for the Fenton reaction [29,30]. To investigate the generation of hydroxyl radical by a Fenton reaction in living systems, ferrous iron was infused through a microdialysis probe, which was implanted in the left ventricular myocardium of anaesthetized rats and perfused with Ringer's solution. The hydroxyl radical was trapped by sodium salicylate and the production of 2,3-DHBA was measured. The following account may be caused by the hydroxyl radical generation in vivo.

2.2.1. Ischemia/reperfusion

A vast number of studies have implicated the hydroxyl radical as one of the mediators of ischemia/reperfusion injury [31,32]. Using a vessel occlusion model of focal cerebral ischemia combined with microdialysis technique, the hydroxyl radical was trapped by 4-HBA (trapping agent) containing perfusate. The ratio of the hydroxylation product 3,4-DHBA to 4-HBA in the brain region increased significantly 30 min after ischemia induction. The increase in the 3,4-DHBA/4-HBA ratios in the brain region coincides with the increased dopamine release, suggesting a role for dopamine oxidation in the increased production of hydroxyl radicals [33]. Hydroxyl radicals trapped by salicylate-generated 2,3- and 2,5-DHBA have also been applied in vivo during myocardial ischemia for 30 min by occluding the left anterior descending in dog heart using, a microdialysis technique coupled with HPLC–ED [34]. While myocardial ischemia and reperfusion stimulates cardiac sympathetic afferent nerve endings of cat, the hydroxyl radical is produced all the way throughout ischemia [35]. Hydroxyl radical generation has also been reported in cases of jejunal ischemic injury [36].

The endogenous catecholamines, norepinephrine and epinephrine are capable of releasing iron from ferritin which significantly enhanced hydroxyl radical generation during ischemia/reperfusion [37]. Althaus et al. (1993) demonstrated that bilateral carotid occlusion in gerbils is useful as a model of brain injury. The conversion of salicylate to DHBA in vivo was employed to study the formation of the hydroxyl radical following central nervous system injury [38]. Recent reports indicate that several endogenous and exogenous compounds are involved in the regulation of the hydroxyl radical in the ischemia/reperfusion status. For example, MAO inhibitors, clorgyline and lazabemide, prevent hydroxyl radical generation caused by brain ischemia/reperfusion in mice [39]. However, glutamate may enhance the hydroxyl radical generation in the standing of ischemia [40,41].

2.2.2. MPP^+ and MPTP

An in vivo microdialysis technique has been applied on the intraperitoneal (i.p.) administration of MPTP, which resulted in a time- and dose-dependent increase in the content of 2,3-DHBA in rat striatum [42–44]. Furthermore, the MPTP metabolite, MPP^+, has also been reported to play a role in the generation of hydroxyl radicals in the extracellular fluid of the rat striatum [45,46], which is suppressed by tamoxifen [47], allopurinol [48], reserpine [49], imidaprilat [50], prazocin [51], L-deprenyl, a monoamine oxidase inhibitor [52] and histidine [53]. However, hydroxyl radical production was enhanced by nitric oxide [54], methamphetamine [55], fluvastatin [56] and potassium chloride [57] in a similar experimental model.

2.2.3. Nitric oxide

Indirect evidence exists that the hydroxyl radical may be generated via nitric oxide synthase (NOS) activation. This pathway is inhibited by fluvastatin, an inhibitor of low-density lipoprotein (LDL) oxidation [58]. Hydroxyl radical levels increased during glutamate perfusion. The radical production was inhibited by the specific NOS blocker, NG-nitro-L-arginine methyl ester [59]. Intranigral administration of sodium nitroprusside (as a nitric oxide donor) to rats induced an acute increase in hydroxyl radical levels [60].

2.2.4. Hepatitis

Hydroxyl radicals have been proposed to be a cause of hepatitis via the accumulation of copper, which has been demonstrated by the higher ratios of 2,3-DHBA/SA in plasma and liver of hepatitic Long–Evans Cinnamon rats [61].

2.2.5. Quinolinic acid

Quinolinic acid (pyridine-2,3-dicarboxylic acid) is a neurotoxic tryptophan metabolite implicated in the pathogenesis of several brain disorders via hydroxyl radical generation. These observations were evaluated by both ESR [62] and microdialysis methods [63].

2.2.6. 6-Hydroxydopamine (6-OHDA)

Toxicity of 6-OHDA in the brain may be caused by the generation of hydroxyl radicals, as analyzed by rat striatal dialysate via microdialysis and HPLC–ED. Hydroxyl radical formation and DNA base alterations are early manifestations of 6-OHDA toxicity and provide clues to the processes that may

be involved in the initiation of cell death in Parkinson's disease [64]. Pergolide presents neuroprotective effects in 6-OHDA-induced hydroxyl radical formation [65].

