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Free radical scavenging reactions of sulfasalazine, 5-aminosalicylic acid and sulfapyridine: Mechanistic aspects and antioxidant activity

RAVI JOSHI[1], SUDHEER KUMAR[2], M. UNNIKRISHNAN[2], & T. MUKHERJEE[1]

[1]*Radiation Chemistry & Chemical Dynamics Division, Chemistry Group, Bhabha Atomic Research Center, Mumbai 400 085, India, and* [2]*College of Pharmaceutical Sciences, Manipal 576119, India*

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Abstract

Reactions of sulfasalazine (SAZ) and its metabolites, 5-aminosalicylic acid (5-ASA) and sulfapyridine (SP), with various oxidizing and reducing free radicals (hydroxyl, haloperoxyl, one-electron oxidizing, lipid peroxyl, glutathiyl, superoxide, tryptophanyl, etc.) have been studied to understand the mechanistic aspects of its action against free radicals produced during inflammation. Nanosecond pulse radiolysis technique coupled with transient spectrophotometry has been used for *in situ* generation of free radicals and to follow their reaction pathways. The transients produced in these reactions have been assigned and radical scavenging rate constants have been measured. In addition to scavenging of various primary and secondary free radicals by SAZ, 5-ASA and SP, 5-ASA has also been observed to efficiently scavenge radicals of biomolecules. 5-ASA has been found to be the active moiety of SAZ involved in the scavenging of oxidizing free radicals whereas reduction of SAZ produced molecular radical anion. The study suggests that free radical scavenging activity of 5-ASA may be a major path of pharmacological action of SAZ against inflammatory bowel diseases (IBD).

Keywords: *Sulfasalazine, 5-aminosalicylic acid, pulse radiolysis, free radicals, antioxidant, kinetics and mechanism*

Introduction

Sulfasalazine (SAZ) is a drug, which is used effectively in the treatment of inflammatory bowel diseases (IBD), such as ulcerative colitis or Crohn's disease [1,2]. Inflammatory diseases, including inflamed intestine and/or colon, are known to be caused by enhanced production of reactive oxygen species (ROS) by phagocytic leukocytes [3,4]. Several anti-inflammatory drugs including aspirin have been shown to reduce the levels of superoxide [5] and hydroxyl radical [6].

After ingestion, SAZ is reduced by coliform bacterial enzyme azoreductase into sulfapyridine (SP) and its active component 5-ASA (Scheme 1) in the intestine and colon [7]. Though SAZ, like other anti-inflammatory drugs, is known to inhibit formation of pro-inflammatory prostaglandins and leukotrienes [8], scavenge free radicals [9], inhibit generation of free radicals [10], possess immunosuppressive activity [11] and inhibit cytokine synthesis [12] by indirect techniques but its pharmacological mode of action is still unresolved.

SP is absorbed from the gut into blood and is metabolized but 5-ASA is not absorbed and its concentration at the site of inflammation in IBD is high. Therefore, the active therapeutic moiety of SAZ in the treatment of IBD is assumed to be 5-ASA, whereas SP functions as a carrier ensuring that 5-ASA is released within the colon. This high concentration of 5-ASA at the site of inflammation in IBD suggests that its major mode of action may be the free radical

Correspondence: Dr. R. Joshi, Radiation Chemistry & Chemical Dynamics Division, Chemistry Group, Bhabha Atomic Research Center, Mumbai 400 085, India. Fax: 91 22 2550 5331/2550 5151. E-mail: rjudrin@yahoo.com; ravij@magnum.barc.ernet.in

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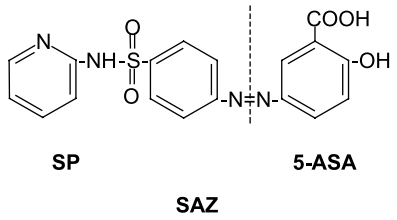

Scheme 1.

scavenging activity. However, the free radical scavenging activity and antioxidant efficacy of any compound depend not only on its high local concentration at the site of radical generation but also on its reaction rate constant with the free radicals.

Though SAZ and its metabolites are shown to scavenge various free radicals [9,13,14] by indirect techniques, no detailed study is reported in the literature to suggest the active moiety of SAZ in its free radical scavenging reactions. Therefore, kinetics and mechanism of reactions of SAZ, 5-ASA and SP with various free radicals have been studied to get direct evidence for the free radical scavenging activities of the individual substrates and their relative radical scavenging activity. The radical-molecule reactions of the drug molecules with tryptophanyl, glutathiyl and lipid peroxyl radicals have also been studied to understand possible *in vivo* free radical reactions. Electron pulse radiolysis coupled with nanosecond kinetic spectrophotometry technique has been used as a clean and direct method to study such reactions [15–18]. Radiation dose deposited in the solution has been measured by thiocyanate chemical dosimeter [19].

Materials and methods

Chemicals

Sulfasalazine (>98%), 5-ASA (99%) and sulfapyridine (99%) were obtained from Sigma and were used as received. All other chemicals were of AR grade. Deionised water (conductivity <0.06 (S cm^{-1}) obtained from a Barnstead nano-pure cartridge filtration system (USA) was used for preparing solutions.

Methods

Pulse radiolysis. The pulse radiolysis system giving pulses of 7 MeV electrons from a linear electron accelerator for *in situ* generation and study of free radicals has been used and is described elsewhere [16,17]. High-energy electrons deposit energy in water to generate its radical and molecular species (Equation 1).

$$H_2O \rightarrow e_{aq}^{-}(0.28)^{\cdot}, H(0.062)^{\cdot}, OH(0.28), H_2, H_2O_2 \quad (1)$$

The values in the parenthesis in Equation (1) are radiation chemical yield in the unit of μmoles per Joule of absorbed energy. The yields of these species depend on the radiation dose deposited by ionizing radiation in the medium, which in turn depends on linear energy transfer of high-energy electrons. The absorbed dose was measured using an air-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} KSCN ($G\epsilon = 2.6 \times 10^{-4}$ m^2 J^{-1} at 475 nm) [19]. The kinetic spectrophotometric detection system covered the wavelength range from 250 to 800 nm. The optical path length of the cell was 1.0 cm. The width of the electron pulse was 50 or 500 ns as per the experimental requirement and the dose used was 16 Gy (1 Gy = 1 J energy deposited in 1 kg of material) or otherwise specified. Equimolar (2×10^{-3} mol dm^{-3}) HPO_4^{2-} and $H_2PO_4^{-}$ were used to prepare solutions at pH 6.8. Acidic and alkaline pH values were adjusted by adding $HClO_4$ or KOH only. High purity (>99.9%) N_2 and N_2O were used for purging solutions as per requirement.

