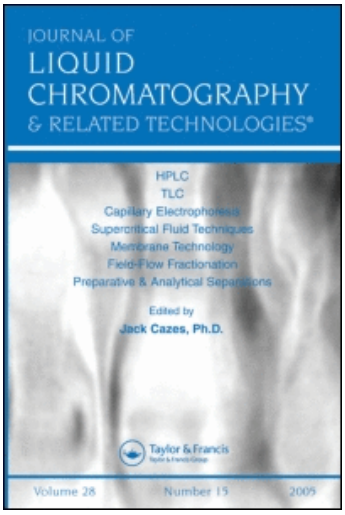


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# Metabolism Studies of Polycyclic Mutagens and Carcinogens Using Liquid Chromatography with Ultraviolet and Electrochemical Detection

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# METABOLISM STUDIES OF POLYCYCLIC MUTAGENS AND CARCINOGENS USING LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET AND ELECTROCHEMICAL DETECTION

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## ABSTRACT

HPLC ultraviolet (UV) and electrochemical (EC) detectors connected in series were used to classify the metabolites of naphthalene, anthracene, acridine, and phenanthridine into phenolic and nonphenolic categories. This method of classification is based on comparison of the relatively high response of the EC detector to electroactive species such as phenols to the more nonselective response of the UV detector. The method is generally useful for many biologically active substances.

## INTRODUCTION

During approximately the past fifteen years, high performance liquid chromatography has become the analytical method of choice for studying metabolism of environmental mutagens, carcinogens, and related substances. The HPLC mode most used, particularly for study



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of compounds such as benzo[a]pyrene, is reversed phase chromatography with UV or fluorescence detection. Reversed phase HPLC is particularly well suited for analysis of mixtures of lipophilic compounds and their metabolites, since polar metabolites elute earlier than nonpolar ones, and during the course of metabolism lipophilic compounds are converted by oxidative enzymes primarily to more polar hydrophilic substances. Thus, metabolites will generally elute before the substrate. In addition, most oncologically active organic compounds absorb strongly in the UV and many are also highly fluorescent.



While researchers investigating the action of chemical carcinogens are familiar with reversed phase chromatography coupled with UV or fluorescence detectors the potential of EC detectors in this area of research does not appears to be well recognized, although they have been employed to monitor metabolism of aniline (1), azo dyes (2), benzene, and phenol (3). When used in series with UV or fluorescence detectors, electrochemical detectors can be very useful for assigning structural class to metabolites formed from polynuclear aromatic hydrocarbons (PAH) and related substances such as the polynuclear aromatic nitrogen heterocyclics (PANH). Mixed function oxidases present in mammalian liver and other tissues metabolize PAH to oxidized derivatives, notably phenols, quinones, and trans-dihydrodiols (4-6). PANH metabolism proceeds to similar compounds although enzymes other than mixed function oxidases appear to be active as well (7-10). Phenol, quinone, and dihydrodiol derivatives of PAH and PANH are readily detected by UV detectors. In

contrast, EC detectors are specific for substances which are electroactive at the applied potential. Of the above compounds only phenols are electroactive under the conditions employed in this account.

This paper describes assignment of structural class to metabolites derived from the PAH naphthalene and anthracene, and the PANH acridine and phenanthridine using reversed phase HPLC with serial UV and EC detectors.



## MATERIALS AND METHODS

### Liquid Chromatography:

A gradient liquid chromatograph equipped with a variable uv-vis detector and an electrochemical detector in series was used in these studies. Spherisorb Hexyl RP columns (4.6 x 150 mm) packed in the laboratory were used with varying linear gradients (11). The gradient system started at 30% strong solvent and progressed to 60% over 20 min. The weak solvent was 10% methanol in 0.02 M $NH_4H_2PO_4$ while the strong solvent was 80% methanol in the same salt solution. A solvent system which will conduct electricty is required for the electrochemical detector and inclusion of methanol in both solvents decreases degassing and bubble formation in the detectors.

The uv detector was operated at 254 or 280 nm depending on analyte absorptivity. The electrochemical detector consisted of a glassy carbon working electrode at a potential of +0.7 V vs. a

Ag/AgCl reference electrode. Under these conditions the electrochemical detector is specific for compounds which will oxidize at this potential. For PAH and PANH derived materials this is primarily limited to phenols and aromatic amines.