2.2.7. Metals

Direct evidence for in vivo hydroxyl radical generation in experimental iron overload by the use of a spin-trapping ESR method was provided by Burkitt and Masson in 1991 [66]. Using salicylate as a trapping agent and HPLC–ED to measure the hydroxyl radical production in vivo, Shen and Sangiah (1995) have demonstrated that cadmium chloride ($CdCl_2$)-induced testicular toxicity may possibly be mediated by a significant increase in hydroxyl free radical formation and a reduction in GSH content and Na^+, K^+-ATPase activity. Vitamin E seems to prevent the $CdCl_2$-induced increase in hydroxyl free radical generation [67]. Infusion of Fe^{2+} into the rat liver resulted in increased formation of 2,3-DHBA [68]. Following intrastriatal Mn^{2+} injection, marked 2,3-DHBA increases were observed time- and dose-dependently as well as the formation of the hydroxyl radical in the brain [69]. The generation of hydroxyl radical by chromium has been reported in vitro [70]. Chromium has also been administered to mice using an ESR spin-trapping technique via DMPO trapping hydroxyl radical in blood. There are indications that hydroxyl radicals are generated in mammals by chromium [71].

3. Hydroxyl radical-related diseases

Oxidative reactions are very important in other biological reactions, and many of these also have the potential for generating free radicals, including hydroxyl radicals under physiological and pathological conditions. It has been postulated that hydroxyl radicals play a direct or indirect role in several diseases, such as aging, brain ischemia, Parkinson's disease, and Alzheimer's disease, rheumatoid arthritis, respiratory distress syndrome, cardiovascular diseases and carcinogenesis. In general, the hydroxyl radical is considered to be a harmful byproduct of oxidative metabolism, causing cell damage in living systems as outlined in the following diseases [72].

3.1. Stroke

Stroke is a common and devastating neurological disorder and is one of the leading causes of death. It is also a major cause of long-term disability. One of the mechanisms implicated in causing neuronal damage is the increased formation of hydroxyl radicals during cerebral ischemia. Hydroxyl radicals are byproducts of cellular physiology generated by specific enzymes, autoxidation, and energy transfer reactions. Under physiological conditions, sophisticated antioxidant defense systems prevent the hydroxyl radicals from injurious interactions with critical cell components. However, under the conditions of stroke events, the equilibrium between free radicals and antioxidant defense systems is lost. This imbalance leads to hydroxyl radical-induced cell damage. When the oxygen supply is limited, the electron transport chain of the inner mitochondrial membrane becomes reduced and the extracellular hydroxyl radical levels consequently increase [73,74]. Several pathophysiological mechanisms for stroke injury have been proposed. An ischemic episode causes the release of excitatory amino acids such as glutamate from the neuronal terminals. Such excitatory amino acid-ischemic injury theory has long received considerable attention. Recent studies have proposed that hydroxyl radicals have a very important role in regulating this mechanism [40,41]. An ischemic episode stimulates the release of glutamate, which in turn increases the production of hydroxyl radicals. Thus, the induced damage is also critical and even worse under the cascades of hypoxia/ischemia or reoxygenation/reperfusion.

3.2. Parkinson's disease

It is generally accepted that progressive, irreversible and regionally specific neurodegeneration and the presence of Lewy bodies are the essential pathological hallmarks of idiopathic Parkinsonism [75]. The causes of these phenomena, however, remain to be elucidated. One of the leading hypotheses is that oxidative stress induced by ROS, such as the hydroxyl radical, damages essential components of the substantia nigra neurons, resulting ultimately in cell death. There are several potential sources of

increased free radicals in Parkinson's disease, including mitochondrial dysfunction, increased free iron levels and impaired free radical defense mechanisms. If the controlling processes are overwhelmed, hydroxyl radicals become highly destructive to nigra neurons. Excess of oxidative stress is the situation in which the prooxidant–antioxidant balance is tipped in favor of the former. This may be worse in the presence of exogenous sources or endogenous stresses such as excess availability of transition metals and limitation (or excess) of local oxygen. As a result of many types of cell injury, hydroxyl radicals are produced in excess of local defense mechanisms. It has been proposed that the hydroxyl radical is one of the major causes leading to this neurodegeneration [76].

3.3. Aging and Alzheimer's disease (AD)

Both the formation of free radicals and the degree of oxidative stress increase with animal age. It is also well known that animal age is accompanied by the accumulation of altered inactive or less active forms of many enzymes. Thus, age is likely associated with an increase in the adventitious production of free radicals and a concurrent decrease in the ability to defend against such free radicals. Aging is also a main element for AD [77]. The direct formation of free radicals from amyloid β (Aβ) protein has been suggested as a key neurotoxic mechanism in AD by ESR spectroscopy using spin traps. Chemical and conformational early modifications of the Aβ peptide are critical steps in AD pathogenesis and have been widely investigated. A Fenton-type hydroxyl radical generating system is capable of generating L-Dopa (3,4-dihydroxyphenylalanine) in the tyrosine residue of Aβ peptide via aromatic ring hydroxylation as a result of hydroxyl radical attack on proteins. Since L-Dopa is not a constituent of mammalian proteins and peptides, the formation of L-Dopa in Aβ in vitro constitutes a possible important modification caused by hydroxyl radical attack. These results lay the groundwork for further studies on modification and damage associated with the degenerative disorders in AD where oxidative stress and inflammation are known to occur [78–80].

