OXIDATION OF ANTHRACYCLINES BY PEROXIDASE

METABOLITES OF SALICYLIC ACID

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d) **Abbreviations**: ASA**,** acetylsalicylic acid (aspirin); SA-BPH and SA-BPQ, biphenol and biphenol quinone forms of SA, respectively; DNR, daunorubicin; DXR, doxorubicin (Adriamycin); LPO, lactoperoxidase; MPO, myeloperoxidase; NSAIDs, nonsteroidal anti-inflammatory drugs; SA, salicylic acid (generic term); HOOC-SA-OH, $^-$OOC-SA-OH, HOOC-SA-O$^\bullet$, neutral and anionic forms of salicylic acid and the respective phenoxyl radical; Q-QH$_2$, Q-QH$^\bullet$ and Q-Q, the quinone-hydroquinone moiety of anthracyclines, and the corresponding semiquinone and di-quinone forms.

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ABSTRACT

Oxidation of anthracyclines leads to their degradation and inactivation. This process is carried out by peroxidases in the presence of a catalytic co-factor, a good peroxidase substrate. Here we investigated the effect of salicylic acid, a commonly used anti-inflammatory and analgesic agent, on the peroxidative metabolism of anthracyclines. We report that at pharmacologically-relevant concentrations, salicylic acid stimulates oxidation of daunorubicin and doxorubicin by myeloperoxidase and lactoperoxidase systems and that efficacy of the process increases markedly on changing the pH from 7 to 5. This pH-dependence is positively correlated with the ease with which salicylic acid itself undergoes metabolic oxidation and which involves the neutral form of the acid (pKa = 2.98). When salicylic acid reacted with a peroxidase and H_2O_2 at acid pH (anthracyclines omitted), a new metabolite with absorption maximum at 412 nm was formed. This metabolite reacted with anthracyclines causing their oxidation. It was tentatively assigned to biphenyl quinone, formed by oxidation of biphenol produced by dimerization of salicylic acid-derived phenoxyl radicals. The formation of this product was inhibited in a concentration-dependent manner by the anthracyclines, suggesting their scavenging of the salicylate phenoxyl radicals. Altogether, this study demonstrates that oxidation of anthracyclines is mediated by peroxidase metabolites of salicylic acid, such as phenoxyl radicals and the biphenol quinone. Given that cancer patients undergoing anthracycline chemotherapy may be administered salicylic acid-based drugs to control pain and fever, our results suggest that liberated salicylic acid could interfere with anticancer and/or cardiotoxic actions of the anthracyclines.

INTRODUCTION

Acetylsalicylic acid (Aspirin, ASA) (Figure 1) is a commonly used analgesic and anti-inflammatory drug. ASA acts by inhibiting cyclooxygenase (COX) and lipoxygenase enzymes and suppresses release of prostaglandins. In vivo, ASA is rapidly metabolized to salicylic acid (SA, 2-hydroxybenzoic acid) (Figure 1) which, although unable to inhibit cyclooxygenase activity, still exerts anti-inflammatory properties. Recent studies suggest that SA and related non-steroidal anti-inflammatory drugs (NSAIDs) possess preventive and therapeutic anti-cancer properties (Thun et al., 2002; Andrews et al., 2002; Gwyn et al., 2002; Sotiriou et al., 1999). These functions are partly due to their inhibition of COX-2, an inducible form of COX that is over-expressed in cancer cells (Hussain et al., 2002).

Studies have shown that SA possesses both anti- and pro-oxidant properties, which are unrelated to its inhibition of COX. The antioxidant functions of SA are to due to its ability to intercept reactive oxygen and nitrogen products. It has been shown that in cell-free systems SA scavenges $^\bullet$OH radicals generated chemically (Fenton system) and radiolytically, to produce hydroxylated products 2,5- and 2,3-dihydroxybenzoic acids (Maskos et al., 1990). These reactions seemed to be so specific that dihydroxybenzoic acids have been used as an index of generation of $^\bullet$OH in vitro and in vivo (Floyd et al., 1986; Ramos et al., 1992; Sagone and Husney 1987; Davis et al., 1989). SA reacts with peroxynitrite (Kaur et al., 1997), quenches singlet oxygen (Kalyanaraman et al., 1993), and inhibits superoxide/NO-dependent LDL oxidation (Herman et al., 1999a).

Similar to other phenolic compounds, SA is a substrate for peroxidases. HRP/H_2O_2 and methemoglobin/H_2O_2 oxidize SA to the corresponding phenoxyl radical, as demonstrated using EPR (Shiga and Imaizumi, 1973, 1975). Incubation of SA with metmyogobin/H_2O_2 affords 2,3-

and 2,5-dihydroxybenzoic acids (Galaris et al., 1988). SA stimulates LDL oxidation by

MPO/H_2O_2 through the intermediacy of SA-derived phenoxyl radicals (Hermann et al., 1999b).

Ascorbate peroxidase and lactoperoxidase metabolize SA at acidic pH (Kvaratskhelia et al.,

1997; Muraoka and Miura, 2005). Stimulated granulocytes induce decarboxylation of SA,

however, no major role for MPO in this process was envisaged (Sagone and Husney 1987).

It has also been reported that NSAIDs modulate the cytotoxic action of anticancer agents

(Duffy et al., 1998; Inchiosa and Smith 1990). This aspect of NSAIDs' biochemistry is of

particular interest given that during chemotherapy cancer patients may also be administered

NSAIDs. Earlier we have reported that acetaminophen, a phenolic compound and the active

ingredient of the popular analgesic drug Tylenol, stimulates oxidation of the anticancer

anthracyclines doxorubicin (DXR) and daunorubicin (DNR) by peroxidases (Reszka et al.,

2004). Because the reaction leads to degradation of anthracyclines and loss of their anticancer

and cytotoxic activities, better understanding of this process and mechanisms' involved may be

important for clinical oncology. It was of interest to find out whether other phenolic compounds

also stimulate oxidative degradation of DNR(DXR). We were particularly interested in SA since

it may be used by cancer patients undergoing anthracycline chemotherapy. We report that at

pharmacologically-relevant concentrations (< 2 mM, Stead and Moffat, 1983), SA efficiently

stimulates oxidative degradation of DNR(DXR) by LPO(MPO)/H_2O_2 systems especially at

acidic pH. We also show that the peroxidative metabolism of SA gives rise to a redox-active

product, presumably of a bi-phenol type, which also mediates oxidation of anthracyclines.

MATERIALS AND METHODS

Chemicals - DNR (hydrochloride form) was obtained from the Drug Synthesis and

Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and

Diagnosis, National Cancer Institute, NIH (Bethesda, MD). Pharmaceutical preparation of DXR

(hydrochloride form) solution for injection (2 mg/mL) (Ben Venue Laboratories, Inc., Bedford,

OH) was purchased from The University of Iowa Hospitals and Clinics Pharmacy (Iowa City,

IA). LPO from bovine milk (EC 1.11.1.7), H_2O_2 (30%), salicylic acid (sodium salt) (SA) and

acetylsalicylic acid (ASA) were from Sigma (St. Louis, MO). MPO (1 mg/mL stock solution),

isolated from human neutrophils was a generous gift from Dr. Jerrold Weiss (University of

Iowa). All chemicals were of the highest purity available. The stock solution of DNR (10 mM)

were prepared in deionized water and stored at 4^oC. The concentrations of the reactants were

determined spectrophotometrically using appropriate extinction coefficients, $\varepsilon_{240} = 39.4$ M^{-1} cm^{-1}

for H_2O_2 (Nelson and Kiesow, 1972), $\varepsilon_{412} = 1.12x10^5$ M^{-1} cm^{-1} for LPO (Jenzer et al., 1986), ε_{480}

$= 1.15$ x 10^4 M^{-1} cm^{-1} for DNR(DXR) (Chaires et al., 1982). Because DNR and DXR tend to

form dimers in aqueous solutions, in the present study they were used in low micromolar

concentrations (< 10 μM) to assure that they were present predominantly as monomers.

Spectrophotometric measurements – Oxidation of anthracyclines was studied by

measuring their absorption spectra at designated time points. The spectra were measured using

an Agilent diode array spectrophotometer model 8453 (Agilent Technologies, Inc., Chesterfield,

MO). Samples were prepared in phosphate buffers (50 mM) for pH 6.0 - 8.0 and acetate buffers

(50 mM) for pH < 6. All measurements were performed at room temperature. Typically the

reaction was initiated by addition of a small aliquot H_2O_2 (5 or 10 μL) as the last component to a

sample consisting of DNR(DXR), SA, and LPO (or MPO) in buffer solution. Time course

measurements were carried out following changes in absorbance at 480 nm (λ_{max} for DNR and

DXR). Data were collected in 2, 5, or 10 s intervals during continuous stirring of the sample in a

spectrophotometric cuvette (1 cm light path). All experiments were repeated at least twice.