Most of the studies have been done at pH ~ 7, which is close to the physiological pH. Non-physiological pH values have been used in some studies to generate secondary radicals as per the standard protocols. It is to be noted that reactions of solutes with glutathiyl and lipid peroxyl radicals at non-physiological pH values are similar to those observed at pH ~ 7. Relative absorption of radicals in the UV–visible region has been observed against time and wavelength to get kinetic and absorption characteristics, respectively. The bimolecular rate constants were calculated by plotting pseudo-first order rate of formation of the transient against the concerned solute concentration. The time points in transient absorption spectra refer to the time elapsed after the irradiation with electron pulse. The uncertainty in the measurement of wavelength, transient absorption and least square fitting to get bimolecular rate constants are ~1 nm, <1 and <3%, respectively.

Results and discussion

The reactions of SAZ, 5-ASA and SP with various oxidizing and reducing radicals have been studied using appropriate chemical systems and following the standard methods for *in situ* generation of the free radicals. The reactions of SAZ and 5-ASA have been studied in detail because of their proposed role in scavenging oxidizing radicals in IBD. UV–visible absorption spectra of SAZ, 5-ASA and SP are shown in Figures 1B, 2B and 3B, respectively to show their effect on transient absorption spectra.

Reaction with the hydroxyl radical

Hydroxyl radical ($^{\cdot}$OH) is the most deleterious oxidizing radical among those physiologically present and reacts with most of the biomolecules at diffusion

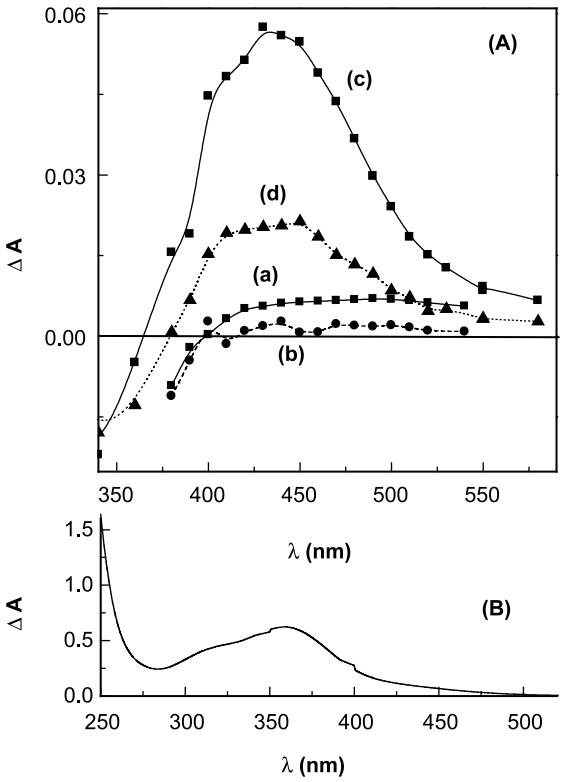

Figure 1. (A) Absorption spectrum of the transients obtained at (a) 10 μs and (b) 820 μs from N_2O-saturated aqueous solutions containing SAZ (8×10^{-5} mol dm^{-3}), dose = 13 Gy; at (c) 40 μs and (d) 850 μs from N_2O-saturated aqueous solutions containing SAZ (1.2×10^{-4} mol dm^{-3}) and NaN_3 (1.0×10^{-1} mol dm^{-3}), dose = 14 Gy, both at pH 6.8. (B) UV–visible absorption spectrum of SAZ (1.1×10^{-4} mol dm^{-3}) at pH 6.8.

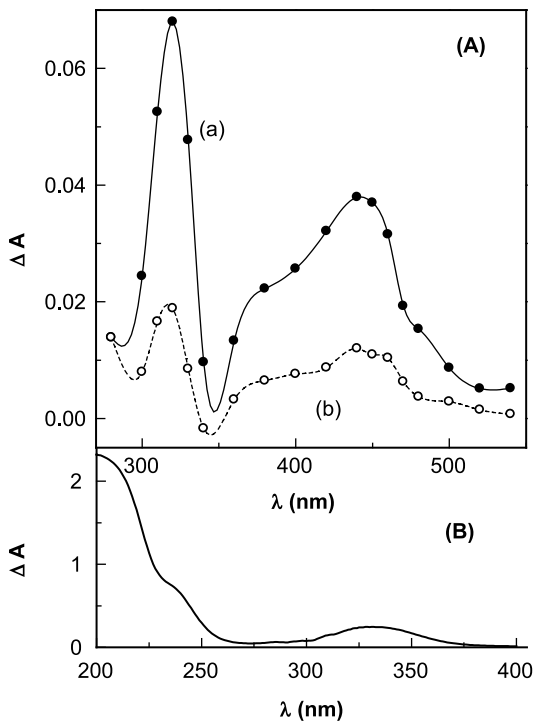

Figure 2. (A) Absorption spectrum of the transients obtained at (a) 70 μs and (b) 1800 μs from N_2O-saturated aqueous solution containing 5-ASA (1×10^{-4} mol dm^{-3}) at pH 6.8. Dose = 13 Gy. (B) UV–visible absorption spectrum of 5-ASA (1.1×10^{-4} mol dm^{-3}) at pH 6.8.

controlled rates [20]. Hydroxyl radical is generated *in situ* by radiolysis of N_2O-saturated aqueous solution (Equation 2).

$$e^-_{aq} + N_2O + H_2O \rightarrow {}^{\cdot}OH + OH^- + N_2 \qquad (2)$$

$$^{\cdot}OH \underset{}{\overset{pK_a=11.8}{\rightleftharpoons}} O^- + H^+ \qquad (3)$$

SAZ has been observed to react with $^{\cdot}OH$ ($k = 5 \times 10^9$ dm^3 mol^{-1} s^{-1}) at pH 6.8 to give transient absorption spectra (Figure 1A; a and b) with maximum at ~450 nm. However, 5-ASA has been found to react with $^{\cdot}OH$ ($k = 6.7 \times 10^9$ dm^3 mol^{-1} s^{-1}) at pH 6.8 (Figure 2A; a and b) to produce maxima at 440 and 320 nm. Transient absorption spectra for SAZ with $^{\cdot}OH$ could not be studied below 380 nm due to its strong ground state absorption. The transient species formed in these reactions have almost the same absorption maxima (440–450 nm) and decay rate constants (~6×10^9 mol^{-1} dm^3 s^{-1}). The phenolic group in both compounds is an easily oxidizable group and is known to react with oxidants to produce a phenoxyl radical ($\lambda_{max} \sim 420$ nm), which decays by second order kinetics [21,22]. Therefore, transient absorption at 440–450 nm observed for SAZ and 5-ASA can be ascribed to their phenoxyl radical. The measured rate constants are of the same order (Tables I and II) as reported earlier [23,24].