4. Analysis of hydroxyl radical

4.1. High-performance liquid chromatography

Many techniques have already been used to detect hydroxyl radicals in vitro and in vivo (Table 1). A colorimetric method was initially described in studies in which the hydroxylated phenols were treated with sodium tungstate and sodium nitrite in an acid medium [81]. The methods for the determination of hydroxyl radical include the ESR system, which measures the ESR spectrum of a spin adduct derivative after spin trapping [82], or chromatographic methods, which determine the trapping products after they have been reacted with hydroxyl radicals [83]. In the use of HPLC systems, salicylate is included in the perfusion buffer at concentrations ranging from 0.1 to 2 m*M*, depending on the detection apparatus employed. However, there is a much higher efficiency (approximately 100%) in a coulometric detector when compared to a standard amperometric detector (approximately 1–5%) [84].

Generally, in advanced oxidation, hydroxyl radicals are produced in relatively large amounts, and the system is usually optimized for a specific pH range, such as 3–4 for Fenton's reaction [85]. It is well known that radical reactions are matrix-dependent and that the formation of derivatives of salicylate in the trapping reaction is also pH-dependent. Actually, the concentration of hydroxyl radicals in biological samples is very low which may be caused when the trapping reaction is carried out at physiological pH (pH 7.4) [86].

Because the ESR methods require a sophisticated and high-cost instrument system, and spin-trapped free radicals are still extremely short-lived, HPLC techniques have been developed to separate and quantify the stable hydroxylated derivatives in biological systems [83,87,88].

4.1.1. Electrochemical detection

In 1984, Floyd et al. developed a sensitive method for the detection of hydroxyl-free radical generation in various systems. Their methodology employs HPLC–ED for the quantification and identification of the hydroxylation products from the reaction of hydroxyl radical with salicylate. The detection limit

Table 1
Methods used for the detection of in vivo hydroxyl radical generation

Animal/organ	Detection method	Enhanced or inhibited hydroxyl radical	References
Generation by ischemia/reperfusion			
Rat/cortex, striatum	Microdialysis/4HB	Enhanced by dopamine	[33]
Dog/heart	Microdialysis/DHBA	Induced by myocardial ischemia	[34]
Cat/heart	Microdialysis/phenylalanine	Decreased by deferoxamine or dimethylthiourea	[35]
Rat/jejunum	Microdialysis/DHBA	Induced by jejunal ischemia	[36]
Rat/kidney	ESR	Decreased by dimethylthiourea	[127]
Rat/cerebral	ESR	Attenuation by oxypurinol	[129]
Rat/brain	ESR	Inhibited by PBN	[128]
Rat/plasma		Enhanced by epinephrine	[17]
Gerbil/brain	Microdialysis/DHBA	Inhibited by U-74006F	[38]
Rat/striatum	Microdialysis/DHBA	Enhanced by glutamate	[40,41]
Generation by MPP^+ or MPTP			
Rat/striatum	Microdialysis/DHBA	Induced by MPP^+	[42,45,46]
Mice/striatum	Microdialysis/DHBA	Induced by MPP^+	[44]
Rat/striatum	Microdialysis/DHBA	Inhibited by tamoxifen	[47]
Rat/striatum	Microdialysis/DHBA	Inhibited by allopurinol	[48]
Rat/striatum	Microdialysis/DHBA	Inhibited by reserpine	[49]
Rat/striatum	Microdialysis/DHBA	Inhibited by imidaprilat	[50]
Rat/myocardium	Microdialysis/DHBA	Inhibited by prazocin	[51]
Rat/substantia nigra	Microdialysis/DHBA	Inhibited by L-deprenyl	[52]
Rat/striatum	Microdialysis/DHBA	Enhanced by nitric oxide	[54]
Rat/striatum	Microdialysis/DHBA	Enhanced by methamphetamine	[55]
Rat/myocardium	Microdialysis/DHBA	Enhanced by fluvastatin	[56]
Rat/striatum	Microdialysis/DHBA	Reduced by histidine	[53]
Rat/striatum	Microdialysis/DHBA	Enhanced by potassium chloride	[57]
Generation by nitric oxide			
Rat/striatum	Microdialysis/DHBA	Enhanced by glutamate	[59]
Rat/substantia nigra	Microdialysis/DHBA	Induced by sodium nitroprusside	[30]
Generation by hepatitic rat liver			
Rat/liver or plasma	Microdialysis/DHBA	Enhanced by hepatitis	[61]
Generation by quinolinic acid			
Rat/striatum	Microdialysis/DHBA	Induced by quinolinic acid	[63]
Rat/brain	ESR	Induced by quinolinic acid	[62]
Generation by 6-hydroxydopamine			
Rat/striatum	Microdialysis/DHBA	Inhibited by pergolide	[65]
Generagy by metals			
Rat/liver	Microdialysis/DHBA	Induced by Fe^{2+}	[68]
Rat/bile	ESR	Induced by Fe^{2+}	[66]
Rat/striatum	Microdialysis/DHBA	Induced by Mn^{2+}	[69]
Mice/testis	DHBA	Induced by Cd^{2+}	[67]
Mice/blood	ESR	Induced by Cr^{6+}	[71]

was less than 1 pmol for the hydroxylation products at that time [83]. HPLC–ED then became the most important method for the in vivo measurement of the hydroxyl radical, and is based on the ability of the hydroxyl radical to attack the benzene rings of aromatic molecules. The rate constant of the in vivo metabolism of salicylic acid producing two main hydroxylated derivatives (2,3- and 2,5-DHBA) is about 5×10^{10} M^{-1} s^{-1} [89]. The in vitro reaction with salicylic acid has a known reaction rate constant of 1.3×10^{10} and 2.4×10^{10} M^{-1} s^{-1} for 2,3- and 2,5-DHBA, respectively [86].

