Aldo-keto reductase 1C2 fails to metabolize doxorubicin and daunorubicin *in vitro*

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A) Running title: Lack of *in vitro* anthracycline metabolism by AKR1C2

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D) List of abbreviations: AKR: aldo-keto reductase; DAUN: daunorubicin; DAUNol:

daunorubicinol; DOX: doxorubicin; DOXol: doxorubicinol; HPLC: high-performance liquid

chromatography.

Abstract

The anthracycline drugs are important for the treatment of a number of malignancies; however, their clinical use is associated with dose-dependent severe chronic cardiotoxicity. While the mechanism for this side effect has not yet been identified, the alcohol metabolites formed during DAUN and DOX therapies have been implicated. The alcohol metabolites of DAUN and DOX, DAUNol and DOXol, respectively, are generated through reduction of the C-13 carbonyl function, which is reportedly mediated by members of the aldo-keto reductase and carbonyl reductase families of proteins. In our search for potential biomarkers for the occurrence of this side effect, we examined the activity of recombinant aldo-keto reductase enzymes, AKR1A1 and AKR1C2, with DAUN and DOX as substrates. Using purified histidine-tagged recombinant proteins and the direct measurement of metabolite formation with an HPLC-fluorescence assay, we did not observe DAUNol or DOXol generation *in vitro* by AKR1C2, whereas AKR1A1 did catalyze the reduction reactions. DAUNol was generated by AKR1A1 at a rate of 1.71±0.09 pmol/min/mg protein and a low level of DOXol was produced by AKR1A1, however, it was below the limits of quantification for the method. These data suggest that the generation of DAUNol or DOXol by AKR1C2 metabolism *in vivo* is unlikely to occur during anthracycline treatment.

The anthracycline antibiotics, DOX and DAUN, are commonly-used, effective treatments for solid tumors and acute myeloid leukemias (Weiss, 1992). Unfortunately, the widespread use of the anthracyclines is limited by the development of dose-dependent chronic cardiotoxicity in patients. This side effect manifests as an irreversible disorder following long-term treatment with approximately 20% of cases leading to congestive heart failure (Singal and Iliskovic, 1998). Though the mechanism by which this side effect develops is not yet identified, evidence implicates metabolism as an important factor (Olson and Mushlin, 1990). Following DOX or DAUN administration, the C-13 alcohol metabolites, DOXol and DAUNol, are the major identified metabolites (Takanashi and Bachur, 1976), and these metabolites are speculated to be responsible for causing cardiac tissue damage (Del Tacca et al., 1985; Boucek et al., 1987; Cusack et al., 1993; Behnia and Boroujerdi, 1999; Olson et al., 2003; Sacco et al., 2003).

Formation of DOXol and DAUNol is mediated by cytosolic oxidoreductase enzymes of the AKR and short-chain dehydrogenase/reductase families, with the AKR1A1, AKR1B10, AKR1C2, and carbonyl reductase enzymes identified as major catalysts (Licata et al., 2000; Martin et al., 2006; Jin and Penning, 2007; Oppermann, 2007). Inhibition of the anthracycline-metabolizing enzymes is suggested as a strategy for avoiding treatment-related cardiotoxicity (Behnia and Boroujerdi, 1999) and chemical inhibitors for reductases and anthracycline derivatives that are metabolically less labile have been explored for their potential roles as safer alternatives to the currently-used anthracycline drug treatments. In part, the success of these strategies relies on accurately identifying the enzymes responsible for anthracycline metabolism.

In this paper, we characterize the involvement of two recombinant aldo-keto reductase enzymes, AKR1A1 and AKR1C2, in mediating the *in vitro* production of DAUNol and DOXol from DAUN and DOX using a specific and sensitive HPLC-fluorescence assay for direct measurement of the alcohol metabolites.

MATERIALS AND METHODS

Chemicals and enzymes. 1-acenaphthenol, *p*-nitrobenzaldehyde, DL-glyceraldehyde, DAUN, DOX, idarubicin, postassium phosphate, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). DNA modifying and restriction enzymes were obtained from Fermentas (Burlington, ON) and New England Biolabs, Inc. (Ipswich, MA). An authentic chemical standard for DOXol was obtained from Qventas Inc. (Branford, CT, USA).