However, SP reacted with hydroxyl radical ($k = 1 \times 10^{10}$ dm^3 mol^{-1} s^{-1}) to give a transient absorption maximum at ~400 nm (Figure 3A: a and b) with low absorption at ~520 nm. It is to be noted that both pyridine [25,26] and benzene [21] rings of SP are known to react with various radicals. Therefore, transient absorption spectrum may have contribution of transients produced in the reactions with both the moieties. The transient absorption observed at 400 nm

Table I. Rate constants for the reactions of various free radicals with SAZ.

Radical	pH	λ_{max} (nm)	k (mol^{-1} dm^3 s^{-1})
$^{\cdot}OH$	6.8	~450	5.0×10^9
$N_3^{\cdot}$	6.8	450	9.8×10^8
$^{\cdot}CCl_3O_2$	11.3	450	4.5×10^8
e^-_{aq}	6.8	420, 560	1.1×10^{10}
$^{\cdot}CO_2^-$	6.8	420, 570	4.0×10^8
$O_2^{\cdot-}$	6.8	420, 560	1.2×10^9
GS$^{\cdot}$	4.6	430, 560	1.5×10^9 (at 560)*
			6.0×10^7 (at 430)*
$LO_2^{\cdot}$	11.0	—	4.0×10^8
SAZ$^{\cdot}$ + AscH$^-$	6.8	—	3.0×10^8

* See text.

Table II. Rate constants for the reactions of various free radicals with 5-ASA.

Radical	pH	λ_{max} (nm)	k ($mol^{-1}\,dm^3\,s^{-1}$)
$^{\cdot}OH$	6.8	320, 440	6.7×10^9
$N_3^{\cdot}$	6.8	320, 440	6.0×10^9
$^{\cdot}CCl_3O_2$	6.8	320, 440	9.0×10^8
e_{aq}^-	6.8	300	6.0×10^9
$^{\cdot}CO_2^-$	6.8	270	5.0×10^9
$GS^{\cdot}$	4.1	330, 430, ~470	7.0×10^7
$Trp^{\cdot}$	6.8	—	7.3×10^7
$L^{\cdot}$	11.0	—	2.0×10^8
$LO_2^{\cdot}$	11.0	—	8.0×10^8
5-$ASA^{\cdot}$ + $AscH^-$	6.8	360, 400, 430, ~460	1.5×10^7

can be ascribed to the reaction of pyridine ring of SP with $^{\cdot}OH$ on the basis of earlier reports for pyridine derivatives [25,26]. The hydroxyl radical adduct of benzene ring is known to give a transient absorption maximum at ~310 nm [21] which is also indicated in the transient spectrum after applying correction due to parent absorption. Since the hydroxyl radical can react by addition, abstraction and one-electron addition [14,18,20] specific one-electron oxidants have been used to unambiguously resolve the absorption of one-electron oxidized transient.

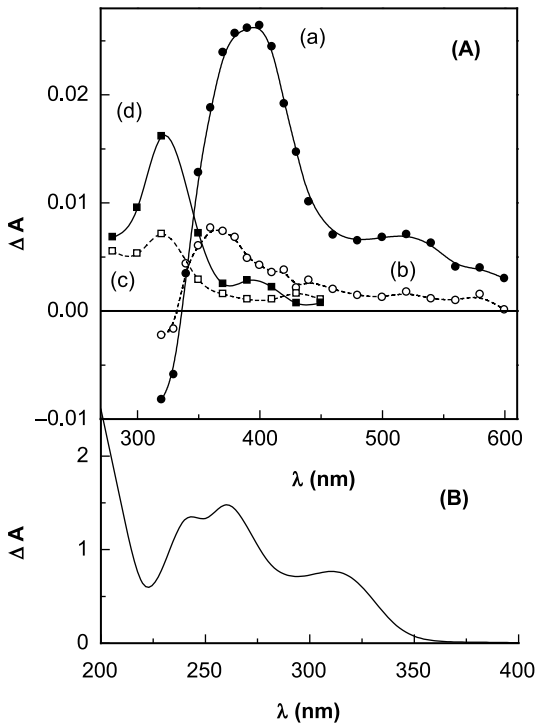

Figure 3. (A) Absorption spectrum of the transients obtained at (a) 10 μs and (b) 860 μs from N_2O-saturated aqueous solution containing SP (1.16×10^{-4} mol dm^{-3}), dose = 16 Gy; at (c) 50 μs and (d) 1810 μs from aerated aqueous solution containing SP (2.32×10^{-4} mol dm^{-3}), tert-butanol (2.5 mol dm^{-3}) and CCl_4 (1.0×10^{-2} mol dm^{-3}), dose = 16 Gy, both at pH 6.8. (B) UV–visible absorption spectrum of SP (6.0×10^{-5} mol dm^{-3}) at pH 6.8.

Reaction with the azidyl radical

The azidyl radical ($N_3^{\cdot}$), generated by reacting hydroxyl radical with azide anion (equations 2 and 4), is known to react selectively with organic molecules to give their one-electron oxidized radical species [22].

$$^{\cdot}OH + N_3^- \rightarrow OH^- + N_3^{\cdot} \quad (4)$$

At pH 6.8, $N_3^{\cdot}$ reacted with SAZ ($k = 9.8 \times 10^8$ dm^3 mol^{-1} s^{-1}) to produce transient absorption spectrum with maximum at 450 nm (Figure 1A; c and d) whereas 5-ASA reacted ($k = 6 \times 10^9$ dm^3 mol^{-1} s^{-1}) to produce transient absorption spectrum with maxima at 440 and 320 nm. The transient absorption spectra obtained for the reactions of 5-ASA with $^{\cdot}OH$, $N_3^{\cdot}$ and $^{\cdot}CCl_3O_2$ (discussed in later section) are similar. These transient absorption maxima (440–450 nm) suggest that phenoxyl radical of SAZ and 5-ASA, $SAZ^{\cdot}$ and 5-$ASA^{\cdot}$, respectively, have been formed. In the reaction of SP with $N_3^{\cdot}$ no transient absorption maximum is observed in the 300–600 nm wavelength region as is the case with pyridine also (figure not shown here) [22] but, the transient absorption spectrum has a tail extending up to 550 nm. Therefore, rate constant has been measured at 480 nm.

