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High-performance ion chromatography applied to free-radical mechanisms in drug design
The problem of ion analysis at high ionic strengths[1]

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Abstract

Putative free-radical intermediates in drug action can be studied by radiolysis of model systems containing low concentrations of drug and much higher concentrations of other solutes to scavenge the primary water radicals and convert them into appropriate oxidants or reductants. The need to employ high ionic solute concentrations (typically >10 mmol dm^{-3}) represents a challenge for the high-performance ion chromatographic detection of drug-derived ions (typically, <50 μmol dm^{-3}). Constraints on the chromatographic method chosen are illustrated with examples of the application of high-performance ion chromatography (HPIC) to radiation chemistry studies in the oxidative decarboxylation of the anti-tumour drugs flavone-8- and xanthenone-4-acetic acids and structurally related aromatic carboxylic acids (CO_2 in the form of CO_3^{2-}), the oxidative denitrification of nitric oxide precursor molecules ($NO^{\cdot}$ in the form of NO_2^-/NO_3^-) and the generation of SO_4^{2-} from novel thiol-based (perthiol) drugs.

Keywords: Radicals; Pharmaceutical analysis; Radiation chemistry; Drugs; Hydroxyarginine; Hydroxyguanidine; Perthiol drugs; Anions; Nitric oxide

1. Introduction

Drugs are frequently metabolized via free-radical pathways [1–3] which can be modelled by radiolysis methods [4–6]. However, the high solute concentrations (typically >10 mmol dm^{-3}) often necessary to generate these putative drug radicals from the initial radicals of water radiolysis represent a significant challenge for high-performance ion chromatography (HPIC) detection of drug-derived ions, which are usually produced in much lower concentrations (typically, <50 μmol dm^{-3}). This paper focuses on the use of HPIC in three areas of drug design: (i) Drugs that can liberate $NO^{\cdot}$, a free-radical species involved in many physiological processes, which may be important in anti-cancer therapy. It is short-lived in oxygenated aqueous solutions, being converted quantitatively to nitrite and, in biological systems, is further oxidized to nitrate. (ii) Decarboxylation reactions, which may be important in the activity of some xanthenone anti-cancer drugs, where the liberated carbon dioxide is most readily determined by conversion to carbonate. (iii) Replace-

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ment of the thiol group in radioprotective drugs with the perthiol group may modify the pro-oxidative effect of the resultant perthiyl ($RSS^{\cdot}$) radical, which can be studied by determination of sulphate.

2. Experimental

2.1. Chemicals and gases

N^G-Hydroxy-L-arginine (NHA, Fig. 1, **1**) and hydroxyguanidine sulphate (HOG, Fig. 1, **2**) were obtained from Alexis Biochemicals (Nottingham, UK). Flavone-8-acetic acid (FAA, Fig. 1, **3**) was donated by Lipha (Lyon, France) and xanthenone-4-acetic acid (XAA, Fig. 1, **4**) by Prof. William A. Denny (Cancer Research Laboratory, University of Auckland School of Medicine, Auckland, New Zealand). Indole-3-acetic acid (IAA, Fig. 1, **5**) was obtained from Sigma (Poole, UK). The radioprotective thiol 2-(3-aminopropyl-amino)ethanethiol (WR-1065, Fig. 1, **6**) was donated by the Drug Synthesis and Chemistry Branch (Division of Cancer Treatment, National Cancer Institute, USA). The symmetrical trisulphide of WR-1065 (Fig. 1, **7**) and WR-1065 perthiol (Fig. 1, **8**) were synthesized according to methods described previously [7]. Acetonitrile was from Rathburn (Walkerburn, UK), potassium dihydrogen orthophosphate and orthophosphoric acid (both of electrochemical grade),

