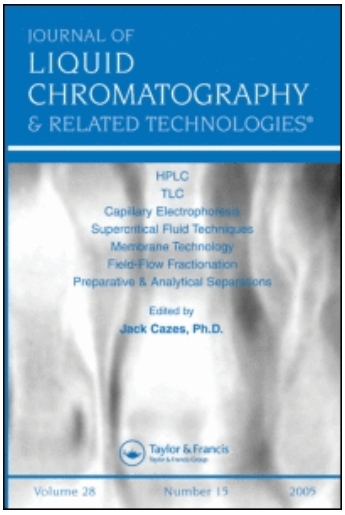


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# Identification of Doxorubicin and an Imine Derivative from Liquid and Solid Samples Utilizing Liquid Chromatography

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# Identification of Doxorubicin and an Imine Derivative from Liquid and Solid Samples Utilizing Liquid Chromatography

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**Abstract:** Doxorubicin is an anthracycline glycoside used to treat a variety of cancer conditions from breast cancer to Wilms' tumor. The appearance of doxorubicin resistance has initiated studies of doxorubicin derivatives. The formation of the doxorubicin imine derivative is shown with means of identification by HPLC. Both the parent doxorubicin and imine derivative can be extracted from aqueous samples into 2-pentanol or 2-methyl-2-butanol by utilizing aggressive salting-out techniques. Both forms of doxorubicin are bright red in color. A mixture of methanol/water or straight ethanol can be used to solubilize from solid samples. Determination of molecular properties indicated the imine group substantially increases lipophilicity and decreases aqueous solubitlity. Doxorubicin and its imine derivative are separated using Alltech Altima 18C 5μ column, UV/Vis detection (200 nm), and a mobile phase of methanol: water (80%/20%) at 1.0 mL/minute. Utilizing non-destructive detection allows the collection of the eluted drug. The resolution achieved was 1.81 minutes, with 784 plates for doxorubicin separation, and 6834 plates for the imine derivative. The lipophilic substituent constant of the imine group is 3.48, indicating increased lipophilicity. The imine derivative is stable for weeks when stored dry at −10°C.

**Keywords:** Doxorubicin, Imine, Anthracycline, HPLC, Anticancer, Adriamycin

## INTRODUCTION

Doxorubicin (adriamycin) is an anthracycline glycoside anticancer drug that is utilized to treat breast cancer, Hodgkins disease, soft tissue sarcoma, and

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cancer of the lung, thyroid, liver, bladder, and stomach.[1] It is a bright red in color and is generally given intravenously. It is often considered as a 14-hydroxy derivative of daunorubicin, and functions as a DNA intercalator,[1] metal ion chelator, or inhibitor of DNA topoisomerase II. The appearance of significant drug resistance has threatened the clinical efficacy of doxorubicin.[2–4] In response, there has been extensive research on the mechanism of this resistance and the potential benefit of derivatizing doxorubicin. Various types of doxorubicin derivatives synthesized include: D-galactose,[5] methylxanthine,[6] cephalosporin,[7] 2-pyrrolino,[8] cyanomorpholino,[9] and peptidyl forms.[10]

This work demonstrates the formation and identification of the imine form of doxorubicin. Imine drugs are utilized for a variety of pharmaceutical purposes, including dendrimer drug delivery systems.[11] The imine functional group appears on other anticancer agents (dioxadet)[12] and antimalarial agents of starch and cellulose structure.[13]

The study of doxorubicin resistance and synthesis of alternate drug types has initiated the investigation of methods to identify and separate the excreted drug and its metabolites. For this purpose, the use of HPLC has been effective and versatile. HPLC has been used to isolate peptide conjugates of doxorubicin,[14] the intact drug,[15–17] and metabolites formed in vivo.[18]

This work presents an effective approach of forming a stable imine derivative of doxorubicin. The separation of the parent doxorubicin from the imine derivative is accomplished by use of HPLC, utilizing an aqueous/alcohol solvent system. Molecular properties, such as Log Kow (1-octanol/water partition coefficient) index of refraction, molar refractivity, molar volume, and solubility are determined for the parent compound and imine derivative. The imine functional group induces significant alteration in some molecular properties. Despite significant change in some properties, it is shown here that HPLC can effectively separate and identify the imine derivative from the parent compound.