A comparison of molar extinction coefficient (ε) values for the transients formed in the reaction of 5-ASA with $^{\cdot}OH$ and $N_3^{\cdot}$ suggest that in both the cases only one-electron oxidized transient is formed. However, in the reaction of SAZ with $^{\cdot}OH$, G($SAZ^{\cdot}$) has been found to be equal to (1/2).G($^{\cdot}OH$) on comparing its reaction with $N_3^{\cdot}$. Therefore, remaining 50% contributes to other reactions like adduct formation, hydrogen abstraction, etc.

Reaction with trichloromethyl peroxyl radical

The trichloromethyl peroxyl radical ($^{\cdot}CCl_3O_2$) has been used as a model peroxyl radical. The reduction potential value of $^{\cdot}CCl_3O_2$, 1.5 V [27], is higher compared to those of the physiologically relevant non-haloperoxides. However, $^{\cdot}CCl_3O_2$ radical has been chosen as a representative one because of its

involvement in CCl_4 induced liver injury as well as for the simplicity in performing the experiments (Equations 5–7) [28,29]. Trichloromethyl peroxyl radical is generated by radiolysis of air-saturated aqueous solution and tert-butanol is added to scavenge the hydroxyl radical.

$$^{\cdot}OH + \text{tert} - C_4H_9OH \rightarrow \text{tert} - {}^{\cdot}C_4H_8OH + H_2O \quad (5)$$

$$e^-_{aq} + CCl_4 \rightarrow CCl_3 + Cl^- \quad (6)$$

$$CCl_3 + O_2 \rightarrow {}^{\cdot}CCl_3OO \quad (7)$$

This radical has also been used earlier to study a very important biophysical phenomenon, namely free radical interaction between vitamins E and C [29].

The shape of the transient absorption spectra observed in the reactions of SAZ (at pH 11.3) and 5-ASA (at pH 6.8) with $^{\cdot}CCl_3O_2$ (figures not shown here) are similar to those observed at pH 6.8 with other oxidants suggesting the formation of the same transients. It should be noted that $^{\cdot}CCl_3O_2$ has not been found to oxidize SAZ at pH 6.8. This suggests that at pH 6.8, 5-ASA can efficiently scavenge peroxyl radical but SAZ is ineffective.

SP reacted with $^{\cdot}CCl_3O_2$, at pH 6.8, to produce transient absorption spectrum with a maximum at ~320 nm and negligible absorption ≥350 nm (Figure 3A; c and d). This absorption maximum is different from those obtained with SAZ and 5-ASA. This can be ascribed to the adduct of $^{\cdot}CCl_3O_2$ with benzene ring of SP [21].

Reaction with hydrated electron

The reactions of SAZ and 5-ASA with hydrated electron (e^-_{aq}) and formate radical anion ($^{\cdot}CO_2^-$) have been studied to find their transient absorption maximum, measure rate constants, understand reduction process and compare their reactions with superoxide radical. The transient absorption spectra for the reaction of e^-_{aq} with SAZ (Figure 4a) showed maxima at 420 and 560 nm whereas for 5-ASA (Figure 5a, 5b) maximum has been observed at 300 nm. The e^-_{aq} adduct of benzene ring is known to have absorption maximum at ~310 nm [21]. However, e^-_{aq} adducts of pyridine and pyridine derivatives produce pyridinyl radical, which is known to have absorption maximum in 300–550 nm region depending on the type and position of substitution [25,26]. Therefore, the absorption maxima produced in the reaction of e^-_{aq} with SAZ and 5-ASA can be ascribed to delocalized molecular anion radical. SP reacted with e^-_{aq} to give transient absorption spectrum with maximum at 360 nm and strong absorption

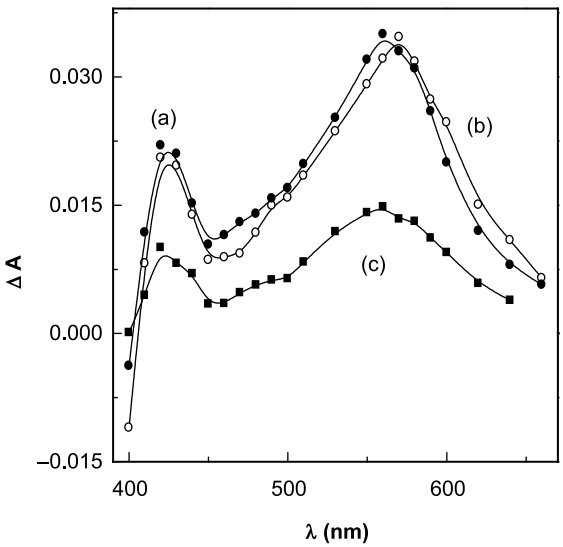

Figure 4. Absorption spectrum of the transients obtained from (a) N_2-bubbled aqueous solution containing SAZ (1×10^{-4} mol dm^{-3}), tert-butanol (3×10^{-1} mol dm^{-3}) at 20 μs and (b) N_2O-saturated aqueous solution containing SAZ (1×10^{-4} mol dm^{-3}), HCOONa (1×10^{-1} mol dm^{-3}) at 90 μs and (c) aerated aqueous solution containing SAZ (1.12×10^{-4} mol dm^{-3}), HCOONa (1×10^{-1} mol dm^{-3}) at 40 μs. All at pH 6.8 and with dose = 16 Gy.

≤350 nm (Figure 6, a and b), which is similar to the pyridinyl radical reported for 2-pyridinecarboxylic acid [25]. The rate constants for the reaction of e^-_{aq} with SAZ, 5-ASA and SP (Tables I–III) agree well with the reported values for similar molecules.

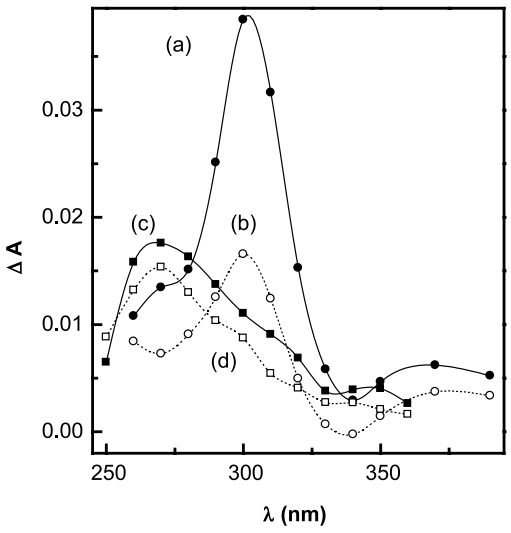

Figure 5. Absorption spectrum of the transients obtained at (a) 5 μs and (b) 114 μs from N_2-bubbled aqueous solution containing 5-ASA (1.73×10^{-4} mol dm^{-3}), tert-butanol (2.1×10^{-1} mol dm^{-3}), dose = 14.7 Gy; at (c) 2 μs and (d) 20 μs from N_2O-saturated aqueous solution containing 5-ASA (2×10^{-4} mol dm^{-3}), HCOONa (1×10^{-1} mol dm^{-3}) dose = 16 Gy, both at pH 6.8.