Cloning of human AKR1A1 and 1C2. The *AKR1A1* and *AKR1C2* genes were sub-cloned from lab vector constructs (p2ZOp2N-*AKR1A1 and* p2ZOp2N-*AKR1C2)* using standard molecular cloning techniques to the prokaryotic expression vector pET28a (Novagen, Madison, WI) as previously described (Bains, O.S.B. et al., 2008). The expression constructs encoded an AKR with a six-histidine affinity tag separated by a 23-amino acid residue linker attached to the amino terminus of the expressed protein.

Expression of recombinant enzymes. The AKR proteins were expressed and purified from *Escherichia coli* cultures. Briefly, pET28a-*AKR* constructs were transformed by heat-shock into BL21 (DE3) pLysS and grown in 500 ml low-salt LB media supplemented with 50 µg/ml kanamycin sulfate and 25 µg/ml chloramphenicol at 37ºC. Expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside administration to a final concentration of 1 mM and the cultures grown for a further 3 hours. Bacterial pellets were collected by centrifugation (4000 rpm for 20 mins at 4ºC), then resuspended at 5 ml per gram wet weight with 300 mM NaCl, 50 mM NaH$_2$PO$_4$, (pH 8.0). Lysis of the cell suspensions and purification of the AKR proteins using Ni-NTA agarose were carried out according to the manufacturer's recommendations (QIAGEN, Mississauga, Ontario). Glycerol was added to the tagged purified protein to a final concentration of 20% and the samples stored at -20ºC.

The recombinant proteins were detected by Western blot analysis using the Odyssey Infrared
Imaging System (LI-COR® Biosciences, Lincoln, NE) with the primary monoclonal AKR1A1
and AKR1C2 antibodies (Abnova Corporation, Taipei City, Taiwan) diluted 1:5000 and 1:3000,
respectively, and secondary IRDye 800CW goat anti-mouse IgG antibody (LI-COR®
Biosciences), diluted 1:5000. Protein concentrations were determined by the Bradford method
using bovine serum albumin as a standard (BioRad, Hercules, CA). Protein purity was assessed
by SDS-PAGE stained with Sypro Ruby (Invitrogen, Burlington, ON).

Measurement of AKR enzyme activities. AKR activity was measured by monitoring the
initial rate of NADP(H) oxidation/reduction reactions using standard conditions (Penning, 2004).
Fluorescence measurements of reduced cofactor were made with excitation and emission
wavelengths of 355 and 460 nm, respectively, using a Fluoroskan Ascent FL (Thermo Fisher
Scientific, Waltham, MA). The assays were carried out with 200-275 ng purified protein,
cofactor (180 µM NADPH for AKR1A1; 2.3 mM NADP$^+$ for AKR1C2) and 1 mM test substrate
(*p*-nitrobenzaldehyde or DL-glyceraldehyde for AKR1A1; 1-acenaphthenol for AKR1C2) in a
reaction mixture of 150 µl 100mM potassium phosphate, pH 7, at 25ºC. Maximal rates (min^{-1})
were calculated from the Ascent® program (version 2.6) using a 5 min interval. Enzymatic
activities (µmol cofactor consumed/min/mg purified protein) were calculated based on a standard
curve constructed from fluorescence measurements for known NADPH concentrations. Organic
solvents at a concentration below 4% (v/v) in the final mixture were used to solubilize some
substrates and were not observed to affect enzyme function. All incubations were conducted in
quadruplicate for each purified protein preparation.

Measurement of anthracycline reduction. Identification and quantification of DOXol and
DAUNol were performed using high-performance liquid chromatography (HPLC; Waters

Alliance 2695, Milford, MA) and fluorescence detection with excitation and emission

wavelengths of 460 and 550 nm, respectively (Waters 2475 Multi λ Detector). Quantification

was based on peak area ratio of DOXol to the internal standard. Linear calibration was

determined over the concentration range 25-500 nM using a $1/x^2$ weighting scheme. Because an

authentic chemical standard for DAUNol could not be obtained, DAUNol was quantified as

DOXol equivalents using a response ratio of 1.0. The limit of quantification for DOXol was

estimated as 25 nM. All data processes were performed with Waters Empower software (ver 2.0).

