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13. ABSTRACT *(Maximum 200*

Identification of the metabolites of DQHS and other artemisinin analogues has been problematic because of the unavailability of standards of the putative metabolites. The following report presents our attempts to circumvent this problem through a combination of mass spectrometry and derivatization of DQHS.

The metabolism of dihydroqinghaosu (DQHS) (dihydroartemisinin) in rat liver microsomes and the isolated perfused rat liver was studied. The metabolites were identified by high performance liquid chromatography/thermospray- (TSP) mass spectrometry. The metabolism of DQHS was found to involve C-hydroxylation at the 2-, 3-, 9-, or 14-position. Deoxygenation of the endoperoxide function together with C-hydroxylation gave rise to different isomers of hydroxy deoxydihydroqinghaosu (hydroxy deoxyDQHS), the prevalent metabolites of DQHS. DQHS also apparently underwent enzymatic oxidation to isomers of both hydroxy deoxyqinghaosu and dihydroxy deoxyQHS. Metabolites apparently resulting from a rearrangement of the A- and B-rings of the molecule have also been tentatively identified.

In the last few weeks we have achieved a minor breakthrough in finding simple routes to the preparation of the putative metabolites of DQHS and other artemisinin analogues. Although this is not exactly a final report on our study of the metabolism of DQHS, with this recent finding, we are only a few more weeks of laboratory work from the final report.

A study of the metabolism of artelinic acid is part of a recent extension to our contract.

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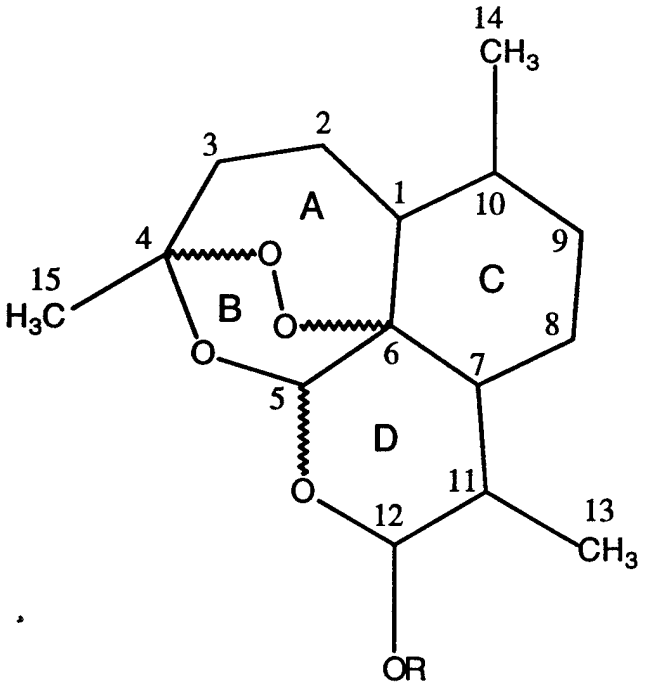
1. Introduction

Qinghaosu (QHS) (also known as artemisinin), a sesquiterpene lactone with an unusual endoperoxide linkage, is the clinically active antimalarial principle isolated from the Chinese medicinal herb, Qinghao or *Artemisia annua* L [1, 2]

For the past ten to twelve years the Steering Committee of the Scientific Working Group on Malaria of the World Health Organization (SWG-CHEMAL) and the Walter Reed Army Institute of Research (WRAIR), have actively pursued the development of new anti-malarial agents based on the lead provided by qinghaosu (QHS).

The development of more potent semi-synthetic derivatives of QHS with improved physico-chemical and/or pharmacological properties has focused on the reduction of QHS to the lactol, dihydroqinghaosu (dihydroartemisinin; Figure 1, DQHS), and subsequent preparation of the ether (or ester) derivatives of DQHS which are soluble either in water or oils and may be administered parenterally in the treatment of cerebral malaria The water-soluble derivatives of DQHS of clinical interest are artesunate (AS) the hemisuccinate ester of DQHS and artelinic acid (AL) the 4-carboxybenzyl ether of DQHS. The oil-soluble derivatives of DQHS of interest are artemether (AM) and arteether (AE), which are the methyl and ethyl ethers of DQHS respectively. DQHS itself has potent anti-malarial activity and was reported to be more active than QHS. The ether and ester derivatives of DQHS have also been shown to produce DQHS as a major primary metabolite and these too seem to serve as pro-drugs of DQHS [3-7]. It is therefore important to understand the metabolism of DQHS as a first step towards understanding the metabolism of the other artemisinin analogues with respect to their rate and extent of conversion to DQHS and its metabolites.

Because of the apparent central role of DQHS in the metabolism and *in vivo* activity of its ether and ester derivatives, the first objectives of the project was to identify the *in vitro* metabolites of DQHS formed in rat liver preparations.



DQHS: R = H

AE: R = CH_2CH_3

AS: R = $C(=O)CH_2CH_2COOH$

AL: R = CH_2 —  —COOH

Figure 1. Structures of dihydroqinghaosu (DQHS), arteether (AE) artesunic acid (AS) and artelinic acid (AL)

Artelinic acid is a semi-synthetic, water-soluble derivative of QHS which was prepared in this institute. The sodium salt of artelinic acid (sodium artelinate) is a water-soluble form of artelinic acid for which the U.S. Army has exclusive rights and patents. Sodium artelinate has been reported to be effective against chloroquine resistant *Plasmodium falciparum* and has superior *in vivo* activity against *P. berghei.* When prepared as a solution in potasium carbonate this compound has been reported to be stable to hydrolysis [8]. This is a desirable advantage over sodium artesunate, the only other water-soluble derivative of QHS, which has

been extensively studied in China and Southeast Asia for the treatment of cerebral malaria. Sodium artelinate also has the advantage of being the only water-soluble derivative of QHS which can be administered by iv infusion [9] Sodiun artelinate is, therefore a promising future drug for the treatment of cerebral malaria. Preliminary studies at this institute have also shown that sodium artelinate has other advantages over the other QHS derivatives including its high plasma levels following iv or im or oral administration, its relatively long elimination half life and high bioavailability in rat and dog.