Subsequently, the method was improved and it is now possible to detect DHBAs at the level of femtomoles [90,91]. Further, the formation of 8-hydroxy-deoxyguanosine (8-OH-dG), of which the hydroxyl radical reacts with DNA or 2-deoxy-guanosine can be effectively separated and detected from deoxyguanosine by HPLC–ED. The sensitivity of electrochemical detection is about 1000 times better than optical detection [92,93], ESR [94] or HPLC with ultraviolet detection [90,95]. During the 1990s, the microdialysis technique was used for hydroxyl radical trapping and sampling, which has been employed in neurodegenerative disease research [42,96].

In vivo, radical metabolism of salicylic acid produces 2,3- and 2,5-DHBAs. However, enzymatic pathways through the cytochrome P-450 system can also produce the latter acid, while the former acid is reported to be solely formed by non-enzymatic hydroxyl radical attack. Therefore, measurement of 2,3-DHBA, following systemic administration of the drug acetyl salicylate or perfusion by microdialysis, could be employed for the assessment of hydroxyl radical formation in vivo (Fig. 1) [95,97–99]. However, others have disagreed, suggesting that the 2,5-DHBA is indeed an accurate reflection of in vivo hydroxyl radical levels. The in vivo levels of 2,3-DHBA are much lower than those of 2,5-DHBA, and it is also possible that the concentrations of 2,3-DHBA are lower than the detection limit in some approaches [100,101].

In our studies, salicylic acid (0.5 m*M*) was used as a trapping reagent for hydroxyl radicals, and the resulting 2,3-DHBA and 2,5-DHBA were measured

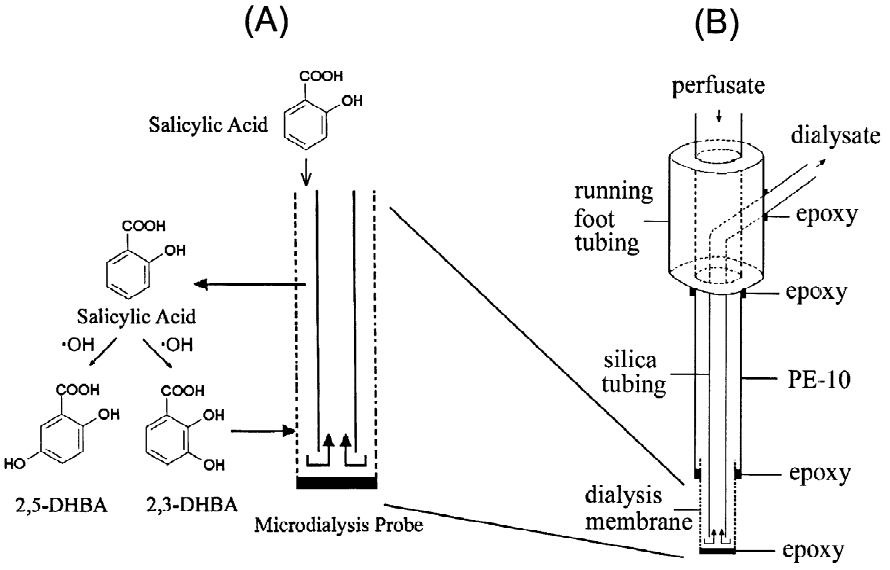


Fig. 1. (A) Enlargement of the tip of the microdialysis probe on the reaction of hydroxyl radicals with salicylic acid through the membrane of microdialysis probe to 2,3- and 2,5-dihydroxybenzoic acids. (B) The skeleton of the microdialysis probe, which was made by our own laboratory (from Ref. [98] with permission from Elsevier Science).

via an on-line microdialysis device directly from the blood vessels. The low flow-rate (1 μl/min) of this continuous microdialysis system provides a high surface to volume ratio, making it particularly sensitive to nonspecific hydroxyl radical production. The mobile phase consisted of 0.1 *M* monochloroacetic acid, 10 μ*M* EDTA, 0.5 m*M* sodium octylsulfate, 20% acetonitrile and 5% tetrahydrofuran in 1 liter (pH 3.0 adjusted using 1 *M* NaOH), and the flow-rate of 0.05 ml/min was found to be optimum. Isocratic separation of these adducts on a microbore RP-C_{18} column (150×1 mm I.D., 5 μm) was achieved within 10 min (Figs. 2 and 3). The optimal applied potential of DHBAs was set at 750 mV based on a hydrodynamic study. This method has a detection limit of 1.3 pmol/ml (or 0.2 ng/ml) for 2,3- and 2,5-DHBAs in Ringer solution (at signal-to-noise ratio = 3). The retention times of 2,3- and 2,5-DHBA depend particularly on pH and sodium octylsulfate (ion pairing reagent) concentration. The best separation was obtained at pH 3.0 and 0.5 m*M* sodium octylsulfate [98].