## EXPERIMENTAL

### Reagents and Instrumentation

All reagents were of liquid chromatography grade and obtained from Aldrich-Chemical Co. (Box 2060, Milwaukee, WI 53201). The HPLC instrument was an Alltech 426 HPLC pump, with Linear UV/VIS 200 detection system, and Altima 18C 5μ column. Infrared spectra was obtained in dimethyl sulfoxide, dried over molecular sieves, and a Mattson Galaxy Series Fourier Transfer IR spectrometer. The doxorubicin was donated from the laboratory of Prof. U.S. Rao (University of Nebraska Medical Center, 42nd and Dewey Avenue, Omaha, Nebraska).

## Molecular Modeling and Property Determination

Determination of molecular properties was accomplished utilizing Chem-Sketch version 8.17 (Advanced Chemistry Development, 90 Adelaide Street West, Toronto Ontario, M5H 3V9, Canada) and EPISUITE property tool (copyright 2000, US Environmental Protection Agency, Washington D.C. USA). All 3-D modeling was accomplished using ADC ChemSketch and CS Chem 3D Pro(CambridgeSoft Corp., 100 Cambridge Park Drive, Cambridge MA 02140).

## Formation of Imine Derivative



For solid samples, an amount of material, which contains approximately 0.8 mg (or less) of doxorubicin is placed into 500 microliters (maximum of 1 mL) of dried HPLC grade acetone that has 1/5 volume of molecular sieve powder to remove water formed during the synthesis. The solid samples should be ground to a fine powder in a mortor and pestle if they coarse or have granules. Also, care should be taken to remove insoluble contaminants or extraneous particulate material. The mixture is allowed to incubate at room temperature for 30 minutes, then placed at 60°C for two hours (with occasional mixing), and finally for one hour at room temperature (yields complete conversion). The liquid supernatant is removed carefully, dried over a rotavapor vacuum or under nitrogen flow, then stored in an air tight container at $-10$°C until use.

For aqueous samples (i.e., IV mixtures, saline mixtures, and urine samples) a volume of an aliquot is made saturated with NaCl, either by addition of solid NaCl or use of a saturated aqueous solution of NaCl prepared previously. Then, the drug can be extracted into either 2-methyl-2-butanol or 2-pentanol (note that common extraction solvents, ethyl acetate and acetonitrile were not effective). The organic phase is collected and dried quickly over anhydrous $MgSO_4$. The organic solvent is removed by a rotavapor or nitrogen gas flow. The residue can be treated as above for solid samples. Normal urine samples were obtained and known amounts of doxorubicin or imine derivative added. These two drugs produce a reddish hue in urine as does doxorubicin in excreted urine from cancer patients. Solid samples for HPLC analysis are initially solubilized into methanol/water (4:1) or straight ethanol.

IR spectroscopy of doxorubicin-imine derivative: While most of the spectral region is identical to the parent compound doxorubicin, two additional unique peaks at $\sim$1400 $cm^{-1}$ and $\sim$1700 $cm^{-1}$. Importantly, these two peaks are indicative for the flexibility introduced in place of the primary amine group of doxorubicin. The IR analysis indicated that alcoholic and phenolic groups within the imine derivative are intact. Stretching peaks for O-H was observed for both drugs at about 3500 $cm^{-1}$ with a C-O signal at $\sim$1050 $cm^{-1}$.

## HPLC Conditions

The sample to be injected is first solubilized into a mixture having a ratio of methanol/water of 80%/20% (the mobile phase). The injected sample is at normal room temperature. The flow rate is 1.0 mL/minute, at 1900 psig, on an Alltech Altima 18C 5μ column. Non-destructive detection is accomplished by a Linear UV/VIS 200 detector. The eluting compounds may collected into suitable glass test tubes. Approximately 10 micrograms of doxorubicin is eluted in the example chromatogram shown here and 1 to 2 micrograms of the imine derivative drug.

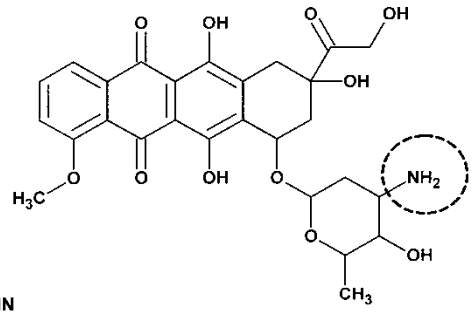
## RESULTS AND DISCUSSION



Doxorubicin is an antibiotic that is utilized for cancer chemotherapy. Utilized in the clinical treatment of many deadly cancer diseases, an analytical method to identify the drug and its derivatives is useful when monitoring pharmacokinetics and patient compliance. The drug and its derivatives are bright red in color and clearly visible in cancer patient urine samples giving a reddish hue. Derivatives of doxorubicin have become important for the study and clinical treatment of drug resistant cancer tissue.