Purified tagged AKR1A1 and AKR1C2 proteins were incubated in 150 μl reaction mixtures

containing 25 mM KH_2PO_4, pH 7.4; and 1 μM of DOX or DAUN at 37ºC. Reaction conditions

were selected to reflect a human physiological environment. Reactions were initiated with the

addition of 1 mM NADPH. Incubations were conducted with up to 1 μg of purified protein for

120 and 240 minutes for DAUN and DOX, respectively, to maximize metabolite generation

given the apparent low activity of these enzymes. At the end of the incubations, reductase

activity was stopped with the addition of 150 μl of ice-cold acetonitrile that contained idarubicin

as an internal standard. Protein was removed from the sample by vortex mixing and

centrifugation at 10,000×g for 10 minutes at 4ºC, and the supernatant was removed for HPLC

analysis. All incubations were conducted in triplicate for each purified protein preparation.

RESULTS AND DISCUSSION

The expression of the recombinant tagged AKR1A1 and AKR1C2 was confirmed by Western

blot analysis showing bands with mobility corresponding to the molecular weight of the tagged

AKRs (~41-42 kDa) (Figure 1). Total protein staining of a SDS-PAGE gel demonstrated that the

only detectable bands for the purified preparations corresponded to the AKR1A1 and AKR1C2

proteins identified by Western blotting (Figure 1).

Activities of the recombinant AKR1A1 using the test substrates p-nitrobenzaldehyde and DL-glyeraldehyde were 5.39±0.35 and 1.56±0.16 μmol/min/mg purified protein, respectively, while the activity of the recombinant AKR1C2 using 1-acenaphthenol was 2.23±0.08 μmol/min/mg. These activities are in good agreement with data reported for purified recombinant and native proteins by other laboratories: AKR1A1, p-nitrobenzaldehyde, 6.0 μmol/min/mg (Palackal et al., 2001); DL-glyceraldehyde, 1.26 μmol/min/mg (O'Connor et al., 1999); AKR1C2, 1-acenaphthenol, 2.5 μmol/min/mg (Burczynski et al., 1998), providing confidence that the recombinant proteins have retained full reductase function.

Formation of DAUNol and DOXol by AKR1A1 and absence of metabolite generation by AKR1C2 were measured by HPLC-fluorescence (Figure 2). The C-13 metabolites were positively identified by identical chromatographic retention times to an authentic chemical standard of DOXol and to the metabolites generated in incubations with human liver cytosolic fractions. Mordente et al. (2001) measured the formation of DAUNol and DOXol by human cardiac cytosol reductases with average rates of 27.5 and 2.3 pmol/min/mg protein, respectively. The high substrate specificity of AKR1A1 was also demonstrated in our results from recombinant enzymes where DAUN was metabolized to a much greater extent than DOX. The levels of DOXol generated were too low for quantification using the HPLC assay, whereas specific reductase activities of 1.71±0.09 pmol/min/mg were determined for DAUN reduction.

Our findings of a lack of AKR1C2 involvement in DOX and DAUN metabolism differ from previously published results (Ohara et al., 1995). This discrepancy may be due to contaminating proteins in previous enzyme preparations or non-specific measurement of reductase activities. SDS-PAGE gel staining for our enzyme preparations shows absence of any detectable contaminating proteins following affinity chromatography. Sypro Ruby total protein staining