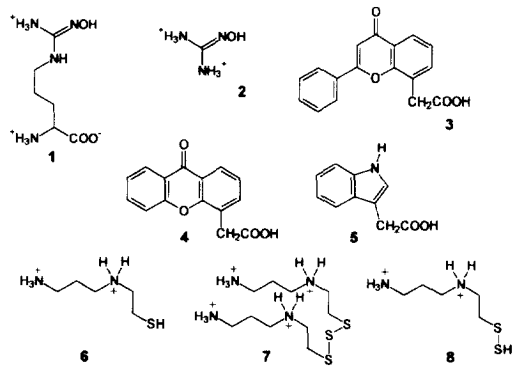

Fig. 1. Structures of compounds described in the text: 1 = N^G-hydroxy-L-arginine; 2 = hydroxyguanidine; 3 = flavone-8-acetic acid; 4 = xanthenone-4-acetic acid; 5 = indole-3-acetic acid; 6 = WR-1065; 7 = the symmetrical trisulphide of WR-1065; 8 = the perthiol analogue of WR-1065.

octanesulphonic acid (OSA) and *tert.*-butylammonium hydroxide (TBAOH) were from Fisons (Loughborough, UK). All other chemicals were from Merck (Poole, UK). All experiments were performed with water purified with a Milli-Q system (Millipore, Watford, UK) and saturated with various gases including nitrogen, nitrogen–oxygen mixtures, nitrous oxide, nitrous oxide–oxygen mixtures or pure oxygen (British Oxygen Company, London, UK) for at least 15 min prior to irradiation.

2.2. Radiolysis methodology and the specific generation of radicals

Steady-state γ-radiolysis generates radicals at a quantifiable rate. The ^{60}Co source in the Gray Laboratory has a current activity of ~500 Ci and the rate of radical production can be varied over ~ two orders of magnitude, by varying the source-to-sample distance. Samples were contained in 4 ml gas-tight vials and irradiated at ~22–30 Gy min^{-1}, as determined by Fricke dosimetry. The formation of CO_2 was quantified by converting it to CO_3^{2-} by the addition of NaOH [8,9] (50 μl of 1 mol dm^{-3} NaOH to a 3-ml sample):

$$CO_2 + H_2O + H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$
$$(pK_a = 6.37) \rightleftharpoons 2H^+ + CO_3^{2-} (pK_a = 10.25) \quad (1)$$

In aqueous solution, the autoxidation of $NO^{\cdot}$ generates nitrite ions stoichiometrically [10,11], although the high solute concentrations employed in radiolysis experiments may modify the aqueous chemistry of $NO^{\cdot}$ to generate some nitrate.

$$4NO^{\cdot} + 4OH^- + O_2 \rightarrow 4NO_2^- + 2H_2O \quad (2)$$

Hydroxyl radicals ($^{\cdot}OH$) and the hydrated electron (e_{aq}^-) are generated by the radiolysis of water. These initial radicals can react with other solutes present in high concentration to generate secondary radicals capable of oxidizing or reducing the drug which is at a much lower concentration. In this contribution, drug-derived ions have been measured following drug interactions with the oxidizing hydroxyl ($^{\cdot}OH$), bromide ($Br_2^{\cdot -}$) and superoxide ($O_2^{\cdot -}$) radicals and the reducing 2-propanol [$(CH_3)_2C^{\cdot}OH$] radical.

2.3. Chromatography

Chromatography systems 1–3 were used to measure NO_2^-/NO_3^-, systems 4 and 5 for CO_3^{2-} and system 1 for SO_4^{2-}.

System 1: HPIC was performed on a Dionex 100 chromatograph equipped with an IonPac anion-exchanger (Dionex, AS4-SC 25 cm analytical and 5 cm guard column). The eluent for this system was 1.8 mmol dm^{-3} Na_2CO_3 and 1.7 mmol dm^{-3} $NaHCO_3$ at a flow-rate of 1.5 ml min^{-1}. Detection was by absorbance at 214 nm using a Waters photodiode array (PDA) detector and by suppressed conductivity (external water) with a Dionex ED40.

System 2: HPLC was performed on a Waters Millennium system equipped with a Hypersil 5 ODS, 125×4.6 mm column, and the eluent consisted of 4% acetonitrile, 5 mmol dm^{-3} TBAOH and 10 mmol dm^{-3} KH_2PO_4 at a flow-rate of 1.5 ml min^{-1}. Detection was by absorbance at 214 nm using a Waters 486 detector.