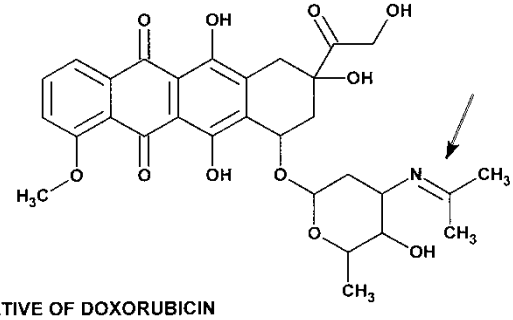
Both the parent doxorubicin and its imine derivative retain the characteristic bright red color known for this drug. Both compounds are solids at room temperature and stable for months when stored dry at $-10^{\circ}$C or less. The molecular structures with corresponding SMILES notation are presented in Figure 1. Alterations in the molecular structure of doxorubicin (i.e., such as an imine group) potentially decreases or eliminates resistance of target cancer cells that is due to expulsion of the drug. The presence of the imine group located on the sugar moiety signifcantly affects the molecular properties of the parent structure, and are presented below in Table 1. Analysis of the 3-dimensional structure reveals the imine group adds signifcant bulkiness to the drug and lies outside the plane of the aromatic portion. Significant changes occur in the derivative induced by the imine group. Primary amine groups are considered Bronsted and Lewis bases (i.e., hydrogen acceptor), however, the strength of the base is greatly affected by any substituents. Primary and secondary amine groups are also weak acids (hydrogen donor).

Synthesis of the imine derivative is accomplished in acetone solvent that is thoroughly dried over molecular sieves prior to use. The presence of water, even absorbed from an ambient environment, does not favor the formation of the imine group. Straight acetone was utilized in this synthesis with powdered molecular sieve dust for removal of water that is generated during the synthesis. The over all synthesis mechanism is presented in Figure 2 for primary amine group targeting. Incubation at an elevated temperature ($60^{\circ}$C) increases the yield of the imine product. Incubation at a lower




DOXORUBICIN

COc1c3c(ccc1)C(c2c(c4c(c(c2C3=O)O)C(CC(C4)(O)C(CO)=O)OC5CC(C(C(C)O5)O)N)O)=O




IMINE DERIVATIVE OF DOXORUBICIN

CC(=N/C1C(C(C)OC(C1)OC5CC(Cc2c5c(c4c(c2O)C(c3c(c(ccc3)OC)C4=O)=O)O)(O)C(CO)=O)O)\C

***Figure 1.*** Molecular structure and SMILES notation shown for the parent compound doxorubicin and imine derivative. The primary amine group (see inset circle) of the parent doxorubicin is a site for hydrogen donor activity. The imine group (indicated by arrow) increases bulkiness of the derivative and increases lipophilicity.

temperature, and for shorter time periods, produces significantly less yields, which vary according to the time of incubation and temperature. Generally, the rate of formation of an imine group is maximal at pH 5 and decreases at higher and lower pH. The reactions and products were protected from light prior to analysis, however, no significant effects were seen if exposure to

***Table 1.*** Molecular properties of doxorubicin and imine derivative

| Property | Doxorubicin | Imine derivative |
|---|---|---|
| Formula weight | 543.5 | 583.6 |
| Log Kow | 1.85 | 5.33 |
| Aqueous solubility | 92.84 mg/L | 0.00170 mg/L |
| Index of refraction | 1.71 | 1.68 |
| Molar refractivity | 131.5 $cm^3$ | 141.9 $cm^3$ |
| Molar volume | 336.6 $cm^3$ | 376.3 $cm^3$ |

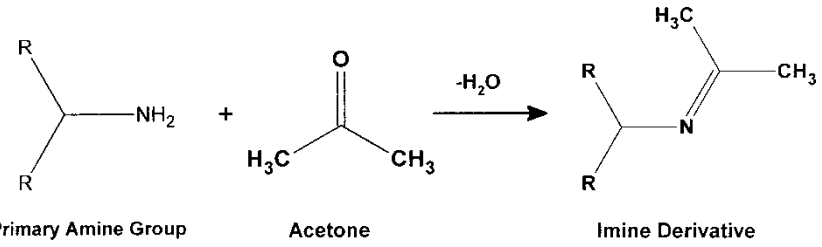



***Figure 2.*** General mechanism of synthesizing an imine group, also indicating the necessary removal of water to favor formation of product.

light occurs during synthesis. Both compounds are considered toxic and should be handled and contained carefully. No odor or vaporization of either compound was observed while handled at room temperature.