**Gas Chromatography/Mass Spectrometry:**

Ethyl acetate residues of PAH and PANH metabolism (see below) or purified metabolites obtained by semipreparative HPLC were analyzed after being derivatized with the trimethylsilyl donar BSA (50 $^{o}$C for 20 min). A 0.24 mm X 30 M DB-5 capillary column (He carrier, 150 to 250 $^{o}$C) was used on a Hewlett-Packard 5985B mass spectrometer which was operated in the electron impact mode.



**In Vitro Metabolism:**

Conditions used in conducting metabolism reactions have been described previously (6,7). Briefly, the 10,000 X G supernatant fractions from rat or guinea pig livers, obtained from animals which had been treated with cytochrome P-448/450 oxidase inducing agents (polychlorinated biphenyls or 3-methylcholanthrene, (12)), were used at a final level of 1 mg/ml of protein in Tris buffer at pH 7.4 and 37.5 $^{o}$C for 20 min. A NADPH reconstituting system was used, and the concentration of substrate was 1 X $10^{-4}$ M. Reactions were stopped by adding 1 volume of cold acetone and the substrate and metabolites extracted into two volumes of ethyl acetate. The organic layers were dried over sodium sulfate, filtered, evaporated to dryness and the residues reconstituted in methanol for HPLC analysis or mixed with trimethylsilyl reagent for GC/MS analysis.

## RESULTS AND DISCUSSION:

### Naphthalene:

Chromatograms of the ethyl acetate soluble metabolites of naphthalene catalyzed by enzymes from PCB induced rat liver enzymes are given in Fig. 1. The UV chromatogram (bottom trace) contains three principal peaks at 5.1, 14.9, and 20.9 min. The 20.9 min peak is residual naphthalene (the substrate) to which the EC gives no response. The large peak at 5.1 min in the UV trace also shows no response in the EC chromatogram, thus it is not a phenol. Gas chromatography/mass spectrometric data indicate that it is a trans-dihydrodiol. The short retention time, indicating high polarity, also suggests a dihydrodiol or other dioxygenated molecule.

The peak at 14.9 min is characterized by a very intense response in the EC tracing. Simultaneous response of UV and EC detectors to a component of PAH metabolism (under the conditions employed) can be taken as an indication that the substance eluting is a phenol. When a phenol is chromatographed using UV and EC detectors in series, EC detector response frequently will be many times greater than that of the UV detector. For the chromatogram shown in Fig. 1 the electrochemical detector was used at a sensitivity of 100 nA full scale which is its **least** sensitive setting, while the UV detector was at 0.02 AUFS, near its **most** sensitive setting. The absolute response of each detector will, of course, be determined by both instrumental parameters and properties of the compound. Changing the applied voltage of the EC or the wavelength of the UV would change the response of these detectors.