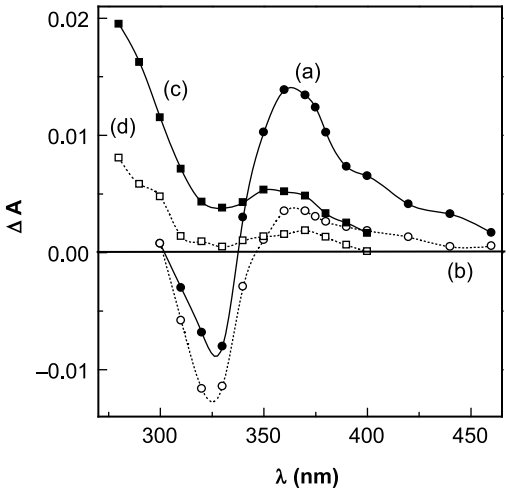

Figure 6. Absorption spectrum of the transients obtained at (a) 21 μs and (b) 590 μs from N_2-bubbled aqueous solution containing SP (1.6×10^{-4} mol dm^{-3}) and tert-butanol (2.1×10^{-1} mol dm^{-3}); at (c) 2.5 μs and (d) 90 μs from N_2O-saturated aqueous solution containing SP (1.16×10^{-4} mol dm^{-3}), HCOONa (1×10^{-1} mol dm^{-3}), both at dose = 16 Gy and pH 6.8.

Reaction with $^{\cdot}CO_2^-$

In the generation of $^{\cdot}CO_2^-$, all of the e_{aq}^- ($G = 0.28$ μmol J − 1), $H^{\cdot}$ ($G = 0.062$ μmol J^{-1}) and $^{\cdot}OH$ ($G = 0.28$ μmol J^{-1}) are converted into reducing species ($^{\cdot}CO_2^-$). But in the study of reaction of e_{aq}^- with the solute, $H^{\cdot}$ also reacts along with e_{aq}^-. Therefore, transient absorption maximum of one-electron reduced solutes can be unambiguously ascribed using $^{\cdot}CO_2^-$ radical provided there is no solute-radical adduct formation. *In situ* generation of $^{\cdot}CO_2^-$ is achieved by radiolysis of N_2O-saturated aqueous solutions of HCOONa (Equations 2 and 8).

$$H^{\cdot}/{}^{\cdot}OH + HCO_2^- \rightarrow H_2/H_2O + {}^{\cdot}CO_2^- \qquad (8)$$

The transient absorption spectra for the reaction of $^{\cdot}CO_2^-$ with SAZ showed transient absorption spectrum with maxima at 420 and 570 nm (Figure 4b), which agrees well with that obtained with e_{aq}^- (Figure 4a). The reaction of $^{\cdot}CO_2^-$ with 5-ASA produced transient absorption spectra with maximum at 270 nm and broad absorption at ~300 nm (figure 5c, 5d). The absorption at ~300 nm is already ascribed to radical anion of 5-ASA but the absorption at 270 nm is speculated to be due to solute-radical adduct. Transient absorption spectrum obtained in the reaction of $^{\cdot}CO_2^-$ with SP shows maximum at ~350 nm (Figure 6, c and d), similar to that observed with e_{aq}^- (Figure 6, a and b), and strong absorption below 300 nm suggesting the formation of similar transient/s.

5-ASA reacts with $^{\cdot}CO_2^-$ to produce solute-radical adduct at higher rate constant as compared to those for SAZ and SP, which make electron-adduct. It is due to the fact that $^{\cdot}CO_2^-$ diffuse to the solute in the first step to make a solute-radical adduct followed by reduction resulting in higher rate for solute-radical adduct formation as compared to the reduction.

Table III. Rate constants for the reactions of various free radicals with SP at pH 6.8.

Radical	λ_{max} (nm)	k (mol^{-1} dm^3 s^{-1})
$^{\cdot}OH$	400, 520	1.0×10^{10}
$N_3^{\cdot}$	—	1.0×10^{9}
$^{\cdot}CCl_3O_2$	320	6.0×10^{6}
e_{aq}^-	360	2.1×10^{10}
$^{\cdot}CO_2^-$	350	3.8×10^{9}
SP + AscH$^-$	—	2.7×10^{9}

Reaction with superoxide radical

Superoxide radical ($O_2^{\cdot -}$) is produced in various metabolic processes, including phagocytosis. $O_2^{\cdot -}$ and its protonated form ($HO_2^{\cdot}$) can cause either reduction or oxidation of the solutes depending upon their reduction potentials. Earlier, indirect techniques have been used to show the reaction of $O_2^{\cdot -}$ with SAZ and 5-ASA [10,30,31]. In the present study, it has been attempted to understand the kinetics of the reaction and characterize the produced transient/s. Superoxide radical is produced by radiolysis of air-saturated aqueous solutions containing HCOONa (Equations 8–10).

$$^{\cdot}CO_2^- + O_2 \rightarrow O_2^{\cdot -} + CO_2 \qquad (9)$$

$$e_{aq}^- + O_2 \rightarrow O_2^{\cdot -} \qquad (10)$$

The transient absorption spectrum for the reaction of $O_2^{\cdot -}$ with SAZ at pH 6.8 (Figure 4c) showed transient absorption maxima at 420 and 560 nm. A comparison of the transient absorption spectra obtained with e_{aq}^- and $^{\cdot}CO_2^-$ (Figure 4a,b) indicates that it produces one-electron reduced transient of SAZ. However, 5-ASA and SP have not been found to react with $O_2^{\cdot -}$ in our experimental conditions of time domain.