provides detection of proteins at 1-2 ng (Berggren et al., 2000) and therefore we estimate protein contamination in our preparations at less than 0.01%. Monitoring NADPH absorbance is the standard assay to characterize reductase enzyme function; however, this method cannot distinguish contaminating protein-catalyzed or non-enzyme catalyzed cofactor usage, potentially leading to erroneous identification of reductase function or substrate use. In our attempts to use the spectroscopic method to measure cofactor usage in AKR1C2-catalyzed DOX and DAUN metabolism, we could not distinguish enzyme and non-enzyme catalyzed signal decrease with substrate concentrations of 10, 50, and 250 μM (data not shown). To specifically measure AKR activities for the anthracycline drugs DOX and DAUN, we have used a direct and sensitive HPLC-fluorescence assay and do not identify any alcohol metabolites formed by AKR1C2 that exceed the limit of quantification of the assay (25 nM). In our *in vitro* system, the initial anthracycline concentrations are 1 μM, a physiologically-relevant concentration for patient cardiac tissue (Stewart et al., 1993).

The failure of AKR1C2 to generate alcohol metabolites *in vitro* suggests that it is unimportant for the generation of DOX or DAUN metabolites *in vivo*. We recognize that *in vivo* studies are needed to clearly define the role of AKR1C2 in anthracycline metabolism; however, the conduct of definitive studies will be difficult. Several reductase enzymes are involved in the metabolism of these drugs and distinguishing their individual contributions to the total metabolism will be a challenge using the traditional approaches of correlation studies and chemical or antibody inhibition. It is with these considerations that we present our *in vitro* findings to focus future work on anthracycline metabolism on the other AKR isoforms and carbonyl reductases.