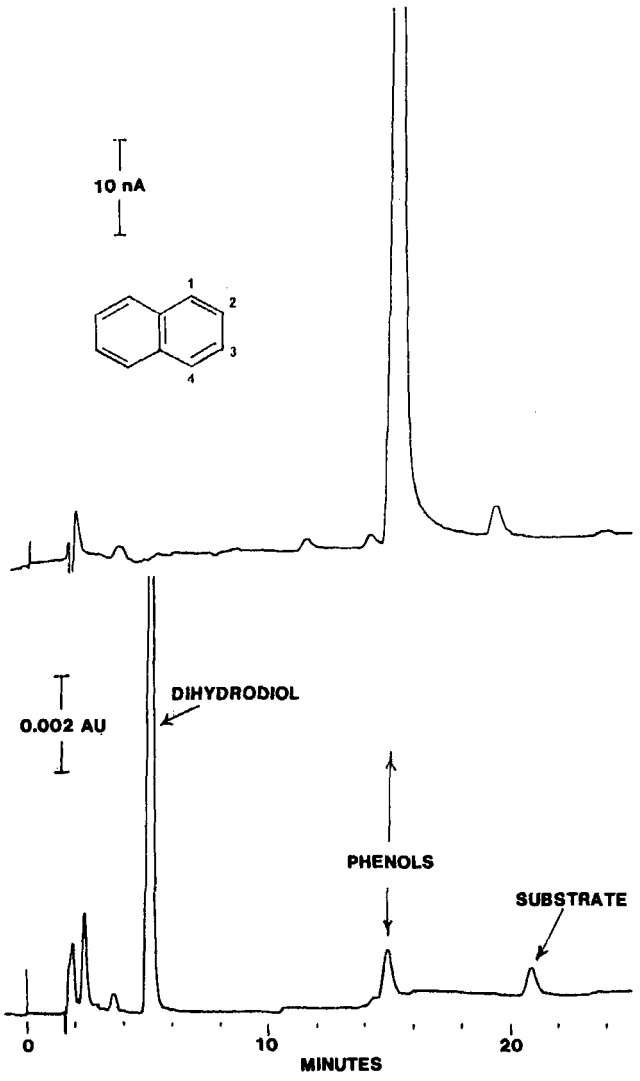


FIGURE 1. EC (top) and UV (bottom) chromatograms of the ethyl acetate soluble metabolites of naphthalene. The identified compounds include: naphthalene-1,2-dihydrodiol (5.1 min), 1- or 2-naphthol (14.9 min), and the substrate, naphthalene (20.9 min).

**Anthracene:**

The EC and UV chromatograms obtained from the ethyl acetate soluble metabolites of anthracene are given in Fig. 2. The UV peak at 13.8 min has no matching peak in the EC chromatogram. Because of this, and because of its relatively short retention time, the compound is tentatively identified as the trans-1,2-dihydrodiol. Gas chromatography/mass spectrometry of material purified by preparative HPLC indicate that this assignment is correct.

The multiplet at 22 to 24 minutes appear to contain two phenols. In this case, the ratio of EC response to UV response is not as great as was the case for naphthalene.

The peak at 31.0 min in the UV chromatogram appears to be matched by a peak in the EC chromatogram. However, the peak in the EC chromatogram actually elutes at 31.5 min. We tentatively conclude that the EC response arises from an impurity of unknown structure.



**Acridine:**

Chromatograms of the ethyl acetate soluble metabolites of acridine obtained from incubation of this PANH with 3-methylcholanthrene induced rat liver enzymes are given in Fig. 3. Comparison of the EC and UV chromatograms indicate that there are two primary phenolic species produced. These are present in the chromatograms at ca. 12 and 18.5 min. Subsequent experiments using combined gas chromatography/mass spectrometry and comparison of HPLC retention times of authentic standards indicate that the peak at 18.5 min is 2-hydroxyacridine and the peak at 12 min is due to 2-hydroxyacridone. Other components which have been identified