System 3: The Waters Millennium system was equipped with a silica-based anion-exchange column (Exsil SAX, 125×4.6 mm) with two guard cartridges (Hypersil 5 ODS and Exsil SAX, 10×4.6 mm), all from Hichrom (Reading, UK). The eluent consisted of 20% acetonitrile, 22 mmol dm^{-3} KH_2PO_4 and 3 mmol dm^{-3} H_3PO_4, at a flow-rate of 1.6 ml min^{-1}. Detection was by absorbance at 214 nm using a Waters 486 detector (Watford, UK) and electrochemically with a Coulochem detector, using a dual porous graphitic electrode, the first electrode was at +0.35 V and the second monitoring electrode was at +0.65 V (ESA, St. Ives, UK).

System 4: Separation was performed on the Dionex DX-100 chromatograph equipped with a Dionex 25 cm IonPac ICE-ASI ion-exclusion column and the eluent was water at a flow-rate of 1 ml min^{-1}.

System 5: This was the same as system 4 with 22% acetonitrile in water as eluent.

3. Results and discussion

3.1. Oxidative denitrification of hydroxyguanidines and analysis of nitrite and nitrate ions

The rational design of stable hydroxyguanidine drugs for the controlled delivery of NO˙ to tumours requires an understanding of the structural features that influence its release by either free-radical or enzymatic pathways. NHA, a stable intermediate in the L-arginine–NO˙ pathway [12], can be oxidized to NO˙ via pathways involving peroxidases and cytochrome P-450 mono-oxygenase [13,14]. Peroxidase reactions can be conveniently studied by model free-radical oxidants generated by radiolysis, since these enzymes oxidise many substrates in a discrete one-electron step [3] and are capable of catalysing the oxidative denitrification of hydroxyguanidines with liberation of NO˙, as measured indirectly by the formation of NO_2^-/NO_3^- [15–17].

Fig. 2 shows typical chromatograms of the separation of an irradiated (100 Gy) N_2O–O_2 (80:20)-saturated aqueous solution containing 100 μmol dm^{-3} NHA and 1 mmol dm^{-3} potassium phosphate buffer, pH 7.4, using chromatography system 1. Under the experimental conditions employed, the ˙OH radical oxidises NHA to liberate NO˙, thus generating NO_2^-/NO_3^- ions. The generation of ˙OH radical does not require high concentrations of a scavenging solute, which could interfere with the chromatography and, consequently, both NO_2^-/NO_3^- ions can be detected by absorbance (214 nm) or conductivity. However, the ˙OH radical is an extremely potent oxidant and the radiation

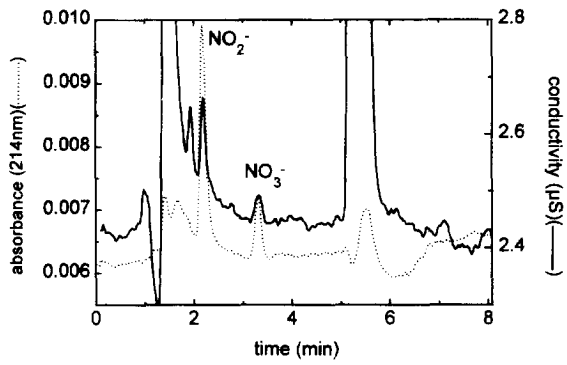

Fig. 2. Chromatogram from the oxidative denitrification of N^G-hydroxy-L-arginine by the hydroxyl radical, ˙OH. Radiolysis (100 Gy) was of an N_2O–O_2 (80:20)-saturated aqueous solution containing 100 μmol dm^{-3} N^G-hydroxy-L-arginine and 1 mmol dm^{-3} potassium phosphate buffer, pH 7.4. Analysis by chromatography system 1 used combined absorbance (214 nm) and conductivity detection.

chemical yield (G) for NO_2^-/NO_3^- is only 50% of the yield expected from $G(^{\cdot}OH)=0.55$ μmol J^{-1}. The indiscriminate nature of the $^{\cdot}OH$ radical oxidation of NHA indicates that this radical is an imperfect model for studying enzymatic drug activation and, instead, a secondary radical may be used to obtain more selective oxidizing conditions and to secure "clean" one-electron oxidation of the drug.