However, artelinate seems to undergo less conversion to dihydroqinghaosu (DQHS), the active metabolite common to the QHS derivatives. If, as has been suggested, both the anti-malarial activity [10] and toxicity [11] of the QHS derivatives are largely due to DQHS, the metabolism of sodium artelinate will be expected to influence its anti-malarial activity and toxicity. It is, therefore, neccesary to understand the metabolism of artelinate. Past or current studies on the metabolism of other QHS derivatives can only provide a limited insight into the metabolism of artelinate because it possesses a benzyl group, a potential target of metabolising enzymes, which is absent in other QHS derivatives. A study of the in vitro metabolism of artelinate has been initiated as one of the initial steps towards the goal of the U.S. Army to develop sodium artelinate as an oral treatment for uncomplicated malarial caused by multidrug resistant *P. falciparum*

2. Identification Of The *in vitro* Metabolites of DQHS

The metabolism of artemisinin analogues has been reviewed [12], and generally involves hydroxylation on the A- and C- rings of the molecule and/or deoxygenation of the endoperoxide moiety to the deoxy- compounds. Possible sites of hydroxylation are the 1,2,3,8,9,10 and 14 positions of the molecule (see Figure 1) Thus the metabolic hydroxylation of these compounds gives rise to isomeric compounds which are difficult to distinguish in the absence of authentic standards. Therefore, the essential problem in studies of the metabolism of artemisinin and its derivatives is the non-availability of authentic standards of the metabolites. The methods which have been adopted to surmount this problem will be described below.

The apparent biotransformation of DQHS by hydroxylation has been reported previously [6].
However, the exact positions of hydroxylation in the isomeric metabolites could not be specified because authentic putative metabolites were not available for comparison.
Because authentic samples of metabolites were not available, the following two approaches were initially adopted in the present study, in an attempt to identify the hydroxylated metabolites of DQHS:

(1). Direct HPLC-Mass spectrometry (HPLC-MS) of extracts obtained following the incubation of DQHS with rat liver preparations.

(2). Preparation of UV/fluorescent derivatives of DQHS and its metabolites to enhance their detection, identification and quantitation during metabolism studies.

Because the above approaches have yielded only tentative information on the structure of the metabolites our present efforts are directed at investigating the metabolism of DQHS by chemical mimics of cytochrome P-450 as a way of obtaining authentic standards of the metabolites to be used for the unambiguous identification of the metbolites of DQHS.

2.1. Identification of the Metabolites of DQHS by High Performance Liquid Chromatography/Thermospray Mass Spectrometry

DQHS was incubated with rat liver microsomes or with the isolated perfused rat liver. After separation by gradient reversed phase liquid chromatography, the metabolites of DQHS were identified by thermospray mass spectrometry. DQHS labeled with carbon-14 in the 13-position of the molecule was used in these studies. In addition, DQHS labeled with deuterium on the position-13 carbon was used to elucidate both the metabolism and mass spectral fragmentation processes of DQHS and its metabolites.
(Experimental details are presented in Appendix 1)
Thermospray ionisation has always been preferred in the mass spectrometry of these compounds because the compounds are much more prone to extensive thermal decomposition under the conditions normally required for acquiring electron impact- or chemical ionisation mass spectrometry.

Tentative schemes of the metabolic pathways of DQHS in the rat liver preparations are shown in Appendix 1

2.1.1. Identification of metabolites of DQHS retaining the endoperoxide function of DQHS

From an observation of the nearly-identical thermospray mass spectra of DQHS and its analogues such as arteether, artemether, artesunate and the propyl ether it has become clear that the mass spectra of these compounds even under this "soft" ionisation technique is determined essentially by the behavior of the thermally unstable endoperoxide group which induces the expulsion of HCOOH from their molecule, pre- or post-ionisation.

Based on this observation it would be expected that the isomers of hydroxy DQHS would have thermospray mass spectra nearly identical to those of the isomers of hydroxy arteether and the available authentic spectra of the isomers of hydroxy arteether may be useful in identifying the isomers of hydroxy DQHS in the absence of authentic samples of the latter. That is, the unknown metabolites of DQHS with spectra which are nearly identical to those of authentic samples of hydroxy arteether must have isomers of hydroxy DQHS. This was one of the approaches adopted in identifying the isomers of hydroxy DQHS formed by the metabolism of DQHS

On this basis, 2-hydroxy DQHS (2-OH-DQHS), 3-hydroxy DQHS (3-OH-DQHS) and 9-hydroxy DQHS (9-OH-DQHS) were tentatively identified as metabolites of DQHS in the rat liver preparations. On the one hand, 9-hydroxy DQHS, in which the hydroxy group is on the C-ring of the DQHS structure is readily distinguished on the other hand from 2- and 3-hydroxy DQHS as a group with their hydroxy function on the A-ring. Overall, each of these compounds have unique features in its mass spectrum which may be related to the position of the hydroxy group.

2.1.2. Identification of deoxy-DQHS and the isomers of hydroxy deoxy-DQHS formed by the metabolism of DQHS

Deoxy-DQHS (DDQHS) was unambiguously identified as a metabolite of DQHS by comparison with the mass spectrum of the authentic compound. Direct deoxygenation of DQHS to deoxy-DQHS was found to take place only in the IPRL.

Most of the metabolites of DQHS result from a combination of deoxygenation of the endoperoxide group and hydroxylation of carbon atoms of the ring structure to give different isomers hydroxy deoxy-DQHS. Deuterium labeling of the 13-position has now confirmed that the carbon atom at this position was not affected by metabolism and that the metabolic hydroxylation of DQHS was restricted to the A- and C-rings. We have been able to show tentatively that 2-hydroxy deoxy-DQHS (2-OH-DDQHS), 3-hydroxy deoxy-DQHS (3-OH-DDQHS), 9-hydroxy deoxy-DQHS (9-OH-DDQHS) and 14-hydroxy deoxy-DQHS (14-OH-DDQHS) are the four isomeric hydroxy deoxy-DQHS metabolites of DQHS resulting from this combination of deoxygenation and hydroxylation.

Although the mass spectra of the isomers of hydroxy deoxy-DQHS are very similar they exhibit predictable differences which make it possible to suggest the position of the hydroxy group on the A- or C-rings of the molecule. Work with deuterium-labeled DQHS confirmed that the carbon atoms of the D-ring of the compounds are retained in the fragment ions in the spectra of these compounds, thus eliminating the possible ambiguity of the mass spectral interpretations which could have arisen from the fact that the D-ring is identical in all the metabolites.

As with deoxy-DQHS, 14-hydroxy deoxy-DQHS (14-OH-DDQHS) was detected only in the bile from the IPRL.

2.1.3. Identification of hydroxy deoxy-QHS and dihydroxy-deoxy-QHS formed by the metabolism of DQHS

3- and 9-hydroxy deoxy-QHS have been tentatively suggested to be metabolites of DQHS bazed on their mass spectra.

9-Hydroxy deoxy-QHS was identified as a metabolite of DQHS based on its mass spectrum.

2.1.4. Identification of ring-opened metabolites of DQHS

Three compounds (designated M222, M224 and M226, respectively) have been identified as metabolites of DQHS resulting from the opening and rearrangement of the A and B rings of DQHS. These rearrangement compounds are analogous to a published microbial metabolite of arteether (AEM1) [3].