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Footnotes

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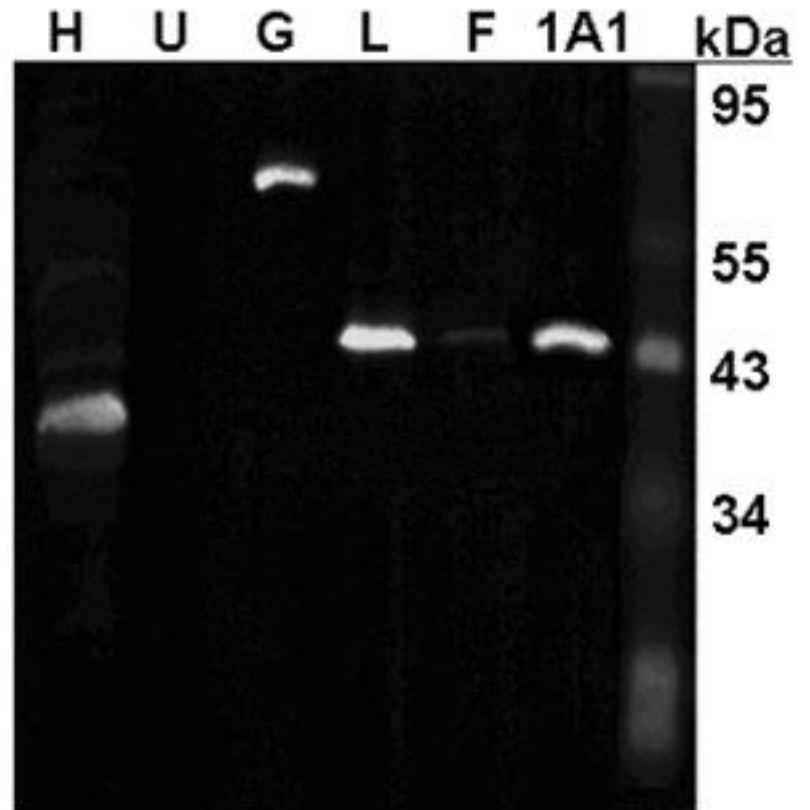Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall
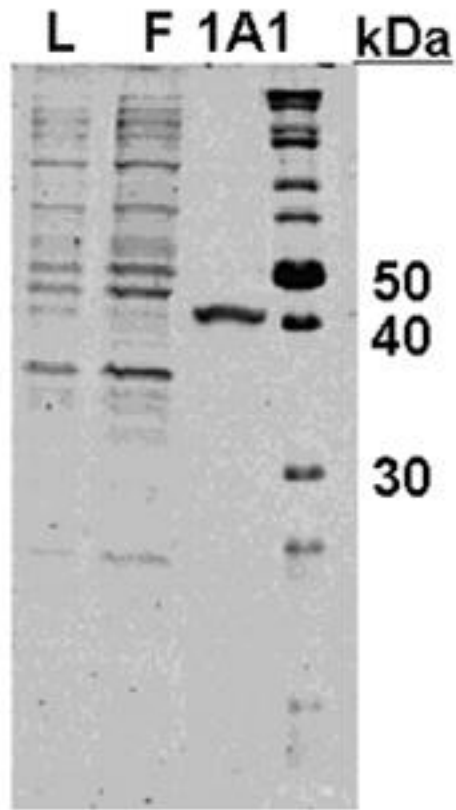
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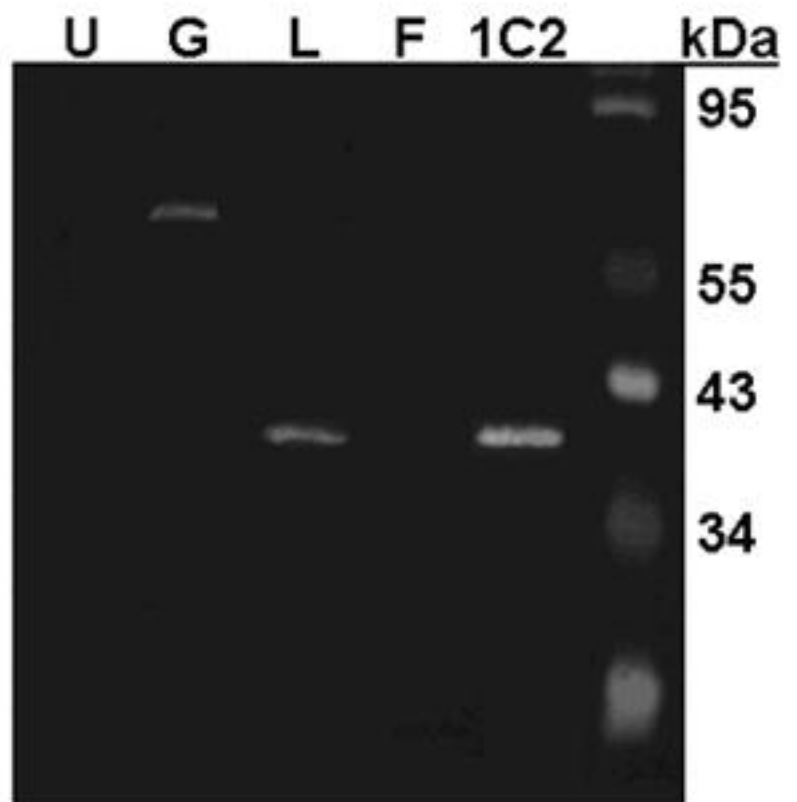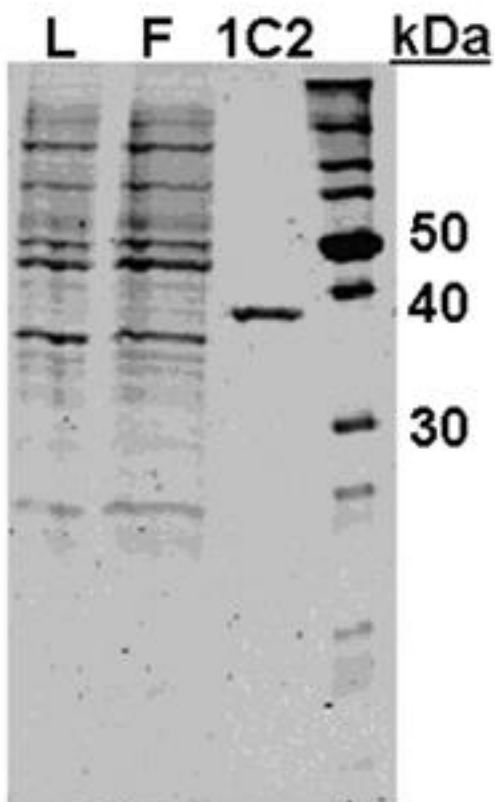
FIGURE LEGENDS