The initial rate of DNR(DXR) oxidation by MPO/H_2O_2/SA, V_i, was determined from the

initial linear portion of the A_{480} versus time traces using the method of linear regression. When

DNR(DXR) was oxidized by LPO/H_2O_2 in the presence of SA, curves of a Z-shape were

recorded. They were characterized by the maximum rate, V_{max}, determined by linear fitting to the

portion of the curve with the largest slope.

Oxidation of SA by LPO/H_2O_2 and MPO/H_2O_2 was determined by recording absorption

spectra of its metabolite showing maximum absorption at 412 nm and by measuring time course

of its formation in buffers of various pH and at various anthracycline concentrations.

Concentrations of the neutral form of SA, HOOC-SA-OH, were calculated using the known total

concentration of the salicylate, the pK_a of the SA carboxylic group of 2.98 (Lide, 2004-2005),

and the actual pH of the sample solution.

EPR measurements. EPR spectra were recorded using a Bruker EMX EPR spectrometer

(Bruker BioSpin, Billerica, MA), operating in X band and equipped with a high sensitivity

resonator ER 4119HS. Samples were prepared in pH 7.1 or 5.1 buffers (total volume 250 µL)

and the reaction was initiated by addition of H_2O_2 as the last component. To facilitate detection

of radicals, 400 µM DNR was used in these experiments. Similar experiments were carried out

with DXR. The sample was transferred to a flat aqueous EPR cell and recording was started 1

min after initiation of the reaction (H_2O_2 addition). The spectra were recorded using microwave

power 40 mW, modulation amplitude 2 G, receiver gain 2 x 10^6, conversion time 40.96 ms, time

constant 81.92 ms, and scan rate 80G/41.92 s. Spectra shown (Figure 10) are average of seven

scans and represent results of typical experiments.

RESULTS

Effect of salicylic acid on oxidation of anthracyclines by MPO/H_2O_2

The effect of SA on the peroxidative metabolism of DXR(DNR) was initially studied

using the conditions previously established for acetaminophen (Reszka et al., 2004). However,

when DXR in pH 7.0 buffer was exposed to MPO/H_2O_2 in the presence of micromolar

concentrations of SA, no changes in the absorption spectrum of DXR were observed, suggesting

that under these conditions DXR was not oxidized.[1] In contrast, oxidation of DXR became

evident when the reaction was carried out at acidic pH. Figure 2 shows spectra recorded at

selected time points following the addition of H_2O_2 to DXR/MPO/SA at pH 5.46. The decrease

in intensity of the drug's characteristic absorption band at 480 nm indicates that the drug

undergoes oxidation. When A_{480} reached a near zero level (indicating that almost all DXR was

consumed), a new absorption band with maximum at 412 nm began to emerge. As will be shown

later, this new band originates not from DXR, but from SA, and the corresponding metabolite is

assigned the symbol X.

Dependence of the reaction on pH was studied next by recording the time course of A_{480}

changes in buffers of various pH at constant initial concentration of SA of 0.5 mM. It may be

seen that the rate of the reaction increases as the pH decreases (Figure 3, inset A). Because the

SA carboxylic group (pKa = 2.98) (Lide, 2004-2005) is the only group that can be affected by

changes in pH in the studied pH range ~ 7 – 5, the observed stimulatory effects are attributed to

the higher concentration of the neutral (non-ionized) form of salicylic acid, HO-SA-COOH, at

acidic pH. Indeed, the initial rate of DXR oxidation, V_i, depends linearly on [HO-SA-COOH],

with the latter being calculated for a given pH (Figure 3, main panel). Similar results were

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obtained for DNR (not shown). This dependence of DXR(DNR) oxidation on pH is completely

opposite to that observed in the presence of acetaminophen, in which the maximum stimulation

was observed at near neutral pH, and no effect was observed at pH ~5 (Reszka et al., 2004). We

emphasize that ionization of the DXR(DNR) hydroquinone group does not change in this pH

range (pKa of first ionization of the drugs' hydroquinone moiety is ~9.5, Razzano et al., 1990)

and, accordingly, its redox potential should remain invariant at these pHs. These results further

support the idea that the observed dependence on pH should be linked to ionization status of the

co-factor and not the anthracyclines.

Simultaneously with measurements of drug oxidation we measured the formation of the

specie X versus pH. Inset B in Figure 3 shows the time course of absorption changes at 412 nm

at various pH. It is apparent that the appearance of the specie X is well correlated with the

complete oxidation of the anthracycline. Figure 4 shows that the initial rate of DXR oxidation

measured at various total [SA] but at one pH (5.25), changes linearly with [HO-SA-COOH],

additionally supporting the idea that the neutral form of SA is involved in the reaction.

Importantly, SA at very low concentration stimulated degradation of substantially higher

amounts of DXR. For example, [HO-SA-COOH] of 0.99 μM (100 μM [SA] total at pH 5.0) was

sufficient to decrease DXR concentration from the initial level of 18.8 μM by 16.1 μM, or by

~85.8 % (N = 2; [LPO] = 24 nM, $[H_2O_2]$ = 35 μM). This suggests that the SA metabolite had to

redox cycle several times to accomplish this level of degradation. When ASA was used instead

of SA, oxidation of DXR(DNR) was observed neither at neutral nor acidic pHs.

Effect of salicylic acid on oxidation of anthracyclines by LPO/H_2O_2

We also studied the capacity of LPO to support the reaction, as this peroxidase is highly

effective in oxidation of phenolic compounds (Monzani et al., 1997). Figure 5 (inset A) shows

that the rate of DNR oxidation by LPO/H_2O_2/SA increases as pH decreases, however, in contrast to the system with MPO, the A_{480} versus time traces assume Z-shape, indicating that the process is autocatalytic. The time course of DNR oxidation recorded at constant pH of 5.0 but at various [SA] also assumes Z-shape (Figure 5, inset B). Figure 5 (main panel) shows that the maximal rate of the reaction, V_{max}, increases linearly with [HO-SA-COOH]. Similar results were obtained for DXR (not shown). Based on these observations we infer that the stimulatory action by SA may also involve its secondary metabolite that is readily formed at acidic pH. This species is presumably formed by recombination of the primary metabolites of HOOC-SA-OH, the SA-derived phenoxyl radicals (HOOC-SA-O$^{\bullet}$), to corresponding bi-phenols (SA-BPH), which then are oxidized to biphenol quinone (SA-BPQ). It is known that oxidation of various phenolic compounds, e.g., phenol (hydroxybenzene), tyrosine or p-cresol leads to formation of the corresponding dimers, which are also substrates for peroxidases (Sawahata and Neal., 1982; Monzani et al., 1997; Bayse et al., 1972; Marquez and Dunford 1995).

Measurements of the position of the enzyme's Soret band during turnover were conducted next. Addition of H_2O_2 (5 μM) to DXR, SA and LPO (0.46 μM) in pH 5.0 buffer caused the peak at 412 nm (ferric LPO) to shift to 430 nm (LPO compound II)[2]. In this form the enzyme lived for ~ 45 s, after which it returned to native LPO. The corresponding spectral lines intersect at 421 nm, consistent with conversion of LPO-II to native LPO (Jenzer et al., 1986). During the LPO-II lifetime, the A_{480} decreased by $\Delta A_{480} = 0.041$, which corresponds to the loss of 3.6 μM DXR (Figure 6A). The presence of LPO in the form of compound II during the reaction suggests that reduction of LPO-II by HOOC-SA-OH is the rate-liming step. When the same experiment was repeated at pH 7.0, the decrease at 480 nm was very small (Figure 6B),

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[2]

(Δ[DXR] = 0.59 μM in 75 s) and the peak at 435 nm was observed for at least 20 min. This result

confirms that reaction of ionized SA (HO-SA-COO$^-$) with LPO-II at pH ~ 7 is very slow. When

SA was omitted, the amount of DXR degraded at pH 7.0 was nearly the same as when SA was

present, further confirming that SA is inactive at this pH (not shown).

Oxidation of salicylic acid by MPO and LPO systems.

We observed that oxidation of SA/DNR(DXR) at acidic pH by either LPO/H_2O_2 or

MPO/H_2O_2 generated species X, but only when the anthracycline was depleted. This suggested

to us that X could be derived from SA and not from DXR or DNR. Therefore we next studied the

formation of this metabolite in the absence of the anthracyclines.

Reaction of SA with MPO/H_2O_2 in pH 5.1 buffer generated spectrum with λ_{max} at 412 nm

(Figure 7A), which is attributed to the specie X. The absorbance at 412 nm, after reaching

maximum starts to decrease, suggesting that X is unstable (Figure 7, inset A). The formation of

this metabolite is pH-dependent as the rate of its formation increases sharply upon changing pH

from 6.4 to 5.0 (Figure 7, inset A). No peak at 412 nm was formed at pH ~ 7.0 and above, during

prolonged observation.[3] Thus, efficient metabolism of SA by MPO occurs only at acidic pH,

with maximum efficiency at pH 5.0, the lowest pH used in our experiments. The initial rate of

the formation of X changes linearly with [HOOC-SA-OH] (Figure 7A, inset B). Similar

observations were made when LPO was used instead of MPO. We tentatively assign the product

X to a SA-derived biphenol quinone (SA-BPQ), and analog of 4,4'-biphenol quinone generated

during enzymatic oxidation of phenol (hydroxybenzene) (Sawahata and Neal, 1982). We note

that the V_i versus pH relationship determined here for SA is opposite to that found for other

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phenolics, for which decrease in pH was associated with decrease in V_i (Monzani et al., 1997;

Marquez and Dunford 1995).