In addition to the absolute DHBA levels, many researchers have used the concentration ratio of DHBA and salicylate to interpret the hydroxyl radical formation, which may exclude the salicylic acid concentration differences in distribution and metabolism after salicylic acid administration [120]. Hall et al. showed a linear increase in 2,5-DHBA in the gerbil brain as the salicylic acid dose was increased, although the concentration ratio of 2,5-DHBA/salicylic acid remained constant [101]. For these reasons, some studies have used the DHBA/salicylic acid ratio to represent the hydroxyl radical formation [95]. The technique of salicylate trapping hydroxyl radical has proven to be simple and extremely sensitive for the study of oxidative stress in Parkinsonian patients, and shows increased levels of both 2,3- and 2,5-DHBA in platelets [102].

Another trapping agent, phenylalanine, has been claimed to react with hydroxyl radicals to form the hydroxylated products *o*-, *m*-, *p*-tyrosines [103]; and, as a naturally occurring amino acid, it would be expected to be nontoxic in physiological environments. However, the disadvantage of phenylalanine may be that its rate constant for reaction with hydroxyl radical, 1.9×10^{9} M^{-1} s^{-1} [104], is somewhat less than that for the DHBA [82,91,105]. Hydroxyl radical trapping using 4-HBA instead of salicylic acid provides several advantages. The benefits of 4-HBA hydroxylation include the fact that there is only one isomer, namely 3,4-DHBA, formed by hydroxylation, high trapping efficiency, and very little systemic production of 3,4-DHBA following 4-HBA administration [106].

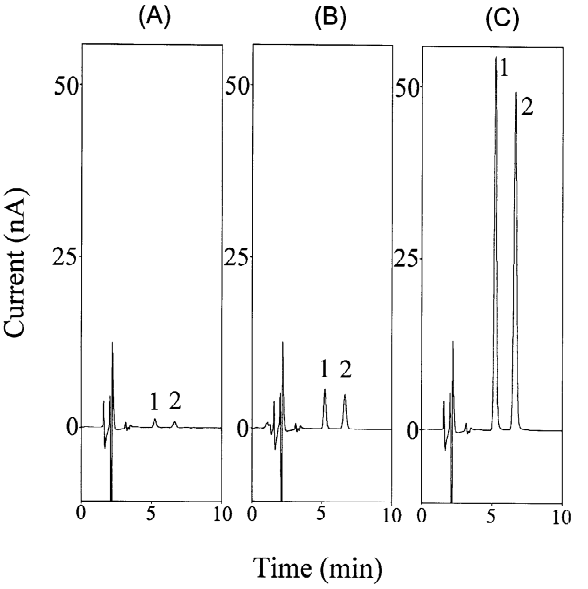
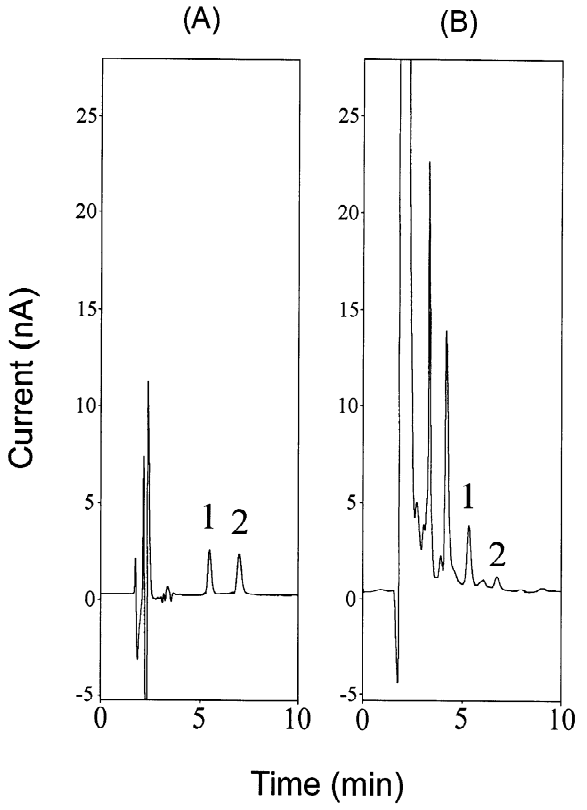


Fig. 2. Typical chromatograms of 2,3- and 2,5-dihydroxybenzoic acids standards: (A) 1 ng/ml, (B) 10 ng/ml, (C) 100 ng/ml. Peaks 1 and 2 represented as 2,5- and 2,3-dihydroxybenzoic acids, respectively (from Ref. [98] with permission from Elsevier Science).

Although HPLC–ED is a very sensitive method for trapping hydroxyl radicals using salicylic acid or 4-HBA, a small amount of hydroxylated contaminants (2,3-DHBA, 2,5-DHBA and 3,4-DHBA) will be traced during the determination of hydroxylation products of the various substrates. This was confirmed by their identification using GC–MS with *tert*.-butyldimethylsilyl (TBDMS) derivative in the microdialysis sampling technique. Plausible factors affecting hydroxylation of DHBAs using microdialysis technique include time, use of metal ions, and reuse of microdialysis probes. The longer the infusion contained 4-HBA, the more 3,4-DHBA was



(A) 2,3- and 2,5-DHBA (5 ng/ml)

(B) Rat blood base-line 2,5-DHBA (8.7 ng/ml)
2,3-DHBA (1.1 ng/ml)

Fig. 3. Chromatograms of (A) 2,3- and 2,5-dihydroxybenzoic acid standards, each 5 ng/ml; (B) the baseline levels of 2,3- and 2,5-dihydroxybenzoic acids at concentrations of 1.1 and 8.7 ng/ml, respectively, in rat jugular vein toward the right atrium. Peaks 1 and 2 represented as 2,5- and 2,3-dihydroxybenzoic acids, respectively (from Ref. [98] with permission from Elsevier Science).

produced per given time period. The liquid switch perfusion with artificial physiological solution may have possibly corroded some metal surfaces. Reusing the microdialysis probe resulted in increased DHBA production [107]. Furthermore, microdialysis is susceptible to nonspecific free radical reactions due to the low flow-rate used, which also prolongs the exposure time contact with the metal surface of stainless steel needles in the perfusate. Transition metal ions are capable of driving the Fenton-type reaction in biological tissues [108]. The detection method using HPLC–ED is summarized in Table 2.