Figure 1—Purification of recombinant histidine-tagged human (A) AKR1A1 and (B) AKR1C2.
(*Left*) SDS-PAGE stained with Sypro Ruby shows purified protein samples, 1A1 or 1C2 (700 ng),
free of contaminating proteins from bacterial lysates (L) (20 µg total protein). Removal of
contaminating proteins is observed in fractions from Qiagen purification procedures (Ni-NTA
column flow through (F), 15 µg total protein). (*Right*) Western blotting of purified protein
samples, 1A1 or 1C2 (700 ng), confirms expression of the desired AKR protein with mobility at
expected molecular weight (~41-42 kDa). Positive controls for antibody immunoreactivity are
human liver cytosol (H) and GST-tagged purified human recombinant AKR enzymes (G; 700 ng).
No antibody immunoreactivity is observed for untransformed bacterial lysate (U; 20 µg total
protein).

Figure 2—Generation of DAUNol and DOXol *in vitro* by purified recombinant AKR1A1
incubated with (A) DAUN and (B) DOX. Measurement of DAUNol and DOXol was performed
using HPLC-fluorescence. Representative chromatograms show clear resolution of DAUNol and
DOXol from DAUN, DOX, and idarubicin (internal standard). Retention times observed for
DOXol, DOX, DAUNol, DAUN, and idarubicin (internal standard) are 4.5, 5.5, 6.0, 6.8, and 7.3
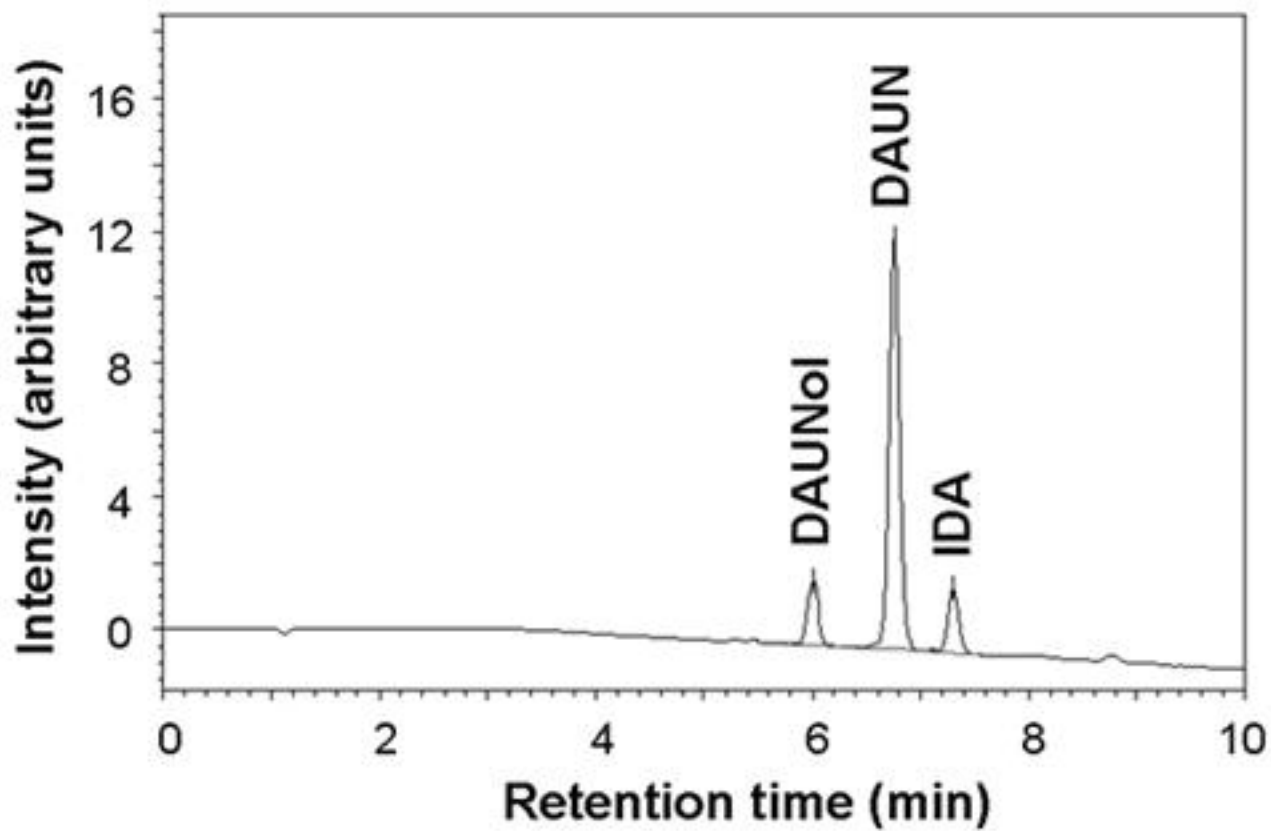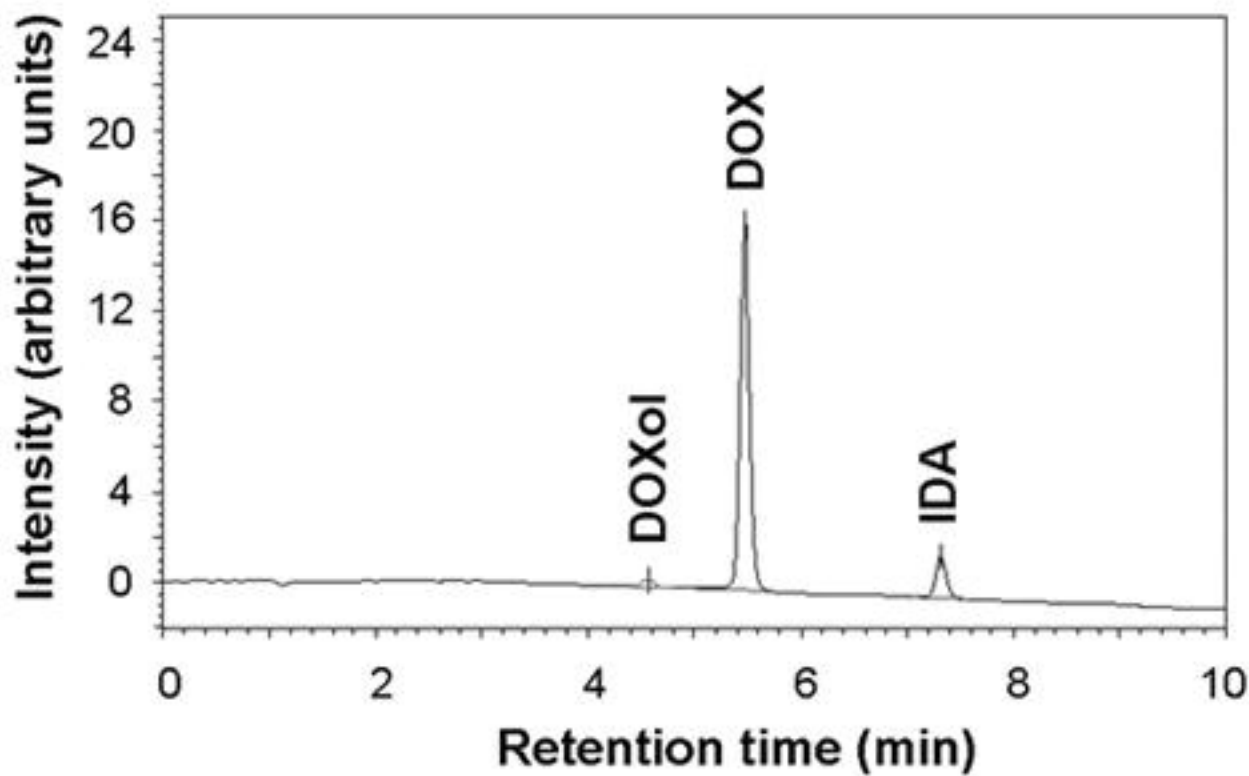minutes, respectively.

Figure 1

(A)

(B)

Figure 2

(A)

(B)