Effect of anthracyclines on the formation of species X.

Based on the observations that MPO(LPO)/H_2O_2/SA oxidizes DNR(DXR), that

MPO(LPO)/H_2O_2 oxidizes SA, and also that the SA metabolite (specie X) appears only when

DNR(DXR) is depleted, we asked how formation of X depends on anthracyclines. We therefore

studied formation of this product as a function of [DNR]. It was expected that if X, and/or its

precursors, reacts with DNR, the appearance of the 412 nm band should depend on [DNR]. In

Figure 8A are illustrated A_{412} versus time traces observed at [DNR] of 0, 3.9, 7.2 and 14.8 μM.

The figure shows that there is a distinctive [DNR]-dependent lag period, preceding the formation

of X. Concomitantly measured changes in absorbance at 480 nm indicate that in contrast to the

formation of X, oxidation of DNR starts immediately after the H_2O_2 addition (Figure 8B). These

results suggest that a precursor of X, or the X itself, may react with DNR. The latter possibility

was studied next.

Oxidation of anthracyclines by the peroxidase metabolite of SA, compound X

Subsequent experiments were designed to find out whether the specie X itself can react

directly with DNR (DXR). The metabolite was prepared by oxidation of SA in the absence of

anthracyclines, and when the absorption at 412 nm reached a maximum level, a small aliquot of

DNR solution was injected. Immediately after the DNR addition, the absorbance at 412 nm

decreased (Figure 9A), suggesting that the metabolite X was reduced. Subsequent addition of

H_2O_2 almost fully recovered X. The cycle, reduction of X by DNR and its re-oxidation by H_2O_2,

was repeated several times, without any significant loss of the compound X. This observation

strongly suggests that X has redox properties similar to that of a quinone/hydroquinone couple.

Simultaneously measured changes in absorbance at 480 nm showed that after DNR addition, A_{480}

momentarily increased and then rapidly returned to the A_{480} initial level, indicating that DNR

was completely oxidized (Figure 9B). Similar responses of X were observed when DNR was

replaced by DXR, reduced glutathione, ascorbate, NADH, or azide (not shown), confirming that
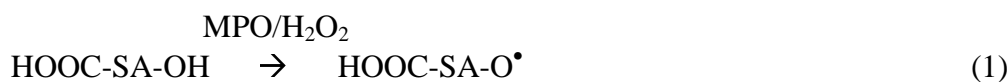
the species is a powerful oxidant.

EPR study

EPR measurements were carried out to find whether oxidation of the anthracyclines by

peroxidases in the presence of SA generates free radicals. When DNR(DXR) was incubated with

MPO/H_2O_2 in the presence of SA in pH 5.1 buffer, the EPR signal shown in Figure 10 (trace A)

was observed. The signal line width of 0.195 mT and g of 2.00479 are close to those reported

previously for DNR(DXR) radicals in other peroxidizing systems (Reszka et al., 2004; 2005a).

No signal was detected when SA was omitted (Figure 10, trace B), or when the reaction was

carried out at pH 7.0 (not shown). The dependence of the signal on pH corroborates our results

of spectrophotometric measurements. These results are consistent with the mechanism whereby

the anthracycline hydroquinone moiety undergoes oxidation to the corresponding semiquinone

by a SA-derived metabolite(s). We did not observe any EPR signals from control samples

consisting of $SA/peroxidase/H_2O_2$ in acidic buffer. Although oxidation of SA by HRP/H_2O_2 or

$methemoglobin/H_2O_2$ generates phenoxyl radicals (Shiga and Imaizumi 1973; 1975), they cannot

be detected using stationary EPR.

DISCUSSION

The structure of anthracyclines contains a hydroquinone moiety (Figure 1, ring B in DXR and DNR), and therefore, they are susceptible to oxidation. However, LPO/H_2O_2 and MPO/H_2O_2 alone do not oxidize the anthracyclines. This is despite the fact that p-hydroquinone itself is a very good substrate for these enzymes. The purpose of our study was to determine whether SA can stimulate oxidation of DXR and DNR by these enzymatic systems. The rationale for this was that: (1) SA is the major metabolite of the commonly used analgesic and anti-inflammatory drug aspirin, ASA. It may be used by cancer patients undergoing chemotherapy, so both SA and the anthracyclines may co-localize in tissues; (2) SA is a phenolic compound, and the peroxidative metabolism of phenols affords reactive metabolites, phenoxyl radicals, which can react with other substrates causing their oxidation. Our earlier study showed that in the presence of acetaminophen, also a phenolic compound, both isolated MPO and MPO-rich HL-60 cells readily oxidize anthracyclines (Reszka et al., 2004); (3) Because oxidation of anthracyclines leads to their inactivation (Cartoni et al., 2003; Reszka et al., 2005b), this reaction may be of clinical importance.

Our study shows that SA stimulates oxidation of DNR(DXR) by peroxidases, but does this in a pH-dependent fashion. The stimulatory effect increases as the pH decreases from 7 to 5, which parallels the dependence on pH of the peroxidative metabolism of SA itself. These observations suggest that oxidation of the anthracyclines is mediated by a SA metabolite, and that the protonated (neutral) form of SA (HOOC-SA-OH) is the preferred substrate for peroxidases. Oxidation of phenols yields the respective phenoxyl radicals (Shiga and Imaizumi 1973, 1975; Monzani et al., 1997; Marquez and Dunford 1995; Hermann et al., 1999b) and, accordingly, oxidation of SA should yield the corresponding phenoxyl radical, HOOC-SA-O$^{\bullet}$, as

described by Eq 1, using MPO as a typical peroxidase. The resulting radicals may dimerize,

forming a corresponding bi-phenol (SA-BPH) (Eq 2), or react with other substrates. We propose

that in the presence of DXR or DNR, the SA-derived phenoxyl radicals react with the quinone-

hydroquinone group (Q-QH$_2$) of the anthracyclines, causing its oxidation to a semiquinone

radical (Q-QH$^\bullet$) (Eq 3). During this reaction the phenoxyl radical is reduced back to HOOC-SA-

OH.

$$\text{HOOC-SA-OH} \xrightarrow{\text{MPO/H}_2\text{O}_2} \text{HOOC-SA-O}^\bullet \tag{1}$$

$$2\ \text{HOOC-SA-O}^\bullet \rightarrow (\text{HOOC-SA-OH})_2\ (\text{SA-BPH}) \tag{2}$$

$$\text{HOOC-SA-O}^\bullet\ +\ \text{Q-QH}_2 \rightarrow \text{HOOC-SA-OH}\ +\ \text{Q-QH}^\bullet \tag{3}$$

This mechanism is supported by the observation that a substantial degradation of DXR

has been accomplished even when [HOOC-SA-OH] << [DXR], since this indicates that SA had

to make several redox cycles HOOC-SA-OH $\rightarrow$ HOOC-SA-O$^\bullet$ $\rightarrow$ HOOC-SA-OH in order to

uphold the oxidation of the much higher concentrations of the drug. Thus, the cycling of the

HOOC-SA-OH/HOOC-SA-O$^\bullet$ couple sustains continuous oxidation of the drug. The scheme in

Figure 11 illustrates the proposed mechanism of this pro-oxidant action of SA. In contrast to SA,

ASA appeared to be inactive. The most likely reason behind this is that in ASA the phenolic

group has been blocked by acetylation (Figure 1) and, accordingly, the compound is not

metabolized by peroxidases and does not generate phenoxyl radicals.

The dependence of the reaction on pH is not unexpected, since there is precedence with

nitrite and acetaminophen (Reszka et al., 2001, 2004). What was unusual was the direction of

these changes, as they do not follow the trend of oxidation of phenols versus pH. It has been

reported that the rate of oxidation of p-cresol decreases on changing the pH from neutral to

acidic values, and this effect was correlated with protonation of an amino acid with pKa ~ 5.8

near the enzyme active site, presumably histidine (Monzani et al., 1997). p-Cresol does not have

carboxylic group. So, its ionization status is not affected by changes in pH. That the dependence

on pH observed in our study is primarily due to protonation of the SA anion, and much less due

to changes in the enzyme' reactivity, is demonstrated by the very good linear correlation between

the rate of SA oxidation at various pH and the actual content of the neutral form of the

compound (HOOC-SA-OH). Similarly good correlation was found between the rate of DXR and

DNR oxidation and the concentration of HOOC-SA-OH at various pH. Thus, we conclude that

oxidation of SA to phenoxyl radicals (Eq 1) involves mostly the neutral form of the compound.