4.1.2. Mass spectrometry (MS)

A mass spectrum is obtained by converting sample species into rapidly moving gaseous ions and separating them based on their mass-to-charge ratios. So far, MS is considered to be the most widely applicable analytical tool available, as it is capable of providing information on the structure of molecular species and the isotope ratios of atoms in sample species. In addition to combination with GC, MS has been coupled with liquid chromatography for the analysis of samples that contain non-volatile constituents. Therefore, MS combined with HPLC has become the most powerful tool for the identification of molecular species in biological samples. In the application of HPLC–MS to determining species of hydroxyl radical in the living systems, Van den Bergh et al. used the products of α-pinene and hydroxyl radical reaction to identify the oxidation products [18]. The HPLC–MS system equipped with atmospheric pressure chemical ionization (APCI) negative ion mode was selected for the measurement of hydroxyl radical products [18]. Tabatabaei and Abbott (1999) used highly sensitive LC–MS and negative electrospray ionization (ESI) to quantify 2,3-DHBA in rat plasma. The limit of detection of 2,3-DHBA was reported to be 0.25 pmol [109].

Hiramoto et al. applied ESI-MS accompanied by HPLC, NMR, and ESR to observe the DNA strand-breaking activity and mutagenicity of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), and to identify hydroxyhydroquinone in coffee as a generator of reactive oxygen species that breaks DNA single strands [110,111]. Tan et al. applied electron impact MS, ESI-MS, and NMR to investigate a potentially new metabolic pathway of melatonin biotransformation from the scavenging of hydrogen peroxide by melatonin [112]. Zhang et al. used HPLC and thermal spray LC–MS to study the mechanism and formation of a new metabolite of benzene in a mouse liver microsomal system [113]. Ali et al. (1997) identified the Chlorella T-1 ingredient enhancing DMPO-OH adduct formation in the Fenton reaction to be lactic acid by NMR and LC–MS [114].

Table 2
Method used for the detection of hydroxyl radical using HPLC–ED

Subject/tissue	Trapping agent/route	Separation column	Detection limit	Mobile phase	Reference
Human/plasma	Salicylate/oral	RP-C_{18} 150×4.6 mm	<1 pmol	30 m*M* sodium acetate, 30 m*M* trisodium citrate, 15% methanol, pH 3.9	[97]
Rat/striatum	Salicylate/microdialysis	RP-C_{18} 100×1 mm	2 fmol	0.1 *M* monochloroacetic acid, 0.01 m*M* EDTA, 0.5 m*M* sodium octylsulfate, 20% acetonitrile, 5% tetrahydrofurane, pH 3.0	[98]
Rat/striatum	Salicylate/microdialysis	RP-C_{18} 250×4.6 mm	<25 pg	50 m*M* sodium acetate, 10 m*M* citric acid, 0.15 m*M* EDTA, 0.43 m*M* sodium octylsulfate, 5% acetonitrile, pH 3.4	[99]
Rat/brain, liver, kidney	Salicylate/i.p.	RP-C_{18} 250×3 mm	1 pg	50 m*M* sodium acetate, 50 m*M* sodium citrate, 8% methanol, 2% propanol, pH 2.5	[95]
Human/platelet	Salicylate/incubation	RP-C_{18} 250×3 mm	5–10 fmol	20 m*M* monosodium phosphate, 30 m*M* sodium phosphate, 75 m*M* EDTA, 150 m*M* octylsulfate, 5% acetonitrile, pH 2.75	[102]
Mice/striatum	Salicylate/i.p.	RP-C_{18} 80×4 mm	3 pg	3 m*M* heptafluorobutyrate, 0.1 m*M* EDTA, 100 m*M* sodium hydroxide, 5% acetonitrile, pH 3.4	[108]
Rat/striatum	4-HBA/i.p.	RP-C_{18} 250×4.6 mm	20 fmol	20 m*M* citric acid, 50 m*M* sodium acetate, 1.875 m*M* sodium heptanesulfate, 0.125 m*M* EDTA, 7% methanol, pH 4.05 (glacial acetic acid)	[106]

4.2. Gas chromatography (GC)

GC is applied in the analysis of volatile and thermally stable constituents in samples. Therefore, in some instances, the derivatization of analytes is required to reduce the polarity of the species or to increase the detection sensitivity prior to chromatographic separation. Because most of the bio-species related to hydroxyl radicals in living systems are hydrophilic and thus have low volatility, the derivatization of species with an appropriate reagent is carried out before GC determination. Most derivatization reactions are methylation with diazomethane and silylation with bis(trimethsilyl)trifluoroacetamide, etc. to increase hydrophobicity and volatility of analytes. Usually the hydroxyl radical is ion-trapped by adding a reagent, or by the species existing in the living system. After appropriate pretreatment processes, such as extraction and derivatization, the adduct-species is injected on to the GC system for separation and detection. Although several detectors are usually applied to monitor the separated species from the GC column, MS is the most applicable detector for bio-species of living systems to identify or re-confirm the detection species. The determination of hydroxyl radicals using salicylate as a trapping agent by GC–MS has also been reported [115]. Castro et al. (1998) describe a procedure where the hydroxyl radical is detected by spin trapping with DMPO following the derivatization of the adduct which formed by silylation with precise and specific identification by GC–MS [116].