3. Preparation of UV/Fluorescent Derivatives of DQHS and Metabolites To Enhance Their Detection, Identification and Quantitation during Metabolism Studies

As mentioned above, metabolism studies with DQHS and other artemisinin analogues are generally hampered by the unavailability of standards of putative metabolites; a problem which arises from the difficulty of synthesising the artemisinin skeleton. Therefore, as a futher means of establishing the identity of the metabolites of DQHS an extensive study was carried out on the preparation of UV-absorbing and/or fluorescent derivatives of DQHS and its metabolites extracted from incubates of rat liver homogenate or rat liver microsomes. The rationale for this aspect of the work on the metabolism of DQHS was as follows: First, it was thought that in contrast to the hydroxy deoxy-DQHS isomers themselves, suitable derivatives of these compounds might have widely different mass spectra, making it easier to distinguish between the isomers. Second, the derivatisation of DQHS to a UV/fluorescent derivative would also serve as a basis for the development of a HPLC-UV/fluorescent method to detect and quantitate DQHS and its metabolites during the metabolism studies. Presently available methods for the artemisinin analogues based on HPLC with electrochemical detection are of limited value in the metabolism studies because the major metabolites lack the reducible endoperoxide moiety on which electrochemical detection depends.

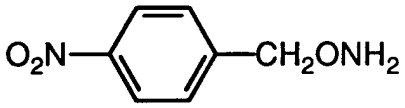
To be satisfactory as a derivatization reaction for the metabolism studies, the reaction should meet certain requirements. Namely: it should be easy to carry out the reaction, without the need for elaborate apparatus; the reaction should be relatively rapid, and should yield a single, stable product; side reactions, if any, should be minor; the reaction should be specific for DQHS and its metabolites, without interference from endogenous co-extractives from the biological sample; if the reagent and the products have similar spectroscopic properties (UV or fluorescence) it should be easy to separate the excess reagent from the products either before or during the chromatographic step of analytical procedure. With these requirements as guidelines, a number of possible approaches to the derivatization of DQHS were investigated. Apart from a study of the reaction of DQHS with conventional carbonyl reagents the investigation centered on the preparation of ester derivatives of

DQHS and metabolites extracted from incubates of DQHS with rat liver homogenate and rat liver microsomes. These reactions and the outcome of the attempts to apply them to the derivatisation of DQHS and metabolites in biological samples are described below

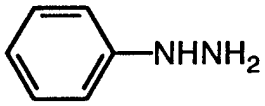
3.1. Derivatization of DQHS as an 'Aldehyde'

DQHS, being a hemiacetal, may be considered a "masked" aldehyde and, opening up the hemiacetal ring of DQHS would unmask the aldehyde group(s) in DQHS. It was thought that if the opening up of the hemiacetal ring(s) of DQHS could be induced in the presence of a reagent which reacts with aldehydes, it should then be possible to derivatise DQHS with such a reagent.

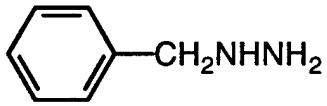
DQHS was found to react with the following compounds, as would be expected of an aldehyde. The reaction takes place in anhydrous pyridine:

(i) 

O-(4-Nitrobenzyl)hydroxylamine (NBOX)

(ii) 

Phenylhydrazine (PHZ)

(iii) 

Benzylhydrazine (BHZ)

As may be expected, NBOX reacts more readily with DQHS than PHZ or BHZ.

Tosyl hydrazine in anhydrous pyridine did not react with DQHS.

The reaction of DQHS with NBOX was found to satisfy the requirements for an acceptable derivatization reaction as stated earlier. The sensitivity of this reagent was, however inadequate and

an attempt was made to prepare fluorescent O-arylmethyl hydroxylamines to use as reagents for the sensitive HPLC-fluorescence detection and quantitation of DQHS and its metabolites. New fluorescent O-substituted hydroxylamines prepared include O-(4-phenylbenzyl)hydroxylamine, O-(4-benzyloxybenzyl)-hydroxylamine, O-(1-naphthalenemethyl)hydroxylamine and O-(9-anthracenemethyl)hydroxylamine. Although the O-arylmethyl hydroxylamines reacted with DQHS and its metabolites extracted from biological fluids, formation of interfering substances by the decomposition of the O-substituted hydroxylamines was a drawback to this approach.

Attention was then turned to an investigation of the esterification of DQHS as means of preparing fluorescent derivatives of the compound.

3.2. Esterification of DQHS

Esterification invariably requires the activation of the carboxylic acid to effect its condensation with the alcohol. This is particularly important with very unreactive hydroxy groups such as that in DQHS [13, 14, 15].

A further difficulty arises when, as in the case with DQHS, mild reaction conditions are required because of the instability of DQHS towards acids and bases and heat. Several attempts to effect the esterification of DQHS using many well-known catalysts and condensing agents. While most of these reactions worked for neat samples of DQHS, they were less successful when applied to DQHS and its metabolites extracted from biological samples. The most successful approach was found in the use of hindered aryl sulphonic acid chlorides as the carboxylic acid activating agent, an approach which had not been previously applied to analytical esterification of alcohols.

To prepare carboxylic acid esters, carbamate esters and carbonate esters of DQHS, the following carboxylic acids, carbamyl chlorides and chloroformates were reacted with DQHS:

[a] Carboxylic Acid Esters

(i) Aromatic Acids

4-Biphenylcarboxylic acids

3,4-Dimethoxybenzoic acids

Naphthoic acid
Diacetyldihydrofluorescein (DADF)

(ii) Arylalkanoic acids
1-Naphthaleneacetic acid
9-Fluoreneacetic acid
9-Fluorenecarboxylic acid
1-Pyrenebutyric acid

(iii) Coumarin acids
Coumarin-3-carboxylic acid
7-Carboxymethoxy-4-methylcoumarin

[b] Carbamates

Carbamyl chlorides
N,N-Diphenylcarbamyl chloride
N-Methyl-N-phenylcarbamyl chloride

[c] Carbonate Esters

Chloroformates
9-Fluorenylmethyl chloroformate
4-Nitrophenyl chloroformate

The different approaches to the analytical esterification of DQHS investigated are reported below, in terms of the catalyst used to activate the carboxylic acid

3.2.1. Esterification of DQHS using Dicyclohexyl-carbodiimide (DCC) as Catalyst

The reaction of DQHS with a carboxylic acid using DCC as a catalyst is illustrated with an example in figure 2.