The observation that the SA-dependent oxidation of anthracyclines occurs at acid pHs is relevant

to the situation in vivo, since the extracellular pH of solid tumors, against which anthracyclines

are frequently used, is acidic. pH as low as 6.1 occurs with some types of tumors (Gillies et al.,

2002).

Oxidation of SA in acid solutions gives rise to a product X, which shows maximum

absorption at 412 nm. Based on the known chemistry of phenoxyl radicals and using the simplest

phenol (hydroxybenzene) as a reference, we tentatively identify this product as the respective

biphenol quinone (SA-BPQ). This compound could be formed by enzymatic oxidation of a

biphenol, which is the product of recombination of phenoxyl radicals. Although oxidation of

phenol can produce 2,2'- and 4,4'- biphenol quinones, only the latter one shows the

characteristic intense absorption at 398 nm (Sawahata and Neal, 1982). Therefore, by analogy to

oxidation of phenol to 4,4'-biphenol quinone, the species X could be assigned to the 5,5'-

biphenol quinone (5,5'-SA-BPQ), in which the >C=O functions are in *para* position. The

structure of this compound and the proposed mechanism of its formation are shown in Figure 11.

The probable reason why this dimeric product is efficiently formed in acid solutions is the low pKa value of the –COOH group in SA phenoxyl radicals, ~ 3 (Neta and Fessenden, 1974). Dimerization of these radicals is facile when their carboxyl group is protonated. In contrast, recombination of SA - derived phenoxyl radical anions ($^\bullet$O-SA-COO$^-$) may be hindered due to repulsion of their negative charges. Thus, as the pH increases from 5 to 7, the proportion of neutral radicals become so low, that any dimers formed are below the detection limit (but see footnote 3).

We found that the SA derived biphenol quinone can be reduced by DNR (Figure 9A). The resulting biphenol was re-oxidized with H_2O_2 and reduced again by a second dose of the drug. The reduction of 5,5'-SA-BPQ was concomitant with DNR oxidation. Thus, in the peroxidase/H_2O_2/SA system, oxidation of anthracyclines can be carried out by both the SA-derived phenoxyl radical and the SA-derived biphenol quinone (Figure 11, step 1 and 2, respectively). The former reaction seems to play a more important role since presence of anthracyclines inhibits formation of biphenols (Figure 11, step 1).

We have previously shown that oxidation of anthracyclines can be inhibited by ascorbate or reduced glutathione (Reszka et al., 2004). Therefore, the efficacy of this reaction in vivo will certainly depend on the presence of endogenous antioxidants, and may become evident under conditions of oxidative stress, when these antioxidants are depleted. We emphasize that redox cycling of anthracyclines might promote development of oxidative stress.

Our results show that oxidation of anthracyclines leads to their irreversible bleaching suggesting a significant modification of their chromophores. In agreement with this, recent studies revealed that oxidation of anthracyclines leads to their degradation to low molecular weight products, 3-methoxyphthalic acid and 3-methoxysalicylic acid (Cartoni et al., 2003;

Reszka et al. 2005b; Bomgaars et al., 1997). This degradation could be mediated by the drugs'-derived semiquinone radicals (Q-QH$^\bullet$), which decay, presumably, by disproportionation to the parent drug and the electron deficient di-quinone, Q-Q, (2 Q-QH$^\bullet$ $\longleftrightarrow$ Q-QH$_2$ + Q-Q). It seems likely that subsequent reactions of this di-quinone species could give rise to the ultimate colorless stable products. We emphasize that semiquinone radicals generated by oxidation of anthracyclines (Q-QH$^\bullet$) differ from the better known radicals formed by metabolic reduction ($^\bullet$QH-QH$_2$). The latter can reduce O$_2$ to superoxide, which restores the drug to its original form (Kalyanaraman et al., 1980). In contrast, semiquinones formed by oxidation undergo structural modifications.

It has been reported that products of anthracycline oxidation are virtually non-toxic to human leukemia HL-60 cells, human prostate cancer PC3 cells and to rat heart cardiomyocytes (H9c2) (Reszka et al. 2005b). These results agree with lower toxicity of 3-methoxyphthalic acid in H9c2 cells reported by another group (Cartoni et al., 2004) and with the lower toxicity of photochemically degraded DXR in P388 murine leukemia cell line (Bomgaars et al., 1997). Altogether, these observations rise the possibility that oxidation of anthracyclines in vivo may suppress their therapeutic activity. Because cancer patients undergoing anthracycline chemotherapy may be administered salicylates to control pain and inflammation, possible complications and decreased anticancer activity of the drugs should be considered. One possible beneficial effect of the drugs' degradation could be reduced cardiotoxicity as suggested by results of in vitro studies on toxicity of anthracycline degradation products in mouse cardiomyocytes (Cartoni et al. 2004, Reszka et al., 2005b). Together these results suggest that it should be possible to modulate inactivation of the anthracyclines in vivo by pharmacological interventions, using stimulants or inhibitors of peroxidative processes.

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Footnotes

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Results of preliminary studies were presented at: Second International Conference on Prostate Cancer Research, Iowa City, IA, October 12-15, 2002. Abstract: Reszka KJ, Britigan LH, McCormick ML, Britigan BE, and Spitz DR (2002) Do pain killers counteract the anticancer action of anthracyclines?

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Numbered Footnotes

In page 9:

1. This refers only to low, pharmacological concentrations of SA, as oxidation of anthracyclines was readily accomplished at pH 7.0 when using high, cytotoxic concentrations of SA, 10 mM.

In page 11:

2. The actually measured λ_{max} was 414 nm for native LPO and 435 nm after H_2O_2 addition
    (assigned to LPO-II). These apparent red shifts in peaks positions must be due to the fact that
    these peaks are on the up-hill slope of the DXR absorption spectrum. When DXR was
    omitted the corresponding spectra, measured before and after H_2O_2, addition showed λ_{max} at
    412 nm and 430 nm, respectively, as expected for ferric and compound II forms of LPO
    (Jenzer et al., 1986).

In page 12:

3. The absence of this metabolite at pH 7 refers to low, pharmacologically relevant
    concentrations of SA. When the concentration of SA was increased to 10 mM, formation of
    this metabolite was apparent even at pH 7 (not shown). Note that at pH 7.0 and 10 mM SA,
    the concentration of the neutral form of SA is nearly the same as from 0.1 mM SA at pH 5.0.

Legends for Figures

Figure 1. Structures of acetylsalicylic acid (aspirin, ASA), salicylic acid (SA) and

anthracyclines. HOOC-SA-OH designates the neutral form of SA.

Figure 2. Absorption spectra recorded during oxidation of DXR by MPO/H_2O_2 in the presence

of SA in pH 5.46 buffer. Spectra 1-14 were recorded 0, 24, 40, 60, 80, 100, 120, 142, 150, 170,

210, 250, 320, and 330 s after H_2O_2 addition. [DXR] = 9.3 μM, [SA] = 0.5 mM, [H_2O_2] = 52

μM, [MPO] = 0.5 μg/mL.

Figure 3. Oxidation of DXR by MPO/H_2O_2/SA at various pH. Plot of the initial rate of DXR

oxidation, V_i , (dA_{480}/dt)$_i$, versus [HOOC-SA-OH]. Mean +/-SD from two independent

experiments. Inset A: Time course of A_{480} changes at pH 5.0, 5.5.22, 5.4, 5.76, 5.82, 7.02 (traces

a – f, respectively). Inset B: Concomitantly measured A_{412} versus time changes attributed to

species X. [DXR] = 9.3 μM, [SA] = 0.5 mM, [H_2O_2] = 52 μM, [MPO] = 0.5 μg/mL.

Figure 4. Oxidation of DXR by MPO/H_2O_2/SA: Plot of the initial rate of DXR oxidation, V_i ,

(dA_{480}/dt)$_i$, versus [HOOC-SA-OH] determined at pH 5.25. Mean +/-SD from two experiments.

Inset: Time course of A_{480} changes at [SA total] of 25, 125, 250, 500, and 1250 μM for traces a –

e, respectively. Typical results. [DXR] = 9.3 μM, [H_2O_2] = 52 μM, [MPO] = 0.25 μg/mL.

Figure 5. Oxidation of DNR by LPO/H_2O_2/SA. Plot of the maximal rate of DNR oxidation,

V_{max}, (dA_{480}/dt)$_{max}$, versus [HOOC-SA-OH] for the reaction at pH 5.0. Inset: Typical A_{480} versus

time traces recorded at [SA] of 25, 50, 100, 150 and 200 μM (total concentrations) (traces a – e,

respectively) at pH 5.0. [DNR] = 10 μM, [H_2O_2] = 25 μM, [LPO] = 22 nM. Inset B: Time course

of A_{480} changes at pH 7.00, 5.97, 5.77, 5.53, 5.45, 5.25 (traces a-f, respectively). [DNR] = 10

μM, [H_2O_2] = 25 μM, [LPO] = 22 nM, [SA] = 0.25 mM (total).