Halliwell and Dizdaroglu (1992) identified 8-hydroxyguanine and a wide range of other base-derived products of free radical attack using GC–MS after acidic hydrolysis of DNA or chromatin and derivatization [117]. In the detection of oxidative stress in the heart by estimating the dinitrophenylhydrazine derivative of malonaldehyde, Cordis et al. (1995) confirmed the collected species from HPLC with GC–MS [118]. In the development of accurate assays for the detection of modified bases from the hydroxyl radical-mediated modifications of nucleo-

bases, Douki et al. (1997) designed a GC–MS assay for the detection of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) [119]. In the photodegradation and phototoxicity studies of furosemide by Vargas et al. (1998), the formation of singlet oxygen through photolysis of the drug was demonstrated by trapping with 2,5-dimethylfuran [120].

In the oxidation reaction between α-pinene and hydroxyl radicals, Van den Bergh et al. (2000) applied GC–MS in addition to HPLC–MS to show that campholenealdehyde and pinonaldehyde were condensable oxidation products for the α-pinene hydroxylation, with pinonaldehyde being the main product [18]. In the study by Kumarathasan et al. of the hydroxyl radical adduct of 5-aminosalicylic acid as a potential marker of ozone-induced oxidative stress, in addition to being identified by an HPLC–ED detection system, oxidation products of 5-aminosalicylic acid were also identified by GC–MS analysis of trimethylsilyl derivatives [121]. As oxidative damage is considered to be an important factor of 6-OHDA toxicity, Ferger et al. analyzed striatal tissue for DNA base alterations using GC–MS [122].

4.3. Capillary electrophoresis (CE)

The application of CE for monitoring of oxidative damage caused by free radicals might be limited by its difficult detection. However, with the improvements of detection and combined techniques, such as with laser-induced fluorescence detection, interfaced on-line to a mass spectrometer (MALDI–TOF, matrix-assisted laser desorption ionization time-of-flight, MS–MS, and ESI-TOF-MS, etc.), CE will become a successful analytical tool with widespread applications in the biological sciences. We believe that fully automated CE instruments will displace cumbersome slab-gel electrophoresis in the studies of oxidative damage caused by free radicals once the appropriate instrumentation is widely available.

CE has been a powerful tool for the analysis of lipids, carbohydrates, nucleic acids, proteins, peptides, and proteomics. However, its application in studies related to the hydroxyl radical in living systems is rare. Coolen et al. (1997) used antipyrine as an exogenous marker for the biomolecular monitoring of oxidative damage caused by free radicals [123]. The ^{60}Co γ-radiation products of antipyrine were separated via micellar electrokinetic capillary chromatography (MECC) and HPLC, and the correlation coefficient was 0.9984 for both techniques. In addition, the dissociation constants of the radiation products were determined by means of capillary zone electrophoresis (CZE). Coolen et al. (1994) also used CZE and MECC to determine the hydroxylated radical products of salicylic acid (2,3- and 2,5-DHBAs) which are often used as a relative measurement in free radical research [124]. The detection limit for these two compounds was 0.5 μ*M*, which was the same as that obtained by the ESR spin trapping method [125].

4.4. Electron spin resonance (ESR)

Endogenous free radicals produced in living systems have extremely short half-lives and in low concentrations. Spin trapping is a technique in which a nitrone or nitroso compound is allowed to react with a free radical to produce a nitroxide whose stability is considerably greater than that of the parent free radical [126]. Instead of aromatic hydroxylation, Kadkhodaee et al. using EPR spectroscopy and spin trapping with the spin traps DMPO and PBN to detect and quantitate the formation of hydroxyl radicals in rat kidney after ischemia–reperfusion in vivo and in vitro in the isolated rat kidney perfused in the absence of leucocytes [127]. The same method has been used to detect and monitor the time course of hydroxyl radical formation in rat cerebral cortex and the PBN spin adduct forming agent is believed to have a protective action in ischemia–reperfusion injury of brain by forming adducts of hydroxyl radical [128]. In addition, oxypurinol has shown the attenuated effect on the hydroxyl radical generation in rat cerebral ischemia–reperfusion [129].