The rate constant for the reaction of SAZ with $^{\cdot}CO_2^-$ is slower than that with $O_2^{\cdot -}$. This is due to the fact that reduction by $^{\cdot}CO_2^-$ is not an outer-sphere electron transfer process but follows adduct formation before causing reduction. In this study direct reaction of $^{\cdot}CO_2^-$ with SAZ can be ruled out on the basis of competition kinetics as shown below:

$$^{\cdot}CO_2^- + O_2 \rightarrow O_2^{\cdot -} + CO_2 \quad (k = 2.4 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}) \qquad (11)$$

$$^{\cdot}CO_2^- + SAZ \rightarrow SAZ^- + CO_2 \quad (k = 4 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}) \qquad (12)$$

Fraction of $^{\cdot}CO_2^-$ reacting with O_2 (2.5×10^{-4} mol dm^{-3}) of aerated solution in

competition with SAZ (1.12×10^{-4} mol dm^{-3}) is:

$$(k_{11} \times 2.5 \times 10^{-4})/(k_{11} \times 2.5 \times 10^{-4} + k_{12} \times 1.12 \times 10^{-4}) \sim 93\%.$$

This calculation shows that ~93% of $^{\cdot}CO_2^-$ reacted with oxygen in aerated solution in presence of SAZ (1.12×10^{-4} mol dm^{-3}) producing $O_2^{\cdot -}$ in the first step followed by a reaction of $O_2^{\cdot -}$ with SAZ. Hence direct reaction of $^{\cdot}CO_2^-$ with SAZ to produce transient absorption maxima at 420 and 560 nm can be ruled out.

The reactions of SAZ, 5-ASA and SP with various radicals show that they efficiently scavenge oxidizing and reducing radicals and can be termed as competitive antioxidants. The reactions of 5-ASA with $^{\cdot}OH$ and $^{\cdot}CCl_3O_2$ produce almost 100% one-electron oxidized phenoxyl radical ($\lambda_{max} = 440$, 320 nm). On the other hand, the reactions of SAZ with $^{\cdot}OH$ and $^{\cdot}CCl_3O_2$ produce ~50% and ~60% one-electron oxidized phenoxyl radical ($\lambda_{max} = 450$ nm), respectively along with other transients. However, SP having both pyridine and benzene rings reacted with $^{\cdot}OH$, $N_3^{\cdot}$ and $^{\cdot}CCl_3O_2$ to produce transients corresponding to both the moieties.

The reactions of SAZ with e_{aq}^-, $^{\cdot}CO_2^-$ and $O_2^{\cdot -}$ produced delocalized radical anion ($\lambda_{max} = 420$, ~560 nm). 5-ASA reacted with e_{aq}^- to produce electron-adduct with benzene ring ($\lambda_{max} \sim 300$ nm) but solute-radical adduct ($\lambda_{max} = 270$ nm) is also produced with $^{\cdot}CO_2^-$. SP reacted with e_{aq}^- and $^{\cdot}CO_2^-$ to produce electron-adduct with benzene ring and pyridinyl radical. The reactions of $O_2^{\cdot -}$ with 5-ASA and SP have not been observed under our experimental conditions. It is clear from the above discussion that more than one transient are produced in the reactions of SAZ, 5-ASA and SP with various oxidizing and reducing radicals. Therefore, the decay rates and relative ratio of the transients produced have not been discussed.

5-ASA is the component that reaches the site of inflammation in IBD and is expected to act as an antioxidant by scavenging free radicals of various biomolecules in addition to primary radicals. Therefore, an attempt has been made to study these biologically relevant reactions directly using pulse radiolysis technique coupled with fast kinetic spectrophotometry. The reactions of 5-ASA and SAZ with lipid, lipid peroxyl, glutathiyl and one-electron oxidized tryptophanyl radical have been studied. The reactions of one-electron oxidized radical of SAZ, 5-ASA and SP with vitamin C have also been studied.

Reaction with lipid radicals

Lipids make the cell membrane and their reaction with $^{\cdot}OH$ radical (physiologically and pulse radiolytically produced) give lipid ($L^{\cdot}$) and lipid peroxyl ($LOO^{\cdot}$) radicals as shown below:

$$LH + {}^{\cdot}OH \rightarrow L^{\cdot} + H_2O \quad (13)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (14)$$

These radicals are produced in the oxidative stress and cause damage in a chain reaction as $LOO^{\cdot}$ can further oxidize lipid molecule to generate a lipid radical and hydroperoxide (LOOH):

$$LOO^{\cdot} + LH/\text{solute RH} \rightarrow LOOH + L^{\cdot}/R^{\cdot} \quad (15)$$

Reactions of solutes with $LOO^{\cdot}$ radical has been studied to investigate their antioxidant action in the lipid phase (Equation 15). Lipid (linoleic acid) has been dissolved in the water at pH 11 to form micelles since it is insoluble at pH 7. $LOO^{\cdot}$ has been generated *in situ* by radiolysis of N_2O and O_2 saturated (3:1) aqueous solution of linoleic acid (2×10^{-2} mol dm^{-3}) at pH 11. N_2O saturation converts e_{aq}^- into $^{\cdot}OH$ (Equation 2), which reacts with lipids (LH) producing lipid radical ($L^{\cdot}$) (Equation 13) followed by reaction of O_2 with carbon-centered lipid radicals ($L^{\cdot}$) producing lipid peroxyl radicals ($LOO^{\cdot}$) (Equation 14). Formation of solute radical (Equation 15) is followed to measure the rate constants. Both SAZ and 5-ASA have been found to scavenge $LOO^{\cdot}$ radical efficiently as measured by the formation of $SAZ^{\cdot}$ ($k = 4 \times 10^8$ mol^{-1} dm^3 s^{-1}) and $5\text{-ASA}^{\cdot}$ (8×10^8 mol^{-1} dm^3 s^{-1}) (see Tables I and II). 5-ASA scavenged $L^{\cdot}$ radical also with $k = 2 \times 10^8$ mol^{-1} dm^3 s^{-1}. This efficient scavenging of $LOO^{\cdot}$ by SAZ and 5-ASA makes them good antioxidant in the lipid phase also. Therefore, they can reduce the damage to lipid bilayer of the cells at least by acting as chain breaking antioxidants. However, SP has not been found to scavenge either $L^{\cdot}$ or $LOO^{\cdot}$.

Reaction with tryptophanyl radical

Tryptophanyl radical ($Trp^{\cdot}$) is produced in the reactions of proteins with oxidants in the first step before producing tyrosine radical followed by protein damage and cross-linking [32,33]. Electron transfer to $Trp^{\cdot}$ by antioxidant can prevent the damage to the protein molecule. $Trp^{\cdot}$ was generated by the reaction of tryptophan (TrpH) with one-electron oxidant, $N_3^{\cdot}$ radical (Equation 16). 5-ASA has been found to efficiently scavenge $Trp^{\cdot}$ at pH 7 (Equation 17) with $k = 7.3 \times 10^7$ mol^{-1} dm^3 s^{-1}.

$$TrpH + N_3^{\cdot} \rightarrow Trp^{\cdot} + N_3^- + H^+ \quad (16)$$

$$Trp^{\cdot} + 5 - ASA \rightarrow TrpH + 5 - ASA^{\cdot} \quad (17)$$

This value has been measured by following both the decay of $Trp^{\cdot}$ (510 nm) and formation of $5\text{-ASA}^{\cdot}$

(440 nm) radicals. However, SAZ and SP have not been found to scavenge Trp$^{\cdot}$ radical. This suggests that 5-ASA can reduce the damage to proteins by acting as radical scavenger as well as repair agent.