The addition of an imine group significantly affects pharmacological properties of the doxorubicin parent structure (see Table 1). The formula weight increases from 543.5 to 583.6, a 7.4% increase. Likewise, there is an increase within the values of Log $K_{ow}$, molar refractivity, and molar volume of 288%, 7.9%, and 11.8, respectively. Molar volume is an indicator of bulkiness, whereas molar refractivity is related to molecular volume and steric effects. Through an increase of bulkiness of the sugar group, it is anticipated this will decrease efflux from the target cancer cell. The large increase in 1-octanol/water partitioning from 1.85 (doxorubicin) to 5.33 (imine derivative) also greatly affects the pharmaceutical properties.[19,20] An increase in Log $K_{ow}$ of this magnitude indicates greater solubility into lipid by-layers and decreased aqueous solubility. Aqueous solubility of the imine derivative decreases to 0.00170 mg/Liter compared to 92.84 mg/Liter for doxorubicin.

An increase of a drug's lipophilicity generally suggests an increase in penetration of cell membranes.[19,20] This outcome also reveals the influence on lipid by-layer solubility due to the primary amine group that remains on the parent structure. A primary amine group provides a site of hydrogen donor and hydrogen acceptor activity, which strengthens aqueous solubility and decreases cell membrane penetration.[19,20] The primary amine group located on the sugar moiety of the parent doxorubicin is a significant nucleophile. There is an 11.8% increase in molar volume, which hints to an increased bulkiness for the imine derivative structure. The lipophilic substituent constant ($\pi$) is a parameter indicating the lipophilic contribution of substituent addition to a parent structure.[19,20] Defined as the difference between the 1-octanol/water partition coefficient of the derivative and the parent compound, a value of 3.48 is obtained, which indicates a substantial contribution of lipophilic property. Loss of hydrogen donor/acceptor capability for the imine derivative may affect: 1) orientation of ligand, 2) recognition of substrates, and 3) affinity of ligand. Molecular dipole of

doxorubicin is calculated to be 3.350 Debye, while the value for the imine derivative is reduced to 2.784 Debye. When considering aqueous solubility, generally, more polar solute molecules will dissolve in water. The decrease of the dipole moment for the imine derivative foretells the significant decrease in water solubility. In addition, the imine derivative shows an increase in formula weight and molar volume, both factors that decrease aqueous solubility.

The two alcoholic organic solvents, 2-pentanol and 2-methyl-2-butanol effectively extracted both compounds from aqueous solution when aggressive salting-out steps were applied and at room temperature. Extraction under these conditions was greater than 90% efficient. Ethyl acetate and acetonitrile are common solvents used for organic solvents but were not effective even under salting-out conditions. The partition coefficient Log $K_{ow}$ values are 0.86 and $-0.15$ for ethyl acetate and acetonitrile, respectively, whereas the Log $K_{ow}$ values for 2-pentanol and 2-methyl-2-butanol are 1.26 and 1.22, respectively. This suggests organic solvents having a Log $K_{ow}$ greater than 1.0 are useful for effective extraction from aqueous solution. An amount of 0.8 mg of doxorubicin was placed into 10 mL of normal urine and effectively extracted by 2-pentanol and 2-methyl-2-butanol. Normal urine samples were clear and straw color in condition with no debris material present. Aqueous samples with and without salt minerals present were likewise effectively extracted following the protocol presented in Experimental.



After determination of the molecular properties of both parent drug and its imine derivative, it is clear that removal of the primary amine group on the sugar moiety and formation of the imine group causes substantial changes in druglikeness charactieristics. Although, this may be beneficial in the clinical aspect, these changes may be problematic in identifying and isolating excreted compounds. A 288% increase in Log $K_{ow}$ value for the imine derivative would suggest a significant increase of partitioning into a lipophilic stationary phase. Conversely, a more lipophilic mobile phase may enhance imine presence. Because the removal of the primary amine group to form an imine group decreases hydrogen donor activity, the expected consequence is a lower solubility in water (which is indeed observed in determined aqueous solubility presented in Table 1).