Figure 6. Absorption spectra and position of the LPO Soret band observed during oxidation of

DXR by LPO/H_2O_2/SA in pH 5.0 buffer (A) and pH 7.0 buffer (B). [DXR] = 11.4 μM, [LPO] =

0.46 μM, [SA] = 25 μM (total), [H_2O_2] = 5 μM. Spectra 1 – 14 in panel A were recorded 0, 5,

10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 75 s after H_2O_2 addition. Spectra 1 – 8 in panel B were

recorded 0, 5, 10, 15, 20, 35, 60, 75 s after H_2O_2 addition. No changes in A_{480} were observed at

longer times of the reaction.

Figure 7. Oxidation of SA by MPO/H_2O_2 in acetate buffer pH 5.1. Absorption spectra (a – i)

were recorded at 0, 10, 30, 50, 70, 110, 150, 190, and 270 s after start of the reaction (H_2O_2

addition). [SA] = 1 mM (total), [MPO] = 0.25 μg/mL, [H_2O_2] = 25 μM. Inset A: Typical time

course of absorption changes at 412 nm recorded in buffers of various pH. Arrow indicates

direction of changes. Traces a - e were recorded at pH 6.47, 5.94, 5.5, 5.32, and 5.06,

respectively. Inset B: Plot of the initial rate of a metabolite formation, V_i , (dA_{412}/dt), versus

[HOOC-SA-OH] based on data in inset A. [MPO] = 0.05μg/mL, [H_2O_2] = 29 μM.

Figure 8. Oxidation of SA by MPO/H_2O_2 at pH 5.06: Effect of DNR. (A) Time course of the

formation of specie X, measured at 412 nm, in the presence of 0, 3.9, 7.2, and 14.8 μM DNR

(traces a – d, respectively). (B) Simultaneously recorded time course of absorption changes at

480 nm showing oxidation of DNR. Note that DNR oxidation starts immediately after H_2O_2

addition. In contrast, the absorption band with maximum at 412 nm appears with a lag time which depends on [DNR], and after A_{480} reaches a minimum. [SA] = 1 mM (total), [MPO] = 0.05 μg/mL, [H_2O_2] = 116 μM.

Figure 9. (A) Quenching of X by DNR at pH 5.06. The species X was generated by oxidation of SA (1 mM) by MPO (0.05 μg/mL) and H_2O_2 (29 μM). When the absorbance at 412 nm reached maximum, aliquots of DNR solution (1.4 μM) and H_2O_2 (5.8 μM; 2nd and 3rd dose) were added (as indicated by arrows). (B) Simultaneously measured changes in absorbance at 480 nm indicate that the added DNR was completely oxidized by X.

Figure 10. EPR spectra generated by oxidation of DXR by (A) MPO/H_2O_2/SA at pH 5.1. [DXR] = 0.4 mM, [MPO] = 4 μg/mL; [H_2O_2] ~ 0.4 mM, [SA] = 1 mM. Spectrum B was recorded with SA omitted. Similar spectra were observed when DXR was replaced by DNR.

Figure 11. Proposed mechanism of the stimulatory action of SA in the oxidation of DNR(DXR) by peroxidases. Q-QH$_2$ and Q-QH$^\bullet$ designate the quinone-hydroquinone moiety of the anthracyclines (rings C and B) and the corresponding semiquinone radical, respectively. HOOC-SA-OH and HOOC-SA-O$^\bullet$ are the protonated (neutral) forms of salicylic acid and the corresponding phenoxyl radical. 5,5'-SA-BPH and 5,5'-BPQ represent the SA-derived biphenol and the corresponding biphenol quinone.

Figure 1

ASA

HOOC-SA-OH
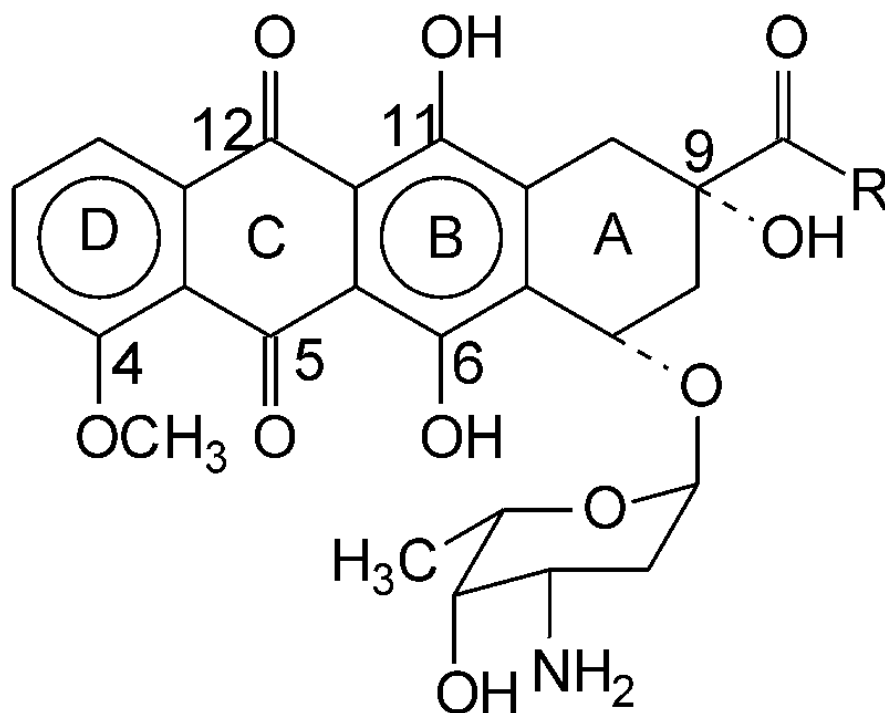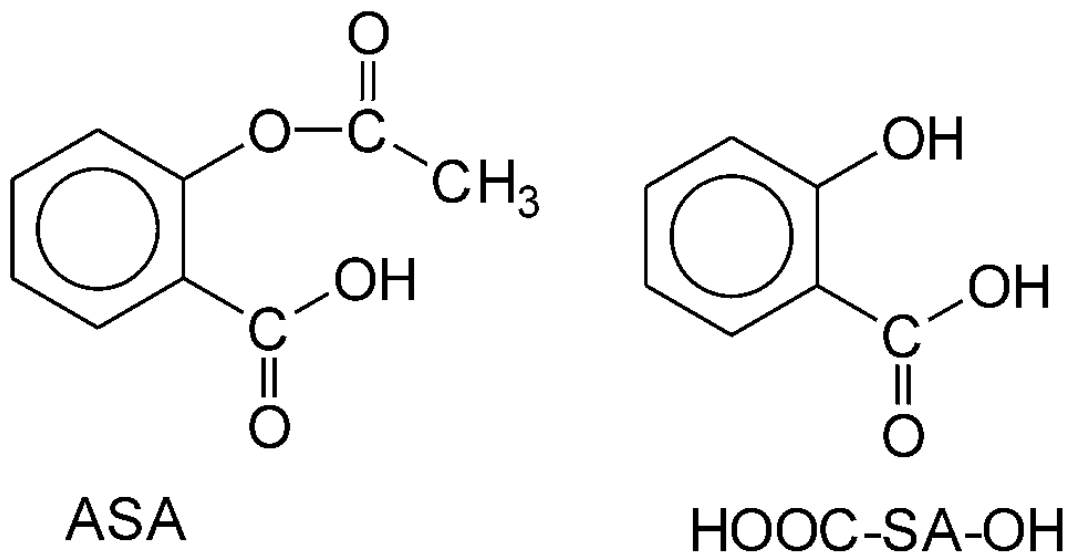
R: -CH$_3$ Daunorubicin (DNR)