Pritsos et al. (1985) proposed a method employing ESR spin-trapping techniques to trap various radicals, which were selected using the spin trap 5,5-dimethyl-L-pyrroline *N*-oxide (DMPO) techniques [130]. Hiraoka et al. (1989) investigated the free radicals produced by the reactions of hydroxyl radicals with six purine nucleoside monophosphates (3′-AMP, 5′-AMP, 5′-dAMP, 3′-GMP, 5′-GMP and 5′-dGMP) using a method combining ESR spin-trapping and HPLC. The separated spin-adducts were

characterized by ESR spectrometry and UV spectrophotometry [131]. Hiraoka et al. (1990) also investigated the free radical reactions induced by hydroxyl radical attack on cytosine-related compounds using a method combining ESR, spin trapping with 2-methyl-2-nitrosopropane and HPLC, and the separated spin-adducts were then examined by ESR spectroscopy and UV photospectrometry [132].

Tosaki et al. (1993) compared the use of ESR spectroscopy and of HPLC for direct detection of hydroxyl radical formation in the ischemic and reperfused heart. They concluded that the HPLC technique may be more suitable for hydroxyl radical detection, based on its convenience, reproducibility and greater sensitivity [133]. Recently, ESR spectroscopy and imaging have evolved considerably and now provide useful information for analyzing the role of free radicals in the living body [134]. The development and application of spin labels make SPR a potential technique for the patho-physiological analysis of oxidative stress in vivo.

4.5. Chemiluminescence

Chemiluminescence has been widely used as a sensitive assay to monitor free radicals and reactive metabolites in living systems [135]. Light emission can be markedly amplified by luminol, which measures a mixture of oxygen-derived species [136]. Lucigenin reacts with superoxide, hydrogen peroxide, or singlet oxygen but not the hydroxyl radical [137]. The hydroxyl radical was generated by the addition of ferrous ion, which produced concentration-dependent luminol chemiluminescence [138].

The sensitivity of chemiluminescence detection has been exploited in combination with HPLC for post-column detection. Ohno et al. (1999) developed a novel high-sensitivity chemiluminescence assay for luminol using thiourea derivatives, and applied it to a HPLC assay for amino compounds [139]. Yoshiki et al. (1996) confirmed the photon emission chemiluminescence of catechins in the presence of active oxygen species (hydrogen peroxide, hydroxyl radical *tert*.-butyl hydroperoxide and *tert*.-butyl oxyl radical) and acetaldehyde to occur non-enzymatically at room temperature in aqueous neutral conditions, which can be used for HPLC detection. There is evidence of chemiluminescence peaking at 430 nm and red chemiluminescence peaking at around 680 nm in HPLC gel filtration chromatography [140]. Agatsuma et al. (1992) proposed that the spectral analyses of clinical samples showing weak chemiluminescence by forced oxidation by active oxygen may provide a new and more sensitive method for diagnosing metabolic disorders [141].

4.6. Miscellaneous

In addition to the GC–MS method, Farmer and Shuker (1999) also discussed the reliability and accuracy of HPLC in combination with ^{32}P-post-labelling and immunoassay methods for DNA and protein adduct determination [142]. Fu and Dean (1997) detected hydroxyleucines in the hydrolysates of tripeptides and proteins, which had been γ-radiolysed and treated with $NaBH_4$ by HPLC using post-column *o*-phthaldialdehyde derivatization [143]. Takeuchi et al. (1996) determined intracellular concentrations of hydrogen peroxide and superoxide by flow cytometry with dichlorofluorescein diacetate and hydroethidine, respectively [144]. Aoyagi et al. measured guanidinosuccinic acid (GSA) using HPLC following a post-column-labeling method using 9,10-phenathrequinone [145]. Fuciarelli et al. (1987) established a correlation between HPLC analysis and an in situ enzyme-linked immunosorbent assay (ELISA) for 8,5′-cycloadenosine formation in a hydroxyl radical evaluation system [146].

5. Conclusion

Because most radicals are inactivated by cellular antioxidant defense systems, only small portions of these radicals play important roles in physiological or pathological processes. However, when hydroxyl radicals are generated in excess or the cellular antioxidant defense is deficient, this can stimulate free radical chain reactions by interacting with proteins, lipids, and nucleic acids causing cellular damage and even diseases.

This paper provides a brief introduction to the hydroxyl radical, the relationships between the hydroxyl radical and a variety of diseases, and the state of the analytical techniques applied in the measurement of the hydroxyl radical. Many analytical tech-

niques, such as HPLC–ED, HPLC–MS, GC–MS, CE, ESR and chemiluminescence, are extensively discussed in this paper. This paper indicates the applications of these analytical tools in clinical diseases and strategies for therapeutic investigation.

Nomenclature

AD	Alzheimer's disease
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
DHBA	dihydroxybenzoic acid
DMPO	5,5-dimethylpyrroline-*N*-oxide
EPR	electron paramagnetic resonance
ESI	electrospray ionization
ESR	electron spin resonance
GC–MS	gas chromatography–mass spectrometry
HBA	hydroxybenzoic acid
HPLC–ED	high-performance liquid chromatography electrochemical detection
HOCl	hypochlorous acid
L-Dopa	3,4-dihydroxyphenylalanine
MALDI–TOF	matrix-assisted laser desorption/ionization time-of-flight
MECC	micellar electrokinetic capillary chromatography
MPP^+	1-methyl-4-phenylpyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMR	nuclear magnetic resonance
NOS	nitric oxide synthase
6-OHDA	6-hydroxydopamine
8-OH-dG	8-hydroxy-deoxyguanosine
PBN	alpha-phenyl-*tert*.-butyl-nitrone
ROS	reactive oxygen species

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