Chromatograms showing elution of doxorubicin and imine derivative are presented in Figure 3. The doxorubicin drug elutes with retention time of about 10.5 minutes and the imine derivative approximately 12.4 minutes. Upon testing the compounds, it was clearly seen that the retention times were sufficiently apart to differentiate doxorubicin and the imine form. Resolution was calculated to be 1.81 minutes and sufficiently large enough to allow separate collection of these eluted drugs. The eluted samples can be subjected to rotavaporization to remove methanol and water solvents to leave the drug residue. Both drugs should be considered a bio-hazard and must be handled accordingly.

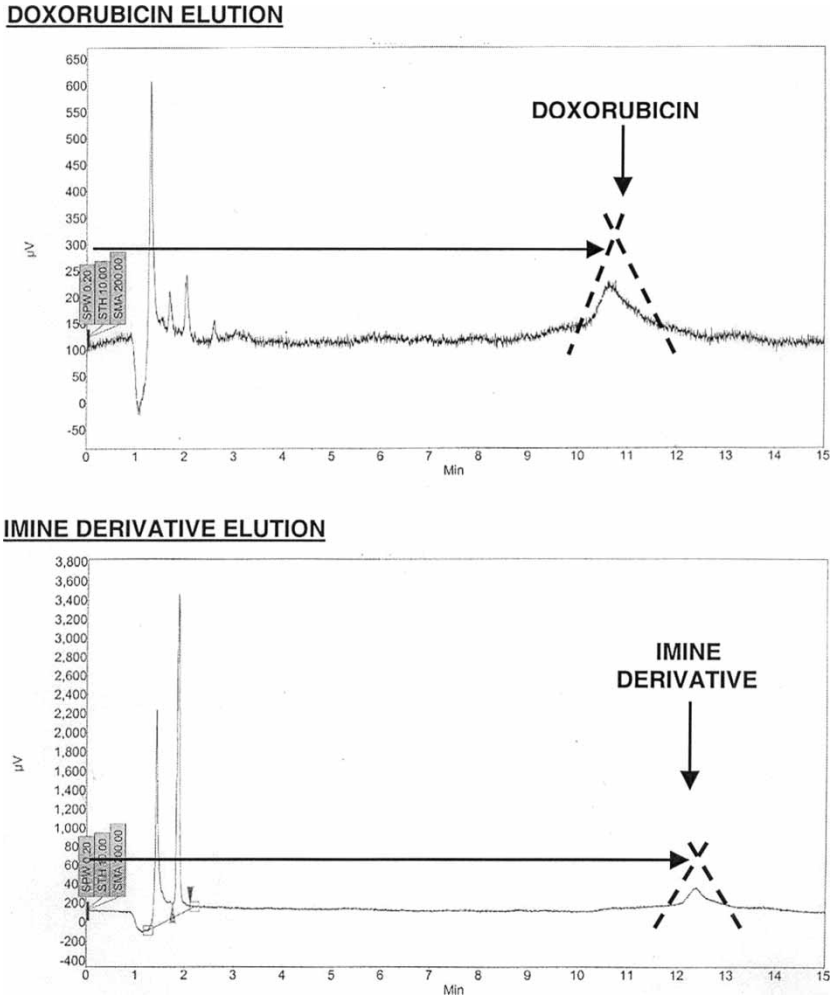



*Figure 3.* Chromatograms show the elution profile of doxorubicin and its imine derivative, with compounds indicated by arrows. The doxorubicin drug elutes with retention time of about 10.5 minutes and the imine derivative approximately 12.4 minutes. Resolution of separation is 1.81 minutes with N number of plates for doxorubicin at N = 784 and the imine derivative at N = 6834.

## CONCLUSION

The imine derivative of doxorubicin was formed in an acetone solvent and found to have similar reddish color as the parent compound. Various molecular properties of the imine compound were determined and showed increases in formula weight, index of refraction, molar refractivity, molar volume, and substantial increase (288%) in Log $K_{ow}$ value. The aqueous

solubility of the imine drug was much less than the parent compound. Both the parent doxorubicin and imine drug were clearly separated by HPLC using a mobile phase of methanol/water at 80%/20%, respectively. Extraction of both drugs for aqueous solutions is feasible when utilizing 2-pentanol or 2-methyl-2-butanol with aggressive salting-out techniques.

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