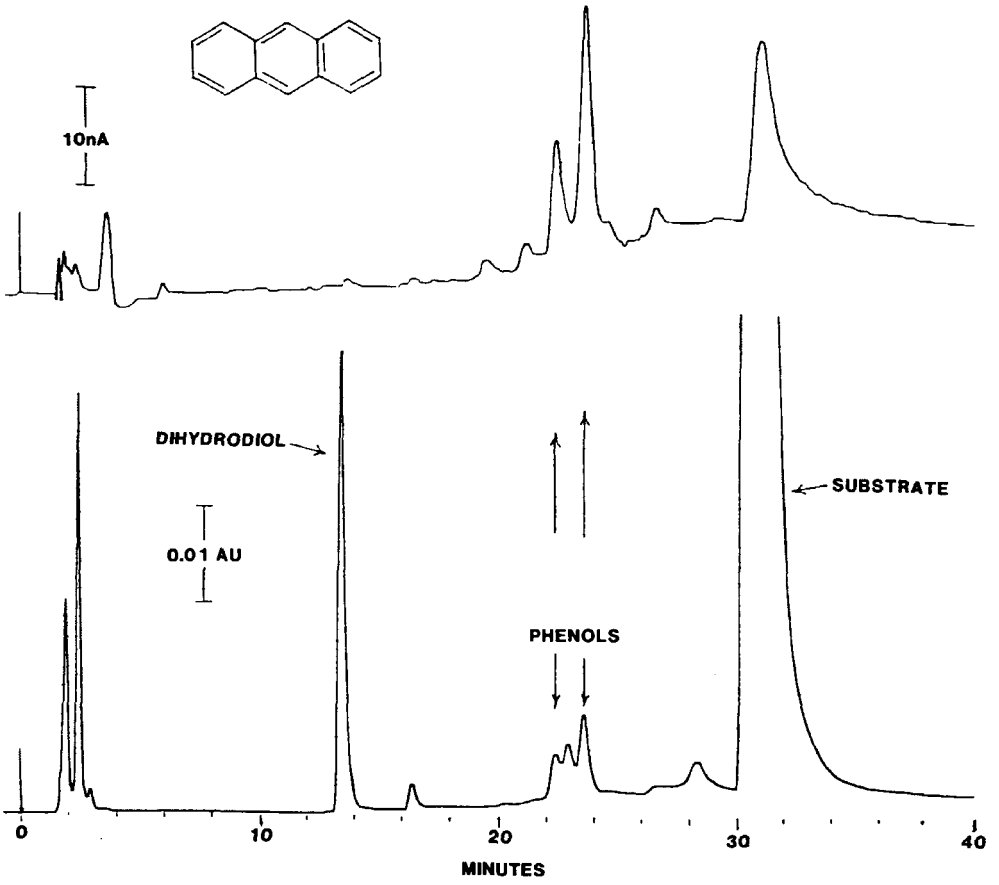


FIGURE 2. EC (top) and UV (bottom) chromatograms of the ethyl acetate soluble metabolites of anthracene. Components include: anthracene-1,2-dihydrodiol (13.8 min), anthracene-1- and -2-ol (22 to 24 min), the substrate (31.0, UV), and an unknown (31.5 min, EC).