The bromide ion is very reactive towards $^{\cdot}OH$ and, if present at high concentration (~10 mmol dm^{-3} Br^-), can prevent $^{\cdot}OH$ reacting directly with the drug of interest, acting as an intermediate in the one-electron oxidation of the drug via the formation of the $Br_2^{\cdot-}$ radical. However, chromatography system 1 is inappropriate for studying the oxidation of NHA by the $Br_2^{\cdot-}$ radical, due to interference of Br^- with the detection of NO_2^-/NO_3^- ions by absorbance and conductivity. Fig. 3 shows a chromatogram of the analysis of an irradiated sample (20 Gy) of an N_2O–O_2 (80:20)-saturated aqueous solution containing 10 mmol dm^{-3} KBr, 100 μmol dm^{-3} NHA and 1 mmol dm^{-3} potassium phosphate buffer, pH 7.4, using chromatography system 2. In this case, the use of a silica-based anion-exchange column (Exsil SAX) in combination with spectrophotometric detection proved an excellent approach to the detection of NO_2^-/NO_3^- ions at concentrations of Br^- between 10–20 mmol dm^{-3}. The $Br_2^{\cdot-}$ radical was found to selectively oxidise the N^G-hydroxyguanidine group of NHA since $G(NO_2^-/NO_3^-)=0.52$ μmol J^{-1} was closer to that expected from $G(Br_2^{\cdot-})=$~0.6 μmol J^{-1}. The $Br_2^{\cdot-}$ radical therefore represents a suitable model one-electron oxidant for studying peroxidase-type catalysis of NHA to generate $NO^{\cdot}$.

The solutes $S_2O_8^{2-}$ and N_3^- can also be used at ~10 mmol dm^{-3} to generate other free-radical oxidants ($SO_4^{\cdot-}$ and $N_3^{\cdot}$, respectively). However, N_3^- interferes with the detection of NO_2^-/NO_3^- ions, while $S_2O_8^{2-}$ is a moderately strong chemical oxidant that oxidises NHA to NO_2^-/NO_3^- ions via non-radical processes, particularly at pH>7.4.

Enzyme systems that are capable of oxidizing hydroxyguanidines to $NO^{\cdot}$ (e.g. NOS and cytochrome P-450) will also generate the $O_2^{\cdot-}$ anion-radical, particularly with sub-optimal concentrations of substrate or poor substrate binding in the enzyme's active site [18]. The $O_2^{\cdot-}$ anion-radical can be generated using radiolysis of oxygen-saturated formate. Fig. 4 shows chromatograms obtained by analysis of an irradiated (50 Gy) O_2-saturated solution containing 0.1 mol dm^{-3} sodium formate, 100 μmol dm^{-3} HOG and 1 mmol dm^{-3} potassium phosphate buffer, pH 8, using chromatography system 3. The $O_2^{\cdot-}$ anion-radical reacts with HOG during radiolysis to generate NO_2^-/NO_3^- ions in a pH-dependent process, in a manner similar to NHA. Once again the high concentration of formate precludes the use of chromatography system 1, but

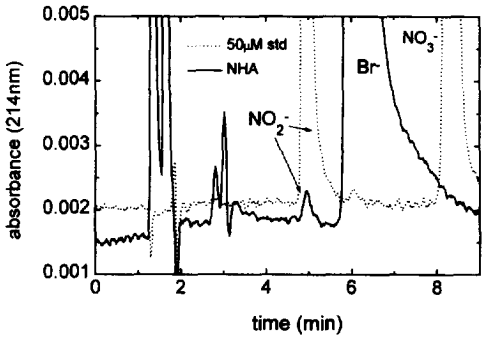

Fig. 3. Chromatogram from the oxidative denitrification of N^G-hydroxy-L-arginine by the bromide radical, $Br_2^{\cdot-}$. Radiolysis (20 Gy) was of an N_2O–O_2 (80:20)-saturated aqueous solution containing 100 μmol dm^{-3} N^G-hydroxy-L-arginine, 10 mmol dm^{-3} KBr and 1 mmol dm^{-3} potassium phosphate buffer, pH 7.4. Analysis by chromatography system 2 used absorbance detection at 214 nm.