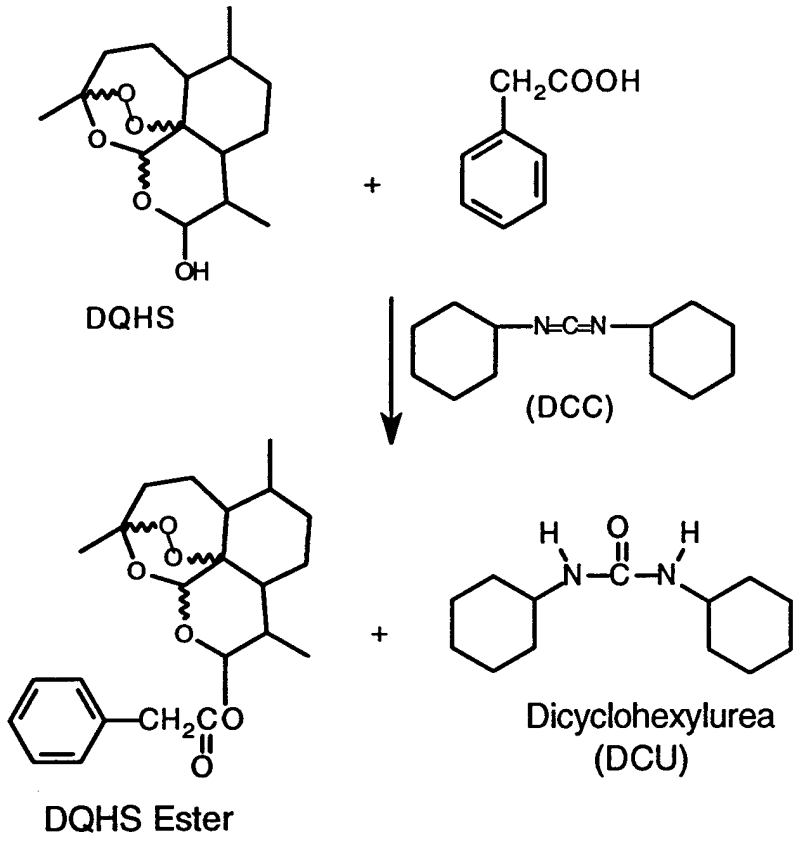


Figure 2: Reaction of DQHS and a carboxylic acid with DCC as catalyst

For example, Luo, Yeh and Brossi [16] reported the esterification of DQHS with diacetyldihydrofluorescein (DADF) and a combination of DCC and 4-dimethylaminopyridine (DMAP) as catalysts. It was suggested that this reaction (illustrated in figure 3) could be a basis for the detection of nanomolar levels of DQHS and its deoxy metabolites.

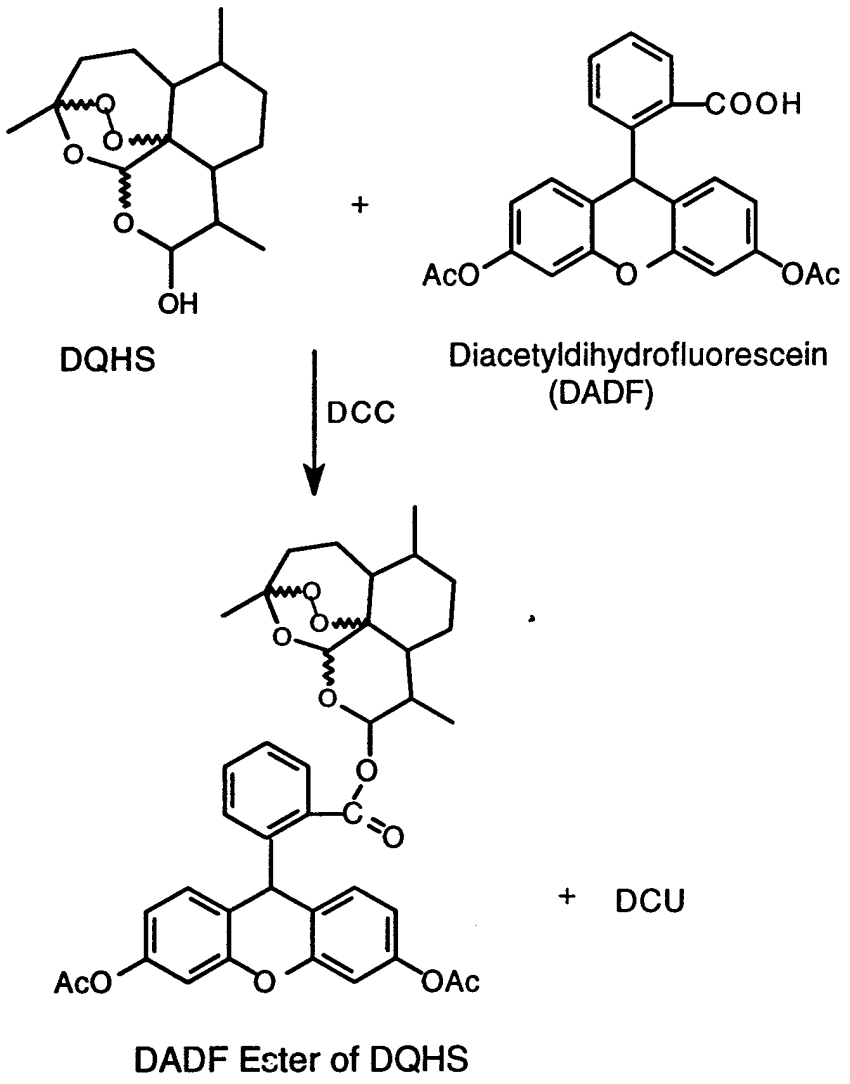


Figure 3: Reaction of DQHS with diacetyldihydrofluorescein

Luo et. al. did not apply this reaction to an actual study of the metabolism of DQHS. Similarly, Luo and Xie [17] reported the detection of subnanogram levels of the DADF ester of DQHS by HPLC-UV but did not apply the reaction to a study of the metabolism of DQHS.

In the present study, esterification of DQHS with a combination of DCC and DMAP (or 4-pyrrolidinopyridine, PPY) as catalysts was found to be inadequate as an analytical reaction because it is beset by the following problems:

(i) Formation of N-acylureas as side products. This side reaction is illustrated in figure 4

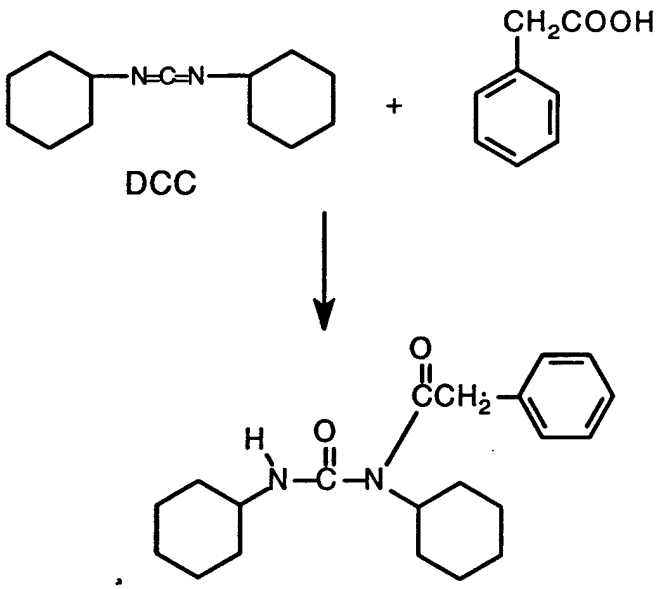


Figure 4: Formation of an N-acyl dicyclohexylurea from DCC and a carboxylic acid

Although the N-acyl dicyclohexyureas were well separated from the respective esters of DQHS, they may interfere during actual metabolism studies.