Reaction with glutathiyl radical

Thiyl radicals (RS$^{\cdot}$) generated in the cellular redox processes and antioxidant action are reactive oxidants [34–36]. Hence repair of thiyl radical is necessary for the storage of thiols (RSH) for their antioxidant activity as well as to protect lipids from thiyl radical attack. Thiyl radical is prepared by irradiation of N_2O saturated 1:1 water–ethanol solution (Equations 2, 18 and 19) at pH below 7 to avoid the formation of unwanted disulfide radical anion (RSSR$^{\cdot -}$) (Equation 20).

$$H^{\cdot}/{}^{\cdot}OH + RCH_2OH \rightarrow H_2/H_2O + RC^{\cdot}HOH \quad (18)$$

$$RC^{\cdot}HOH + RSH \rightarrow RS^{\cdot} + RCH_2OH \quad (19)$$

$$RS^{-} + RS^{\cdot} \rightleftharpoons RSSR^{\cdot -} \quad (20)$$

The transient absorption spectrum recorded for an aqueous ethanolic (50% v/v) solution containing 2.0×10^{-4} mol dm^{-3} 5-ASA, 1×10^{-3} mol dm^{-3} glutathione (GSH) at pH 4.1 is shown in Figure 7. This shows absorption maxima at 330, 430 and ~470 nm. The bimolecular rate constants for the growth of absorptions as measured at 330 and 430 nm are same ($\sim 7 \times 10^{7}$ dm^3 mol^{-1} s^{-1}). The transient absorption at 330 and 430 nm decay at the same rate also suggesting that both are due to the same radical (5-ASA$^{\cdot}$) as observed with other oxidants also. An additional absorption maximum at ~470 nm is also observed in this case. The transient absorption at 470 nm decays slowly (inset of Figure 7). We speculate that the absorption maxima at ~470 nm is due to solute-radical adduct (5-ASA···GS)$^{\cdot}$ produced in addition to phenoxyl radical (absorption at 430 nm). The absorption at 470 nm has not been observed to produce phenoxyl radical (430 nm) at a later stage. However, formation of product/s having absorption in this wavelength region has been observed by gamma radiolysis.

SAZ reacted with GS$^{\cdot}$ to produce transient absorptions at 560 and 430 nm (Figure 8). The transient absorption maximum at ~560 nm is formed rapidly ($k = 1.5 \times 10^{9}$ dm^3 mol^{-1} s^{-1}). There is also a simultaneous formation of transient absorption at 430 nm but at a much slower rate ($k = 6 \times 10^{7}$ mol^{-1} dm^3 s^{-1}) (inset of Figure 8). Transient absorption spectrum observed in this reaction is different from those observed in the reaction of SAZ and 5-ASA with azidyl radical. Therefore, the absorption at 560 nm is speculated to be due to solute-radical adduct (SAZ···GS)$^{\cdot}$ and that at 430 nm is due to phenoxyl radical on 5-ASA group. Transformation of this solute-radical adduct (560 nm absorption) into solute radical has not been observed in this case also. We presume that GS$^{\cdot}$ reacts with SAZ and 5-ASA to produce solute-radical adduct in addition to cause oxidation. SP, however, has not

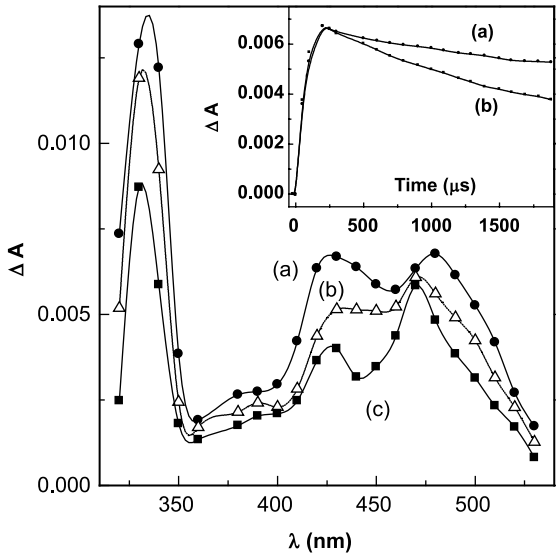

Figure 7. Transient absorption spectrum obtained from N_2O-saturated aqueous solution containing 5-ASA (2×10^{-4} mol dm^{-3}), ethanol (50% v/v) and glutathione (1×10^{-3} mol dm^{-3}) at pH 4.1 at (a) 300 μs, (b) 1000 μs and (c) 1880 μs. Dose = 16.2 Gy. *Inset*: kinetic traces at (a) 470 nm and (b) 430 nm under identical conditions.

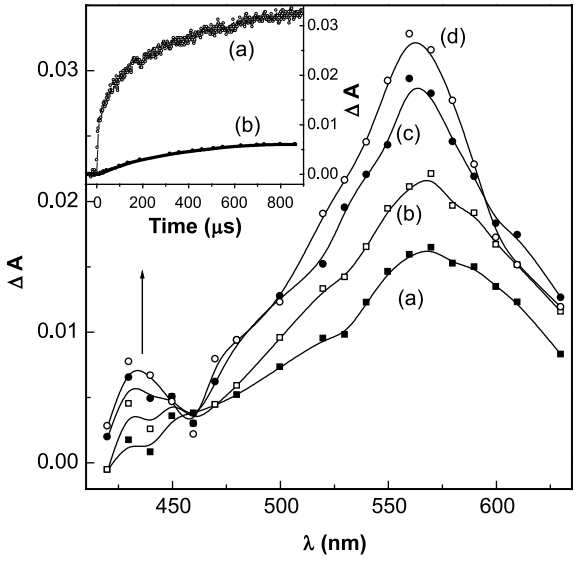

Figure 8. Transient absorption spectrum obtained from N_2O-saturated aqueous solution containing SAZ (1×10^{-4} mol dm^{-3}), ethanol (50% v/v) and glutathione (1×10^{-3} mol dm^{-3}) at pH 4.1 at (a) 45 μs, (b) 137 μs, (c) 410 μs (d) 865 μs. Dose = 18 Gy. *Inset*: kinetic traces at (a) 560 nm and (b) 430 nm under identical conditions.

been found to scavenge $GS^{\cdot}$ radical. It is to be noted that the pH value used in this study is low enough to avoid the reaction of $RS^{\cdot}$ with RS^{-} [37,38].