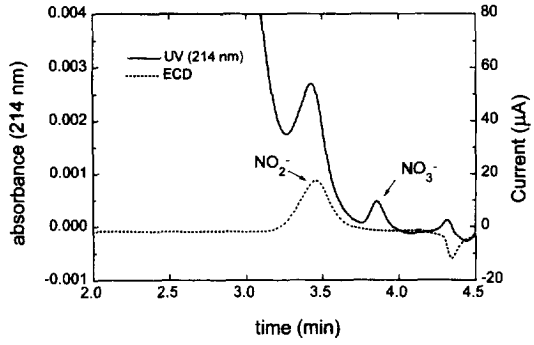

Fig. 4. Chromatogram from the oxidative denitrification of hydroxyguanidine by the superoxide, $O_2^{\cdot-}$ anion-radical. Radiolysis (50 Gy) was of an O_2-saturated aqueous solution containing 100 μmol dm^{-3} hydroxy-guanidine, 0.1 mol dm^{-3} sodium formate and 1 mmol dm^{-3} potassium phosphate buffer, pH 8. Analysis by chromatography system 3 used absorbance detection (214 nm) and electrochemical detection.

chromatography system 3 does allow the combination of absorbance (214 nm) and electrochemical detection of NO_3^- and NO_2^-, respectively.

3.2. Oxidative decarboxylation of N^G-hydroxy-L-arginine and anti-tumour aromatic carboxylic acids

We have established that $^{\cdot}OH$ radicals do not react exclusively with the N^G-hydroxyguanidino group of NHA to liberate $NO^{\cdot}$ and that other competing reactions, possibly oxidative decarboxylation, may be involved. Fig. 5a shows a typical chromatogram of an irradiated (100 Gy) N_2O–O_2 (80:20%)-saturated aqueous solution containing 100 μmol dm^{-3} NHA and 1 mmol dm^{-3} potassium phosphate buffer, pH 7.4, as analysed by chromatography system 4. The oxidation of NHA by $^{\cdot}OH$ radical was found to liberate CO_2 (measured in the form of CO_3^{2-}), the $G(CO_3^{2-})=0.21$ μmol J^{-1} plus $G(NO_2^-/NO_3^-)=$ 0.25 μmol J^{-1}, accounting for 84% of the expected $G(^{\cdot}OH)=0.55$ μmol J^{-1}.

FAA and related xanthenones have been extensively evaluated as anti-tumour agents [19–22]. Apart from important observations of $NO^{\cdot}$ and cytokine induction there have been remarkably few suggestions as to the mode of action at the molecular level. We have previously shown that one-electron oxidation of both FAA and a series of analogues of XAA by radiolytically generated $SO_4^{\cdot-}$ radical leads to efficient decarboxylation [9], as measured by chromatography system 4. The resultant carbon-centred radical may generate peroxyl radicals, which are known to induce cellular damage by lipid peroxidation. One problem in these studies on the ion exclusion ICE-AS1 column has been the retention of the aromatic carboxylic acids, which, on subsequent injections, can co-elute with the CO_3^{2-} peak. As a consequence, lengthy run times of ca. 50 min were often required to allow clearance from the column. This problem has been circumvented using chromatography system 5, which utilizes an acetonitrile–water eluent to promote faster elution of the organic acids and to prevent interference with the CO_3^{2-} peak. Fig. 5b shows a typical chromatogram of the oxidative decarboxylation of IAA by the $Br_2^{\cdot-}$ radical, as analysed by chromatography system 5. IAA eluted at a significantly shorter time of ca. 14 min. The retention time of CO_3^{2-} ion (~10 min) and the peak shape were similar to those observed in a water eluent and it was concluded that any potential resin swelling on introduction of acetonitrile had a negligible effect on the efficiency of separation.