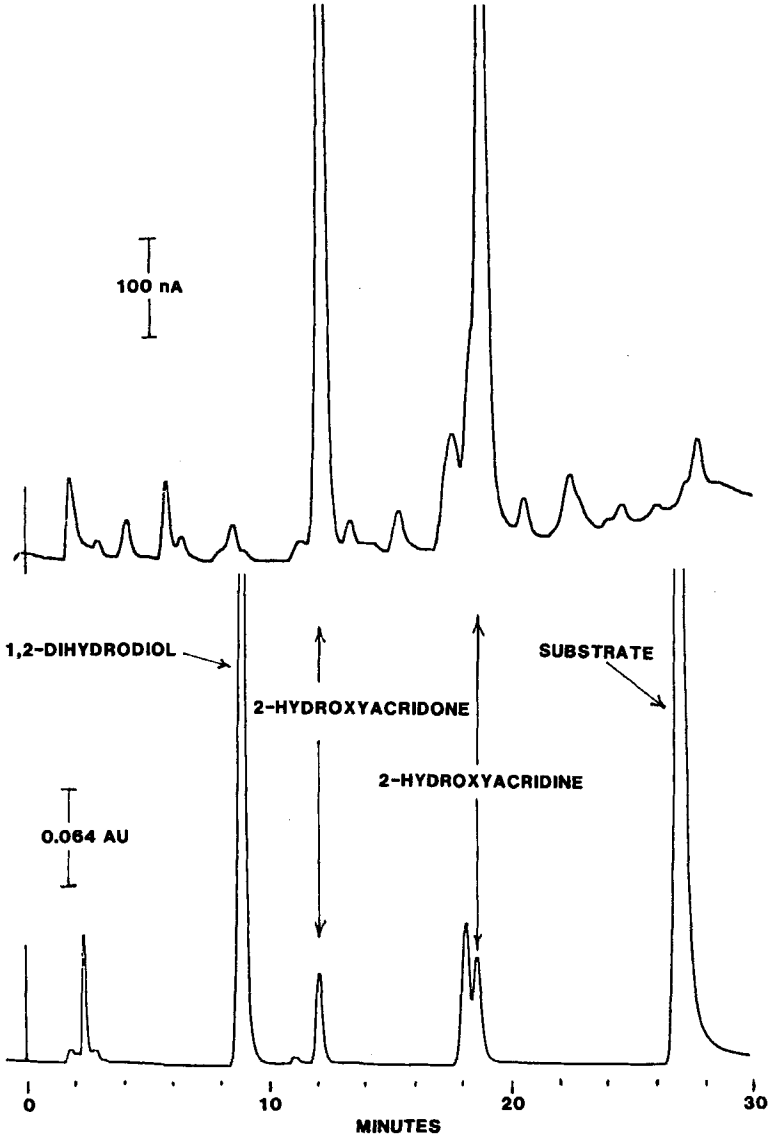


FIGURE 3. EC (top) and UV (bottom) chromatograms of the ethyl acetate soluble metabolites of acridine from 3-MC induced rat liver enzymes. Phenolic components are: 2-hydroxyacridone (12.0 min) and 2-hydroxyacridine (18.5 min).

include acridine-1,2-dihydrodiol (9.0 min), acridone (18.0 min), and the substrate (27.5 min). As expected, these three compounds are not electroactive.

Metabolism of acridine by guinea pig liver enzymes induced by polychlorinated biphenyls gave ethyl acetate soluble residues which provided the chromatograms in Fig. 4. As can be seen, the metabolism of acridine by these enzymes gives a pattern which is different than that provided by the enzymes used to obtain Fig. 3. The principal metabolic pathway is through acridone (19.5 min). Subsequently we have determined that acridone is produced by catalytic action of aldehyde oxidase (EC 1.2.3.1), a soluble hepatic enzyme system (13). Further metabolism of acridone gives 2-hydroxyacridone (13.5 min), and a diphenol, labelled 'B' in the UV chromatogram which we have tentatively identified as the 2,7-isomer. Other highly oxygenated metabolites, which also appear to be phenolic, include 'A' (4.5 min) and 'C' (8.8 min). The peak at 9.5 min in the UV chromatogram (which does not appear in the EC tracing) is due to the 1,2-dihydrodiol.



**Phenanthridine:**

The metabolism of phenanthridine by 3-methylcholanthrene induced rat liver enzymes is represented by the chromatograms in Fig. 5. Inspection of these two chromatograms indicate that at least one major and two minor metabolites are phenols (11.5, 18.2, and 23.5 min). The large phenolic peak at 11.5 min appears to be derived from metabolism of phenanthridone (the metabolite evident in the UV chromatogram at 20.5 min). Phenanthridone, like acridone, is a product of aldehyde oxidase catalysis. The relatively large peak at