(ii). Dicyclohexylurea (DCU), the other product of the reaction is poorly soluble in organic solvents and was difficult to remove. Unexpected precipitation of DCU in the solution to be chromatographed made sample handling difficult.

Although low nanogram levels of pure samples of some of the DQHS esters prepared in the present investigation could be detected by HPLC-UV, application of the DCC-catalysed esterification of DQHS to extracts of liver homogenate incubated with DQHS was unsuccessful. Even DQHS itself could not be detected in such samples, probably because of interference in the reaction from endogenous nucleophilic compounds co-extracted from the samples.

3.2.2. Esterification of DQHS using N,N-Carbonyl-diimidazole (CDI) as Catalyst

Esterification of DQHS with CDI as catalyst is illustrated in figure 5:

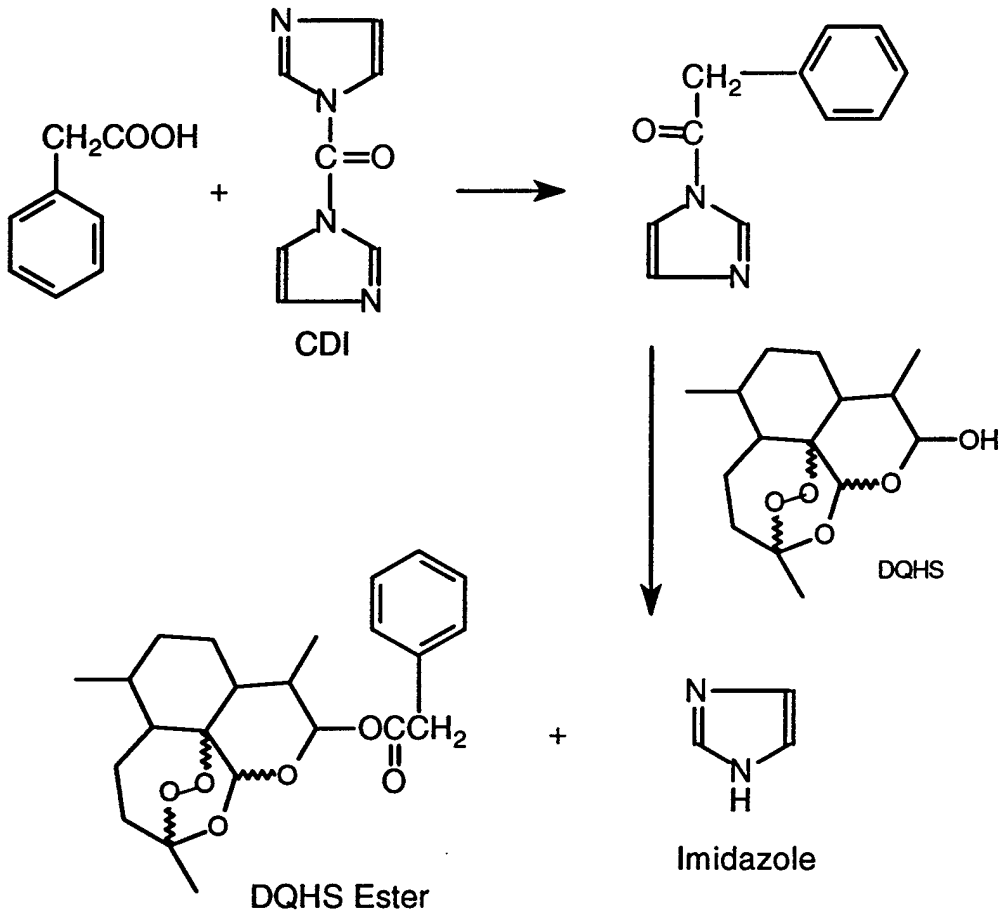


Figure 5: Reaction of DQHS and a carboxylic acid with CDI as catalyst

This approach has the advantages that the reaction takes place under mild conditions and that imidazole formed as a side product is easy to remove, being soluble in water. Although esterification of DQHS with CDI as catalyst has not been previously reported, it was found to work, with pure samples of the esters being readily obtained. However, application of the reaction to extracts of DQHS and metabolites from liver homogenate and liver microsomes was not successful, probably due to interference from nucleophilic co-extractives.

3.2.3. Esterification of DQHS with Carboxylic Acid Chlorides, using 4-Dimethylaminopyridine (DMAP) or Pyrrolidinopyridine (PPY) as Catalyst

Esterification of DQHS with acid chlorides or anhydrides is a well-known procedure [13, 14, 15]. The reaction is illustrated in figure 6:

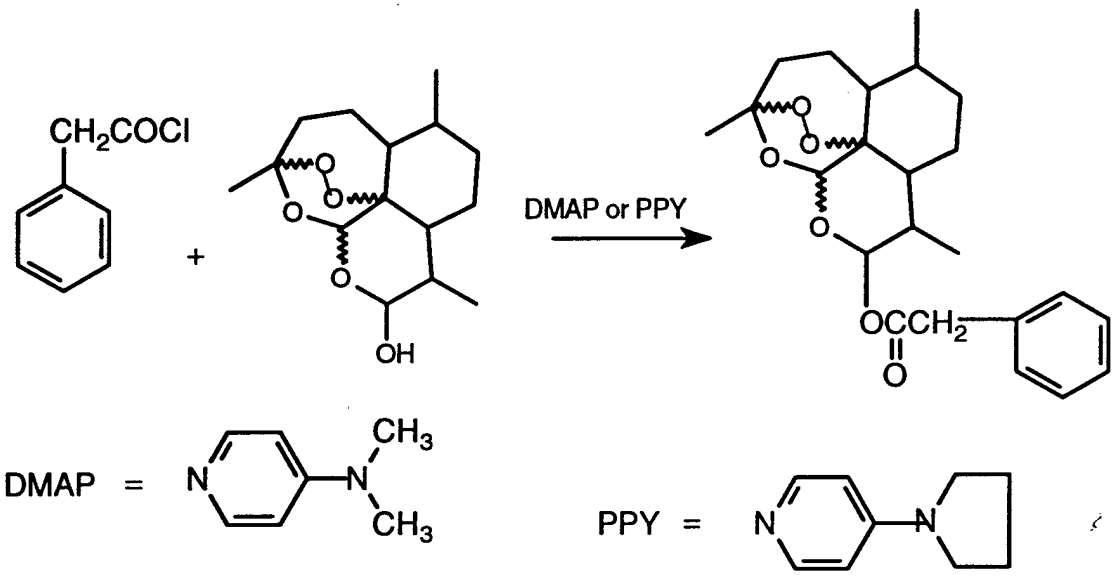


Figure 6: Reaction of DQHS with an acid chloride using DMAP or PPY as catalyst

It was found that the ester derivatives of DQHS are much easier to prepare by this procedure than by the DCC method. PPY was found to be a stronger catalyst than DMAP. With PPY as catalyst, reaction was apparently complete in 1h or less. With DMAP, the reaction mixture had to be kept for 12 to 14h.
Application of the reaction to extracts of DQHS and metabolites was not wholly successful as only DQHS itself could be detected. Different extraction methods, including solvent extraction, solid phase extraction with commercial extraction cartridges and charcoal adsorption, were adopted without any improvement in the result.

3.2.4. Esterification of DQHS with Carbamyl Chlorides or Chloroformates using DMAP or PPY as Catalyst

Formation of carbamate and carbonate derivatives of DQHS have been reported [13]. These reactions are illustrated in figure 7:

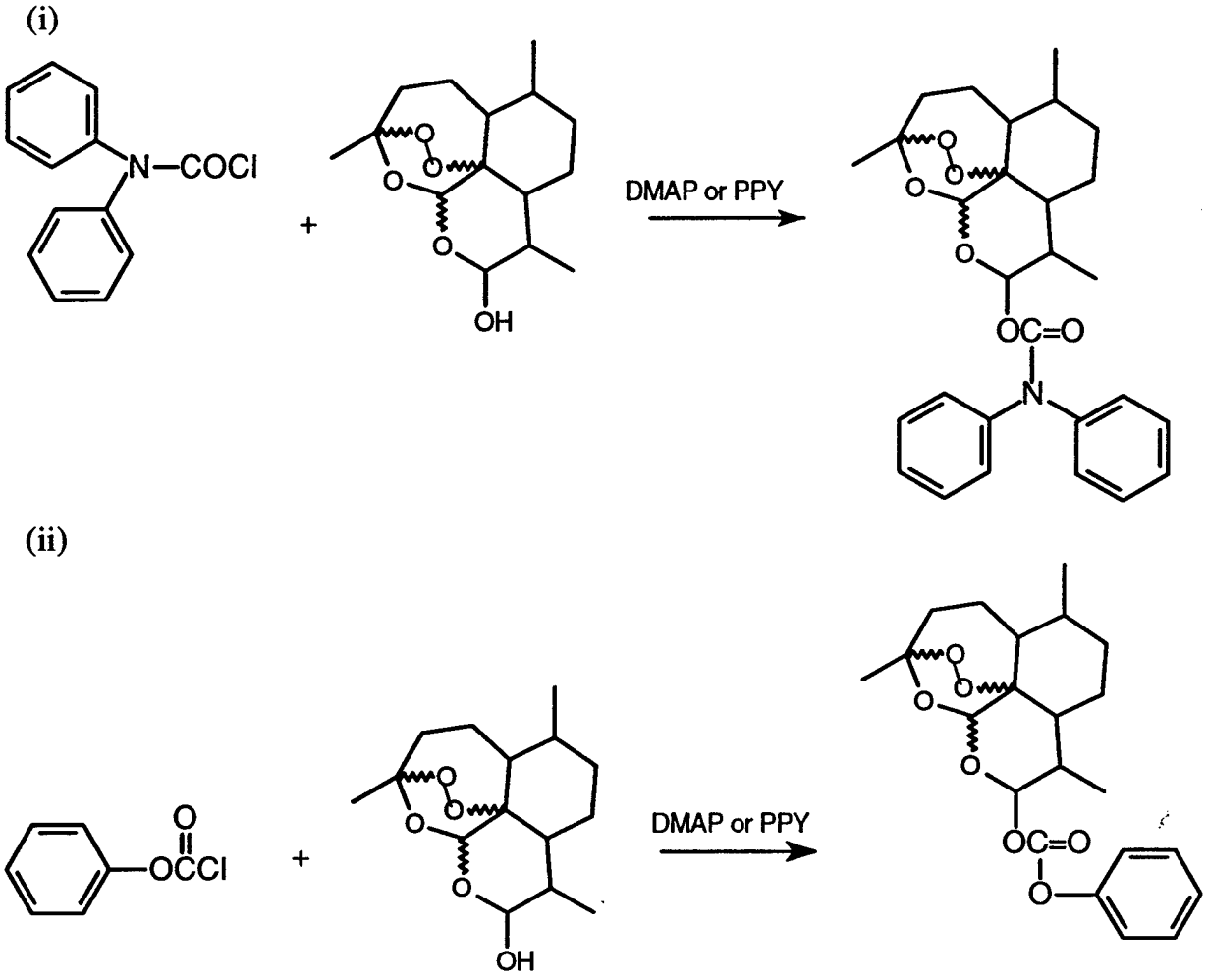


Figure 7: Reaction of DQHS and (i) a carbamyl chloride and (ii) a chloroformate with DMAP or PPY as catalyst.

Application of the reaction to the HPLC-UV analysis of extracts of DQHS and metabolites was not successful because diphenylurea or diphenylcarbonate formed respectively as side products from the excess reagents interfered with the chromatography of the derivatives. These side products would result from the excess reagents as illustrated in figure 8:

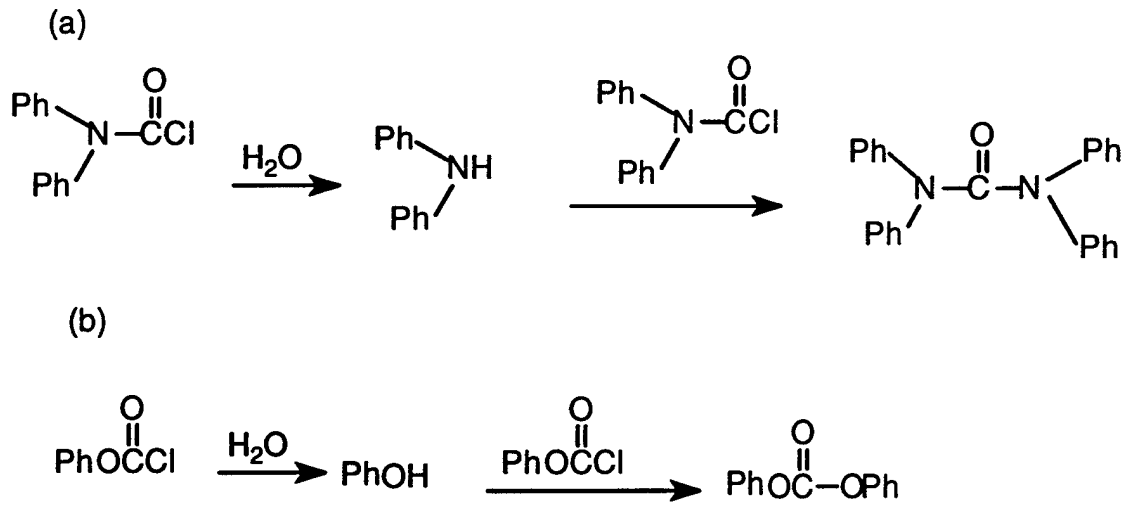


Figure 8: Formation of (a) diphenylurea from diphenylcarbamyl chloride and (b) diphenylcarbonate from phenyl-chloroformate