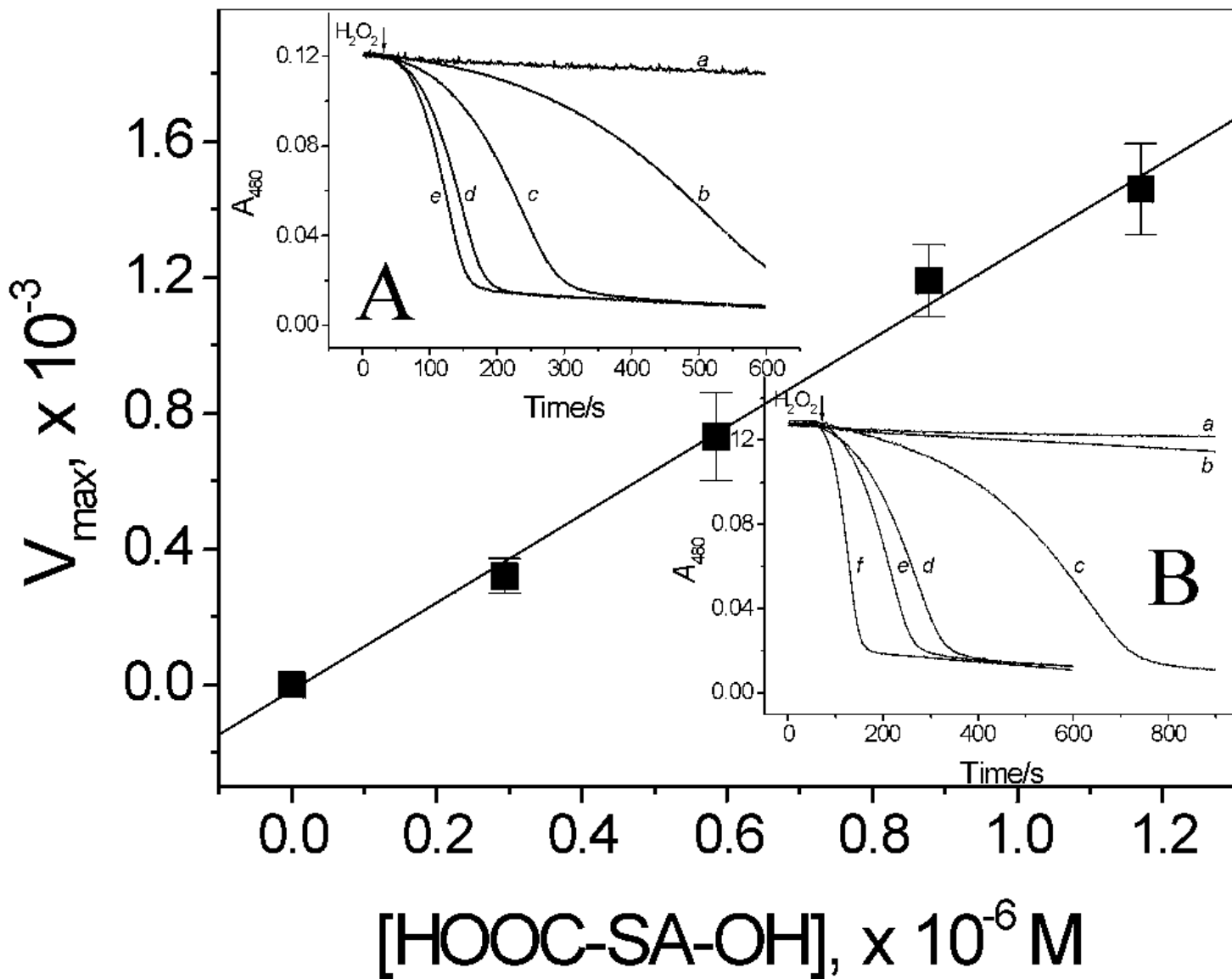R: -CH$_2$OH Doxorubicin (DXR, Adriamycin)

Figure 2
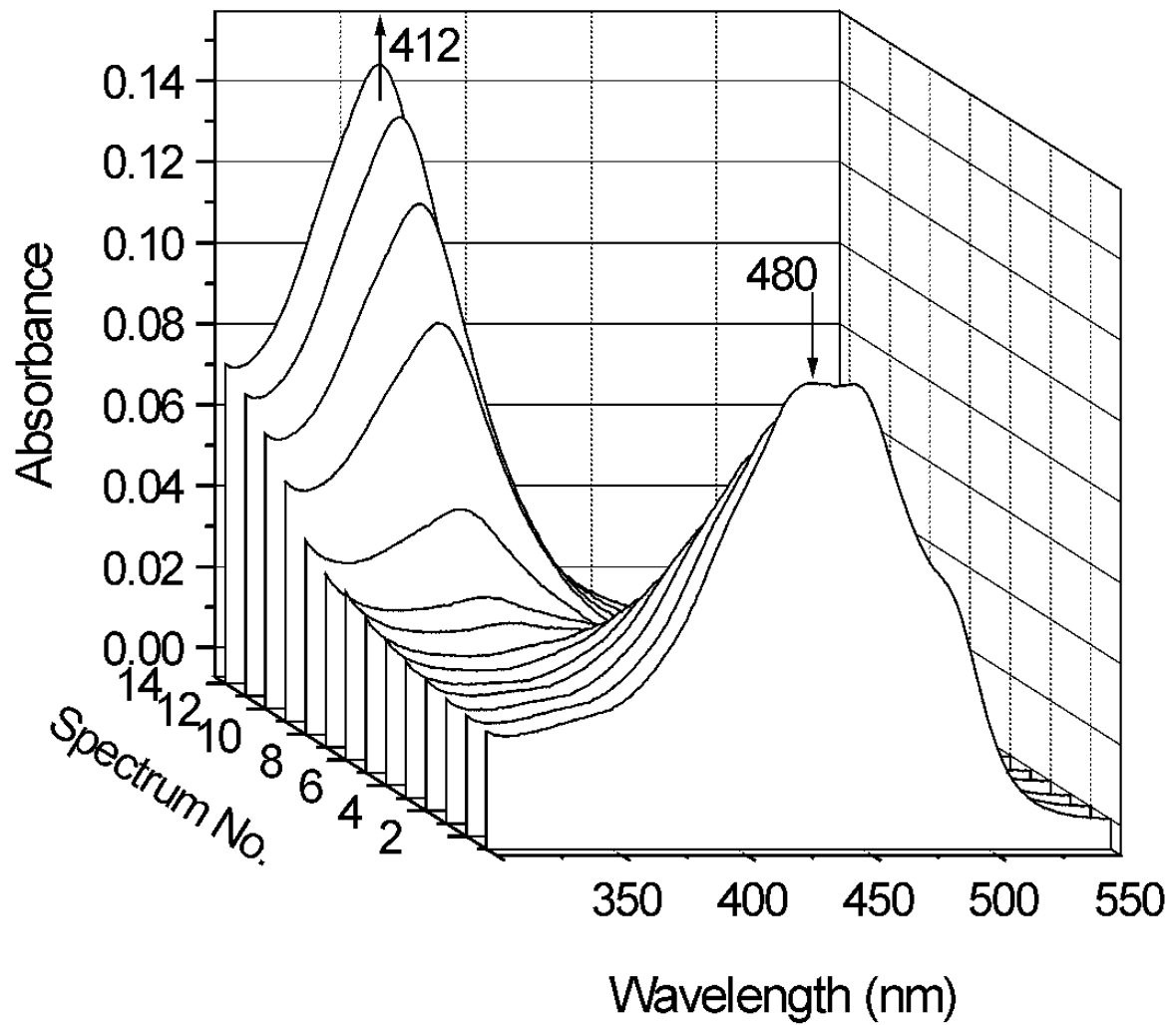
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