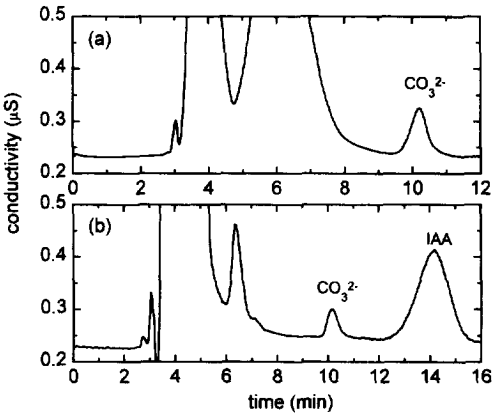

Fig. 5. (a) Chromatogram from the oxidative decarboxylation of N^G-hydroxy-L-arginine by the hydroxyl, $^{\cdot}OH$, radical. Radiolysis conditions as per Fig. 2. CO_2 was converted to CO_3^{2-} ions by addition of NaOH post-irradiation. Analysis by chromatography system 4 used conductivity detection. (b) Chromatogram from the oxidative decarboxylation of indole-3-acetic acid by the $Br_2^{\cdot-}$ radical. Radiolysis conditions as per Fig. 3. CO_2 was converted to CO_3^{2-} as for (a). Analysis involved chromatography system 5 with a water to acetonitrile eluent to prevent prolonged retention of the aromatic carboxylic acid.

3.3. The generation of SO_4^{2-} ions from novel perthiol drugs

Thiols have been employed as potential therapeutic agents in the prevention of free-radical mediated pathological disorders and as radioprotective adjuncts to clinical cancer radiotherapy [23–25]. Structural modification of thiols to their disulphur perthiol analogues represents a novel strategy whereby differences in S–H bond energies form the basis for manipulation of thiol anti-radical properties [7,26]. In the classical repair reaction, the thiol restores free-radical damaged sites on biomolecules by hydrogen atom transfer from the S–H moiety. Thiol and perthiol repair of carbon-centred alcohol radicals results in the formation of thiyl ($RS^{\cdot}$) and perthiyl ($RSS^{\cdot}$) radicals, respectively [7], which could exhibit pro-oxidative effects through their reaction with molecular oxygen to generate a (per)thiyl peroxyl radical [$RS(S)OO^{\cdot}$]. Ion-chromatography detection

of SO_4^{2-} ions has provided useful mechanistic insights into the fate of the RSSOO˙ radical [27]. RSS˙ can also be generated by ˙OH radical attack on the symmetrical trisulphide of WR-1065. Fig. 6 shows chromatograms of SO_4^{2-} ion formation from radiolytically generated RS˙ and RSS˙ radicals in the presence of molecular oxygen, using chromatography system 1. The mechanism of SO_4^{2-} anion formation has been suggested to proceed by analogy with the well-characterized reactions of RSOO˙ and peroxyl radicals [27] via Eqs. (4–6):

$$RSSOO^{\cdot} \leftrightharpoons RSSO_2^{\cdot}(+O_2) \rightarrow RSSO_2OO^{\cdot} \quad (4)$$

$$2RSSO_2OO^{\cdot} \rightarrow 2RSSO_3^{\cdot} + O_2 \quad (5)$$

$$RSSO_3^{\cdot} \rightarrow RS^{\cdot} + SO_3(+H_2O) \rightarrow 2H^+ + SO_4^{2-} \quad (6)$$

The RSS˙ radical produces significantly greater quantities of sulphate than its thiyl radical counterpart.