Derivatives of DQHS and metabolites with diphenylcarbamyl chloride were found to be detectable by TLC with UV light or after heating the plate in an oven (purple spots) or spraying the plate with dilute nitric acid and drying in an oven (yellow-green or blue-green spots). However, this result was difficult to reproduce.

3.2.5. Esterification of DQHS using 1-methyl 2-chloropyridinium iodide as condensing agent

The use of 1-methyl 2-halopyridinium salts as condensing agents in the formation of ester and amide bonds has been studied extensively by Mukaiyama *et. al.* [18].

Although this method has not been applied to the analytical esterification of alcohols, we investigated the esterification of DQHS with various carboxylic acids using 1-methyl 2-chloropyridinium iodide as the condensing agent. The esterification of DQHS with 1-methyl 2-chloropyridinium iodide as the condensing agent is illustrated below (figure 9):

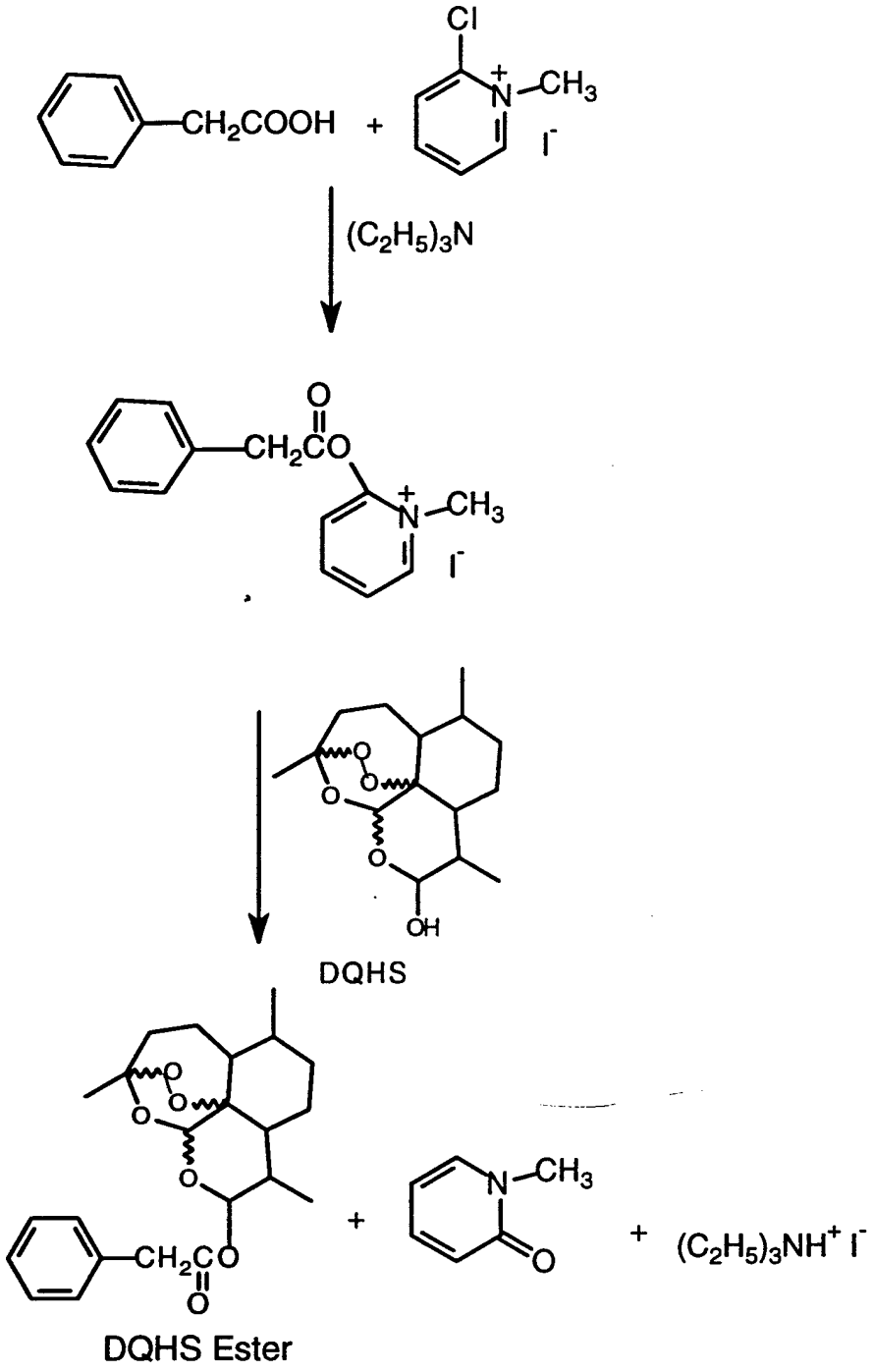


Figure 9: Esterification of DQHS using 1-methyl-2-chloropyridinium iodide as catalyst

DQHS was readily esterified with several fluorescent acids using 1-methyl 2-chloropyridinium iodide as the condensing agent. Application of this method to the derivatisation of DQHS and it metabolites extracted from biological samples was not successful. The poor solubility of the reagent made it difficult to control the quantity to be used for analytical derivatization where the need to avoid too much of excess reagent is paramount. Attempts to dansylate DQHS with dansyl glycine and dansyl butyric acid with 1-methyl 2-chloropyridinium iodide as the condensing agent were also not successful. This may have been due to the possible self-condensation of the dansyl amino acids. With the mixed anhydride method, such self-condensation is precluded by the ethoxycarbonylation of the sulfanamido nitrogen of the dansyl amino acid during the preparation of the mixed anhydride. It is also likely that the dansyl amino acid condenses with 1-methyl 2-chloropyridinium iodide via the sulfanamido nitrogen to give an intermediated which cannot acylate DQHS.