These studies clearly show that physiologically relevant oxidizing radicals ($LO_2^{\cdot}$, $Trp^{\cdot}$, $GS^{\cdot}$) are scavenged by the phenoxyl group of 5-ASA whereas the superoxide radical is efficiently scavenged by SAZ only.

Reaction of one-electron oxidized solutes with vitamin C

Vitamin C is a well-known water-soluble physiological antioxidant [29]. Vitamin C (ascorbic acid or $AscH_2$) has pk_a at 4.1 and 11.8 and exists as anion ($AscH^{-}$) at pH 7. Its one-electron oxidized radical ($Asc^{\cdot-}$) has absorption maximum at 360 nm with $\varepsilon = 3300\,mol^{-1}\,dm^3\,cm^{-1}$ [39]. Transient absorption spectrum for the reaction of 5-ASA$^{\cdot}$ with $AscH^{-}$ at pH 6.8 showed various maxima (Figure 9). The absorption maxima at ~360 and ~430 nm are due to $Asc^{\cdot-}$ [39] and 5-ASA, respectively. The absorption maxima observed at ~400 and 440–460 nm are new and also decayed at a slower rate (inset of Figure 9). We speculate that the absorption at 400 and ~460 nm are due to solute–radical complex, $(5\text{-}ASA\cdots AscH)^{\cdot-}$, in the transient state. The change in absorption is not much but transient absorption spectrum and decay rate of 5-ASA$^{\cdot}$ ($k = 1.5 \times 10^7\,mol^{-1}\,dm^3\,s^{-1}$) suggest that vitamin C scavenges 5-ASA$^{\cdot}$. Similarly, $SP^{\cdot}$ and $SAZ^{\cdot}$ have been found to be repaired by vitamin C ($AscH^{-}$) at a rate of $k = 2.7 \times 10^9\,mol^{-1}\,dm^3\,s^{-1}$ and $3 \times 10^8\,mol^{-1}\,dm^3\,s^{-1}$, respectively. In the reaction of $SP^{\cdot}$ with $AscH^{-}$, a simultaneous decrease in the transient absorption of

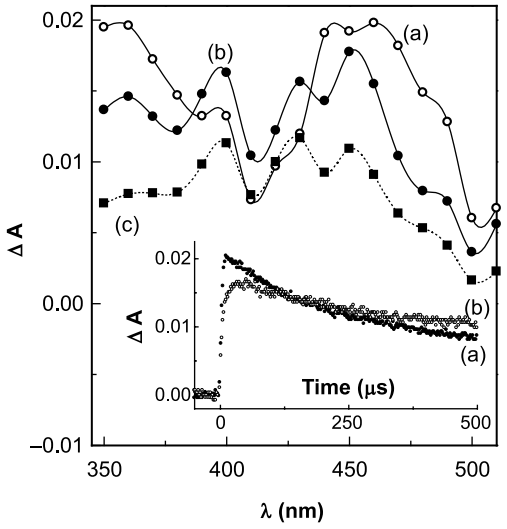

Figure 9. Transient absorption spectrum obtained from an aerated aqueous solution containing 5-ASA ($4 \times 10^{-4}\,mol\,dm^{-3}$), ascorbic acid ($4 \times 10^{-4}\,mol\,dm^{-3}$), tert-butanol ($2.5\,mol\,dm^{-3}$) and CCl_4 ($4 \times 10^{-2}\,mol\,dm^{-3}$) at pH 6.8 at (a) 10 μs, (b) 100 μs and (c) 400 μs. Dose = 21 Gy. *Inset*: kinetic traces at (a) 460 nm and (b) 380 nm under identical conditions.

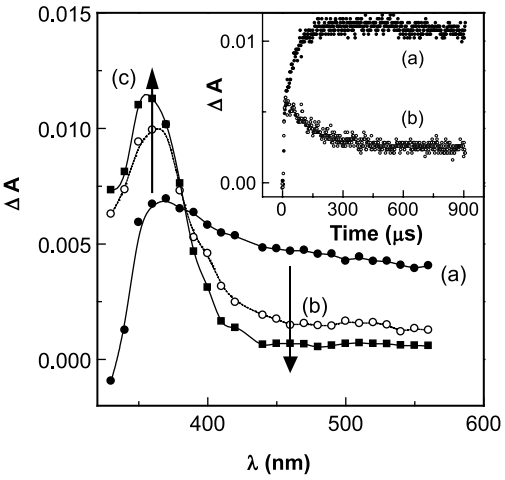

Figure 10. Transient absorption spectrum obtained from N_2O-saturated aqueous solution containing SP ($3.25 \times 10^{-4}\,mol\,dm^{-3}$), ascorbic acid ($1 \times 10^{-5}\,mol\,dm^{-3}$), and NaN_3 ($1 \times 10^{-1}\,mol\,dm^{-3}$) at pH 6.8 at (a) 25 μs, (b) 140 μs and (c) 285 μs. *Inset*: kinetic traces at (a) 360 nm and (b) 400 nm under identical conditions. Dose = 14 Gy.

$SP^{\cdot}$ (400–500 nm) and increase in $Asc^{\cdot-}$ (360 nm) is also clearly observed in the spectrum and kinetic traces (Figure 10).

Some new absorption bands have been observed in the reactions of 5-ASA$^{\cdot}$ with $AscH^{-}$ (400 nm, 440–460 nm); and $GS^{\cdot}$ radical with SAZ (at 560 nm) and 5-ASA (at 470 nm) in addition to known absorption bands of the transients. They have been speculated to be due to solute–radical adduct on the basis of absorption and decay characteristics only and need further detail investigation.

Conclusion

SAZ, 5-ASA and SP efficiently scavenged e_{aq}^{-}, $^{\cdot}CO_2^{-}$, $^{\cdot}OH$, $N_3^{\cdot}$ and $^{\cdot}CCl_3O_2$ radicals. 5-ASA scavenged glutathiyl, tryptophanyl and lipid peroxyl radicals also. SAZ scavenged lipid peroxyl and superoxide radical. However, 5-ASA and SP have not been found to scavenge superoxide radicals. It is evident from this study that 5-ASA is the most effective moiety involved in the antioxidant action of SAZ and its metabolites. In the antioxidant activity of SAZ, phenoxyl group of 5-ASA acts as electron (or H-atom) donor. One-electron oxidized SAZ, SP and 5-ASA are scavenged by ascorbate. This study suggests that free radical scavenging activity of 5-ASA may be the major mode of action of SAZ in IBD.

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