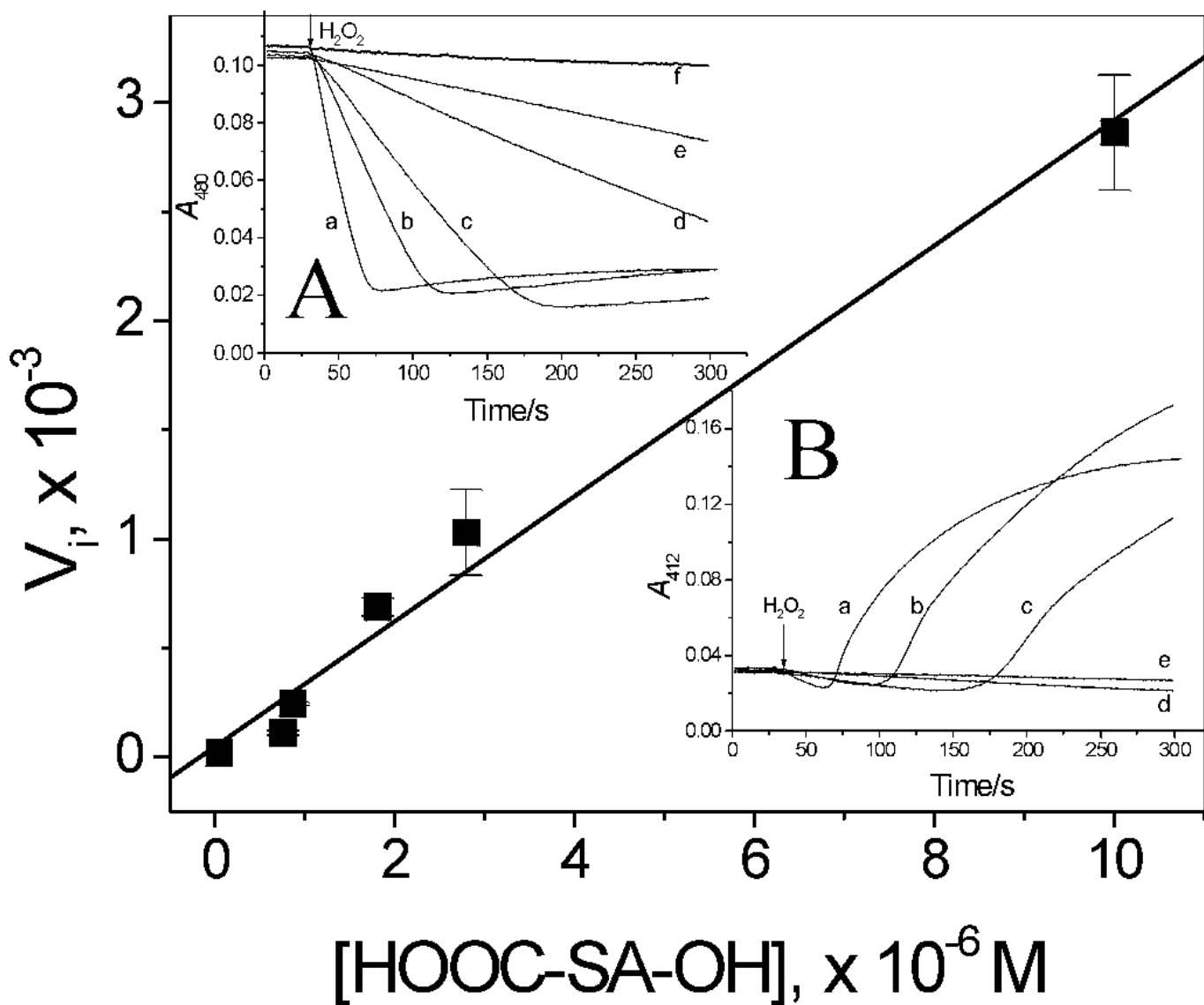
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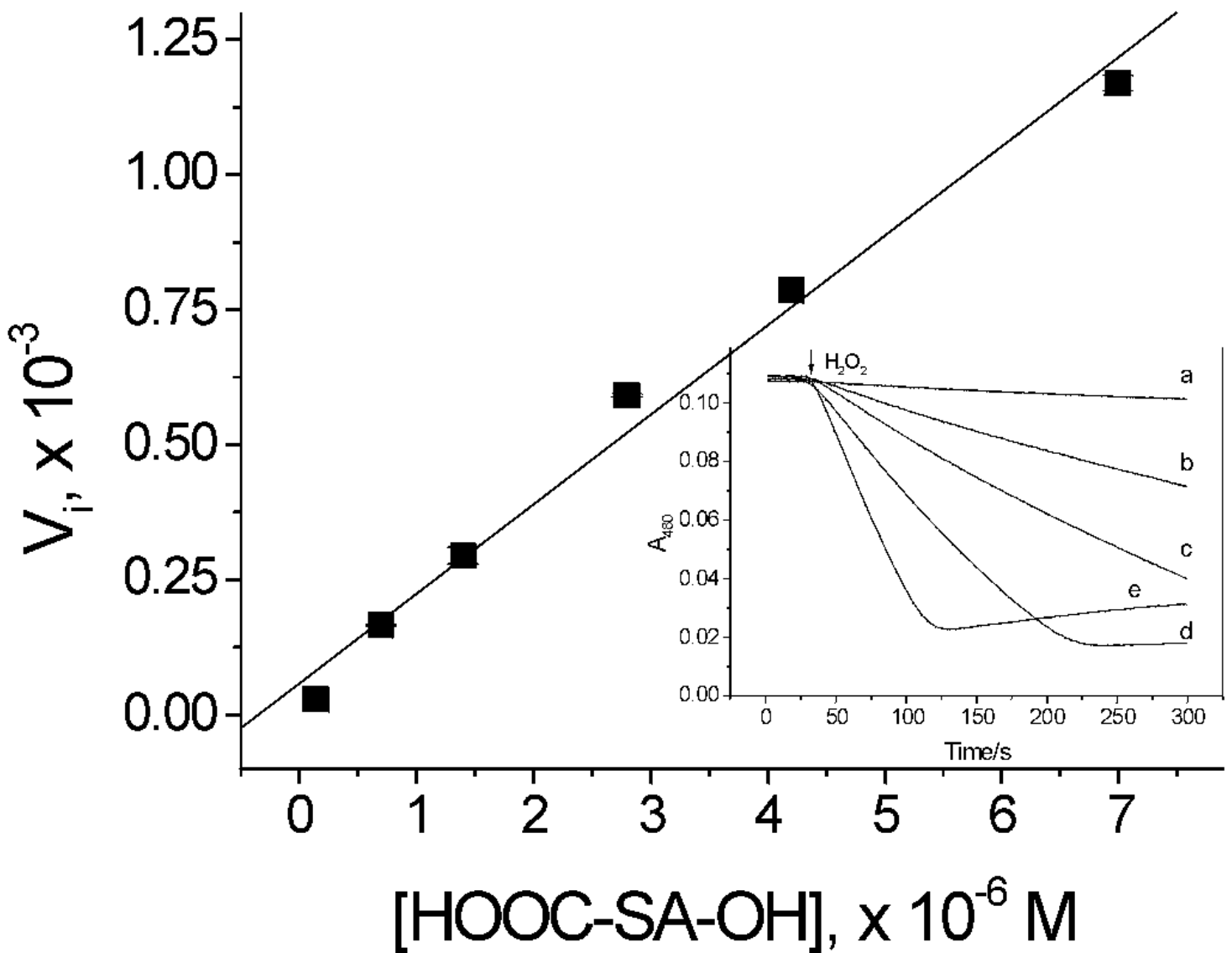
Figure 5