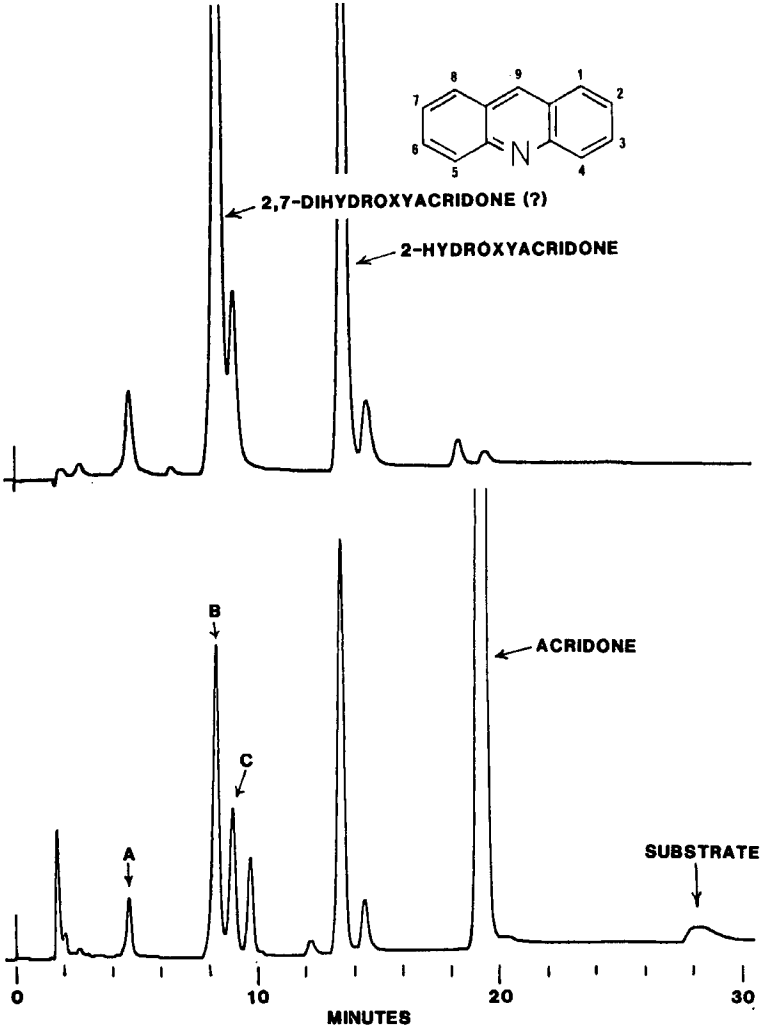


FIGURE 4. EC (top) and UV (bottom) chromatograms of the ethyl acetate soluble metabolites of acridine from PCB induced guinea pig liver enzymes. Phenolic components include 2-hydroxyacridone and a polar metabolite tentatively identified as 2,7-dihydroxyacridone.

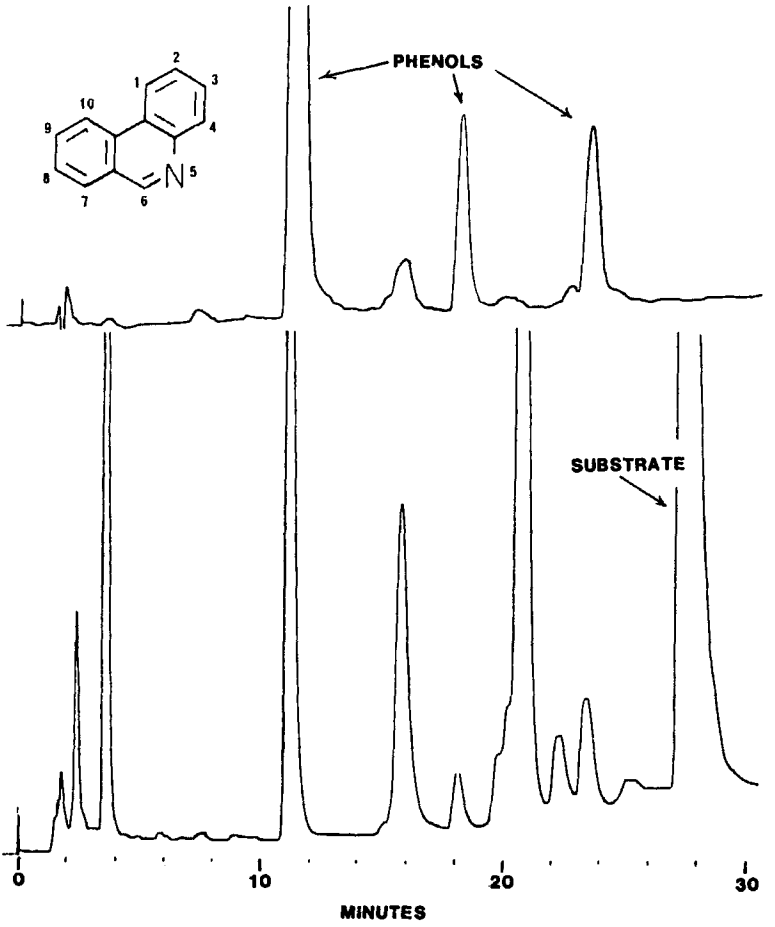


FIGURE 5. EC (top) and UV (bottom) chromatograms of the ethyl acetate soluble metabolites of phenanthridine from 3-MC induced rat liver enzymes. Components include a phenolic metabolite of phenanthridone (11.5 min), two phenols of unknown structure (18.2 and 23.5 min), phenanthridine-N-oxide (16.0 min), phenanthridone (20.5 min), and substrate (28 min).

16.0 min has been identified as phenanthridine-N-oxide by comparison with authentic material.

## SUMMARY

As described above, coupled UV and EC detectors can give information which allows the metabolites of PAH and PANH to be classified rapidly as being phenolic or nonphenolic. When combined with other information (relative HPLC retention, gas chromatography/mass spectrometry, etc.) results from combined UV-EC chromatography can greatly facilitate research on metabolic pathways.



## ACKNOWLEDGEMENT

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