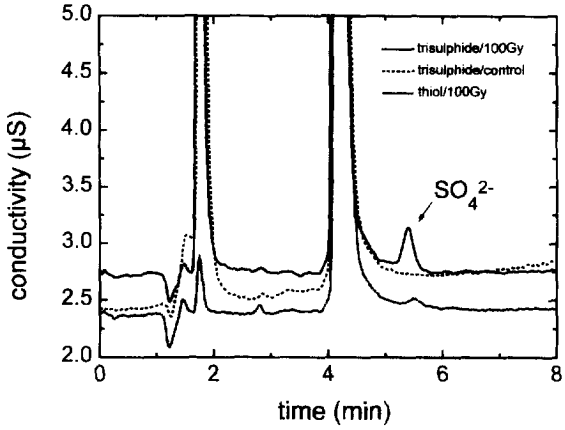

Fig. 6. Chromatogram comparing the generation of sulphate ions by sulphur-centred perthiyl, RSS˙, and thiyl, RS˙ radicals in the presence of molecular oxygen. The RSS˙ radical was generated by the radiolysis (100 Gy) of an N_2O–O_2 (80:20)-saturated aqueous solution containing 100 μmol dm^{-3} WR-1065 trisulphide, and 1 mmol dm^{-3} potassium phosphate buffer, pH 7.4. The corresponding RS˙ radical was generated by radiolysis (100 Gy) of a N_2O–O_2 (80:20)-saturated aqueous solution containing 100 μmol dm^{-3} WR-1065, 1 mol dm^{-3} 2-propanol, and 1 mmol dm^{-3} potassium phosphate buffer at pH 7.4. Although the yield of both radicals are similar, RSS˙ radicals generate significantly greater quantities of sulphate than RS˙ radicals. Analysis was by chromatography system 1.

4. Conclusions

Radiation chemistry provides the means to generate specific free radicals in known concentration, thus giving quantitative information of value in understanding drug action. Within this context, IC can contribute to rational drug design by characterizing and quantifying free-radical processes that result in ion formation. The application of IC in three areas of drug design has been addressed: The oxidative denitrification of hydroxyguanidines as a source of NO˙, the oxidative decarboxylation of anti-tumour aromatic carboxylic acids and the generation of sulphate from novel perthiol drugs. In radiolysis studies where the oxidant is the hydroxyl radical, there are few constraints on the mode of analysis, and suppressed conductivity (or absorbance) detection with the widely available Dionex AS4A-SC column can be used. Equally satisfactory are the silica-based anion-exchange or reversed-phase ion-pair separations, where absorbance or electrochemical detection are feasible. Where high concentrations of secondary radical scavengers are present, conductivity detection may be compromised and, in the case of bromide, we found the reversed-phase ion-pair system was most satisfactory. The use of formate to generate the superoxide radical caused severe problems with both conductimetric and absorbance detection, but electrochemical detection was unaffected by the formate peak. The use of this detection mode precludes the use of the carbonate eluent with the AS4 column because its conductance is too low.

Detection sensitivity for the studies described here is not limiting for practical reasons. As described in Section 2, 1 Gy of radiation generates ~0.6 μmol dm^{-3} radicals. It is simple to design experiments involving (as here) 100 μmol dm^{-3} drug being studied. Thus, doses of a few tens of grays decompose sufficient of the drug to permit the determination of the rate of loss, while generating sufficient product(s) for facile determination. At higher radiation doses, the products may begin to scavenge the reactive radicals. Thus, in typical studies where yield of product is plotted against dose, sub-micromolar sensitivity is rarely required, and the detection limits required are easily attained by any modern detector. The only system where

detection limits might be expected to be higher is that using the ICE-AS1 ion-exclusion column, which has a 9-mm I.D., with consequently increased peak dilution. However, in the case of the carbonate analysis, the NaOH added to the sample to convert the carbon dioxide liberated to carbonate always contained significant amounts of carbonate. This gives a zero dose intercept, which is the major limitation to the sensitivity of this particular analysis.

In all cases, the challenge for the experimenter is to balance the design of the radiolysis experiment with the appropriate methodology for ion separation. When the balance is struck, IC can make a significant contribution in radiation chemistry applied to drug design.

Acknowledgments

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