Figure 6
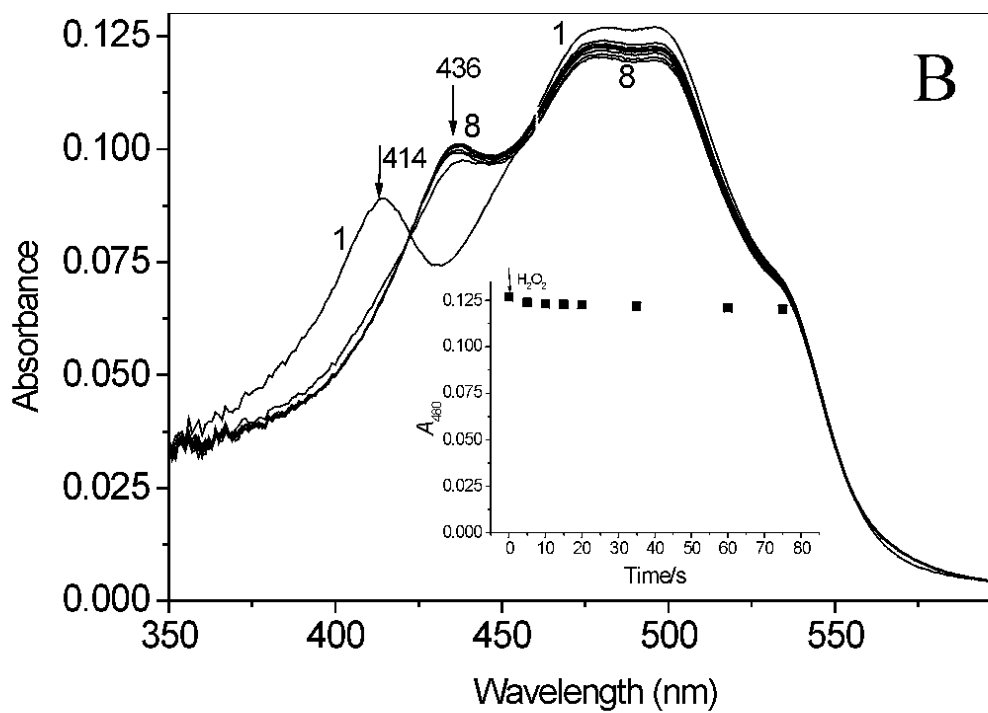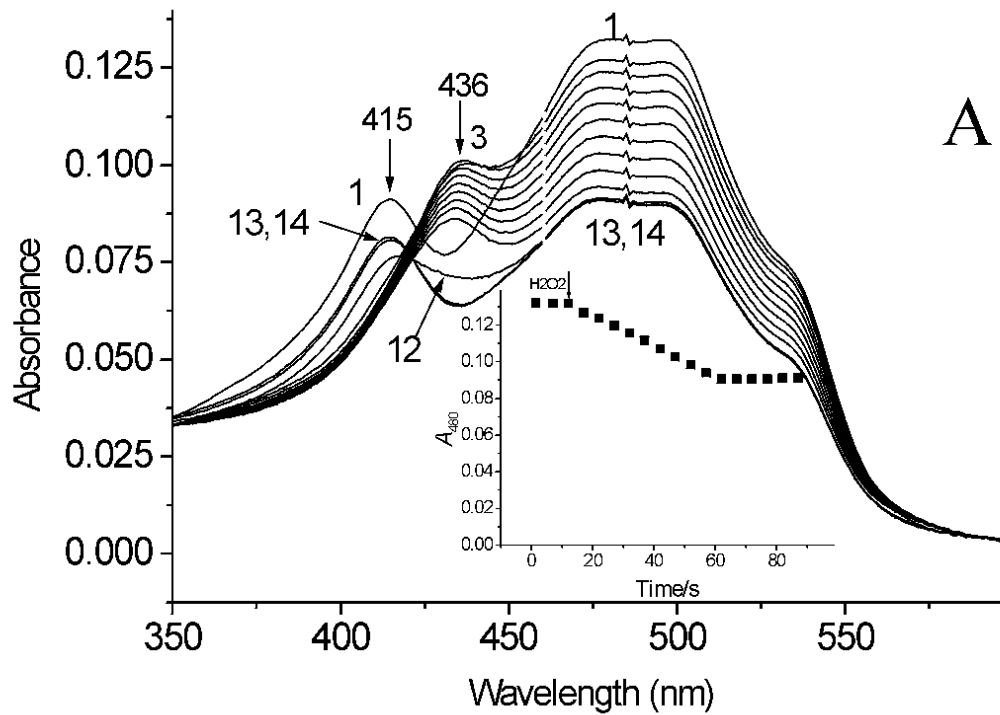
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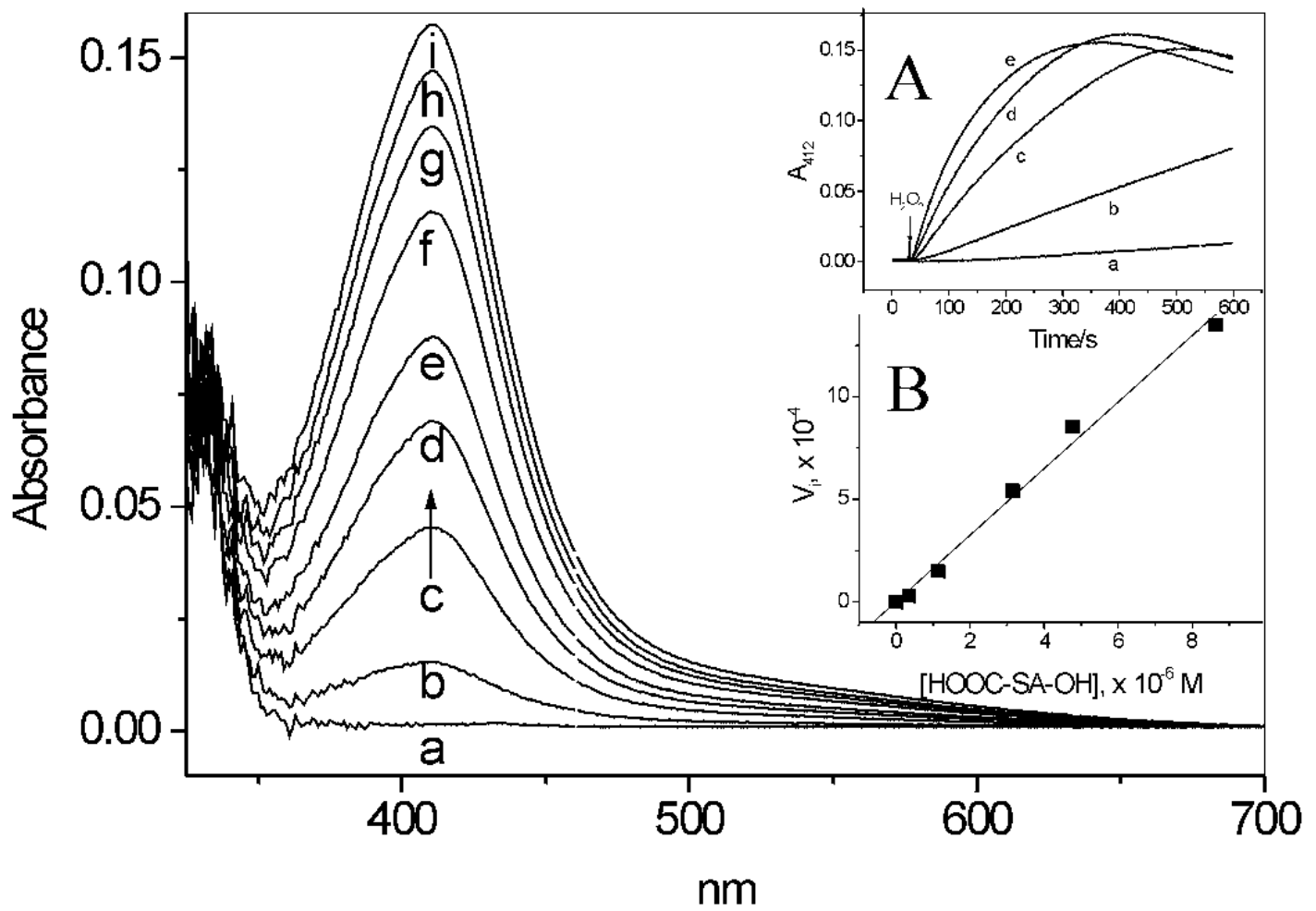
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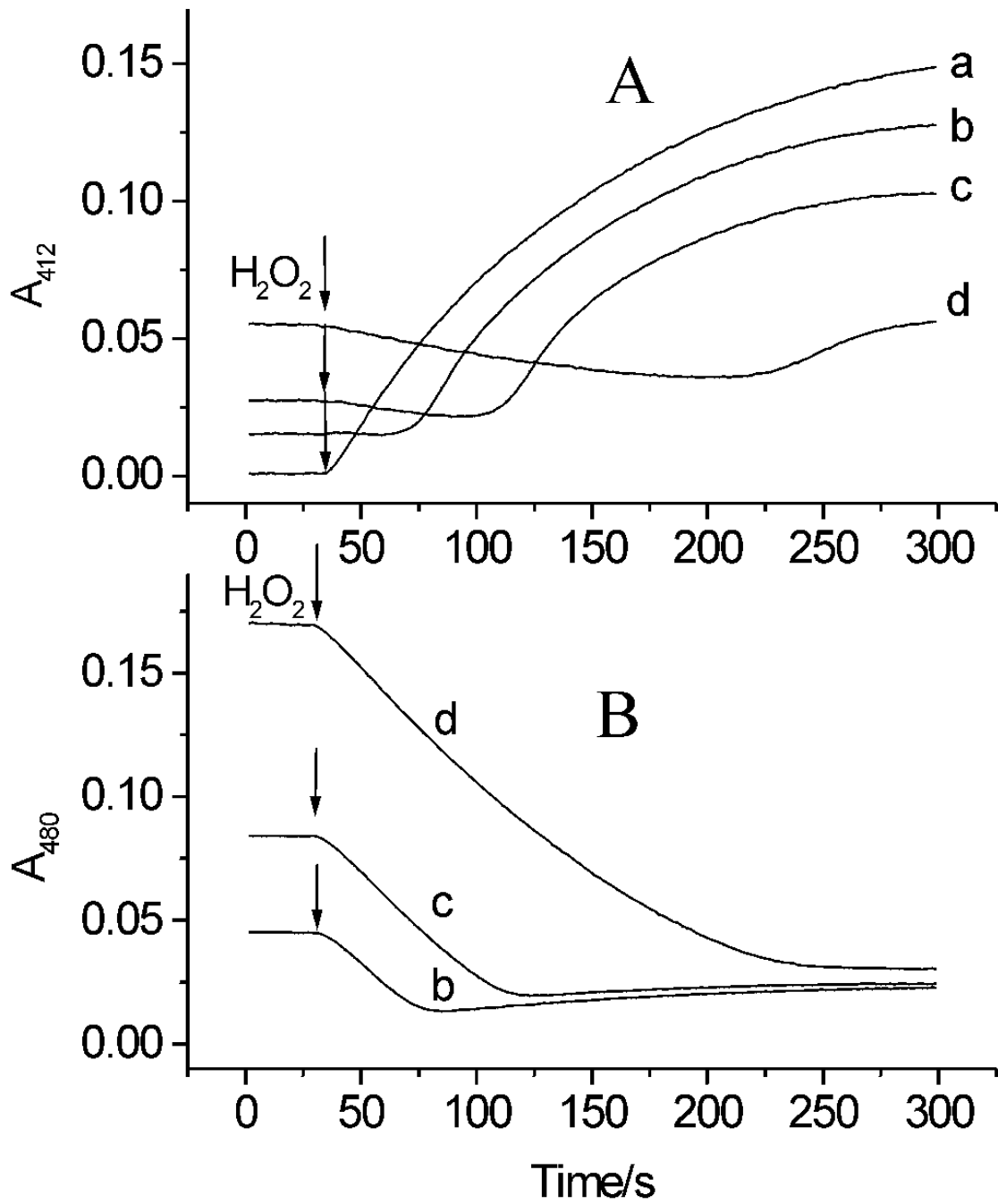
Figure 9

JPET # 89417
Figure 10

8.0 Gauss

JPET # 89417

Figure 11