3.2.6. Esterification of DQHS with mixed carboxylic-carbonic anhydrides

The most well-known application of mixed carboxylic-carbonic anhydrides is the formation of peptide bonds [19]. This technique has never been applied to the analytical esterification of an alcohol. We have found that DQHS may be readily esterified by a variety of carboxylic acids after the acids have been converted to the mixed anhydrides with ethyl chloroformate. The reaction of DQHS with a carboxylic acid using the mixed ethoxyformic anhydride is illustrated below (figure 10):

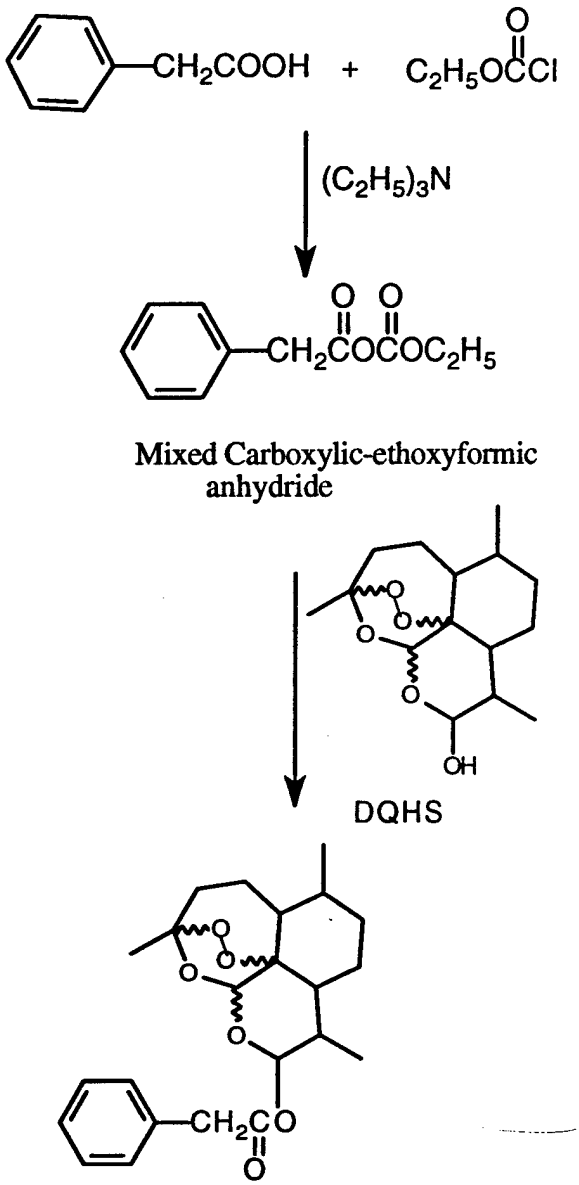


Figure 10: Reaction of DQHS and a mixed carboxylic-ethoxyformic anhydride

Esterification of DQHS with 10 different acids, including seven fluorescent arylalkanoic acids, has been investigated. All the acids, as the mixed ethoxyformic anhydrides, reacted smoothly and rapidly with DQHS, except 9-anthracenecarboxylic acid which reacted only very slowly and in poor yield. A novel approach to this method was also developed, which involves the preparation of the mixed anhydride by a two-phase reaction of ethyl chloroformate in chloroform with a sodium hydroxide solution of the acid, in presence

of a phase transfer reagent such as tetrabutyl ammonium hydrogen sulphate. After evaporating the excess ethyl chloroformate, the resulting mixed anhydride is re-dissolved in chloroform and reacted with a chloroform solution of DQHS. This method was found to give a cleaner "analytical" reaction in contrast to the homogeneous reaction used in peptide synthesis, which was found to give rise to some unwanted side-products. With our two-phase approach to the preparation of the mixed anhydride, the only significant side product identified by mass spectrometry is the ethyl ester of the carboxylic acid. However, the ethyl esters elute very early on HPLC of the reaction mixture and, in most cases do not interfere with the desired ester derivative of DQHS.

The ester derivatives of DQHS with the arylalkanoic acids, such as biphenyl acetic acid, were detectable at low nanogram levels by HPLC-UV. Application of this method to extracts of DQHS and its metabolites from liver homogenate was successful. However, with some of the acids, formation of the anhydride was observed when the application of the reaction to biological extracts necessitated the use of a large excess of the reagent. Also, in some cases the anhydride also interfered in the chromatography of the desired derivatives.

This novel approach also made it possible to dansylate DQHS with dansyl amino acids such as dansyl glycine thus establishing a basis for the detection and determination of DQHS at sub-nanogram, or lower, levels on the basis of the well-known dansyl fluorophore. Although esterification of alcohols with dansyl amino acids has not been previously attempted, it was thought that the method being developed for the dansylation of DQHS will be of value in the HPLC-fluorescence analysis of alcohols as their novel dansyl amino acid esters. Although the dansyl group is one of the most fluorescent , as well as the most studied of all fluorigenic reagents, analytical dansylation of alcohols has not been achieved with dansyl chloride (or even with dansyl fluoride) because of the inadequate reactivity of the alcohol. Although this approach to the dansylation of alcohols was of limited success when applied to DQHS and metabolites extracted from incubates of rat liver preparations, it may be a useful development in the analysis of alcohols in general.

3.2.7. Esterification of DQHS with mixed carboxylic-sulfonic anhydrides

The partial success observed in the esterification of DQHS with mixed carboxylic-carbonic anhydrides encouraged further investigation of other mixed anhydrides, in particular, mixed carboxylic-sulphonic anhydrides which have been applied in other condensation reactions which are analogous to esterification. 2,4,6-Trimethylbenzenesulfonyl chloride (mesitylene sulfonyl chloride) and 2,4,6-triisopropyl benzenesulfonyl chloride and 2,4,6-trichlorobenzoyl chloride were examined as the condensing agents. These hindered sulphonyl chlorides were developed only as condensing agents in the formatiom of internucleotide bonds, although there are two reports on the use of mesitylene sulphonyl chloride in the formation of large-ring lactams (cylisation of amino acids). Use of mixed carboxylic-sulphonic anhydrides prepared from these reagents was found to be a ready approach to the analytical esterification of DQHS and its metabolites, as well as other alcohols in general. A paper based on this work has been accepted for publication in the *Journal of Liquid Chromatography and Related Technologies.* A copy of the manuscript is attached (Appendix 2).

4. Work in Progress

4.1. Preparation of Metabolites of DQHS or Artelinic Acid by Reaction of DQHS or Artelinc Acid with Chemical Models of Cytochrome P-450

As discussed above, the principal obstacle in the study of the metabolism of artemisinin analogues is the non-availability of standards of the putative metabolites. This problem arises because total synthesis of the artemisinin skeleton is involved and expensive. So far the only known approach to making the metabolites of an artemisinin analogue is through fermentation of the compound with fungi. The fungi fermentation method is unpredictable in terms of the right microorganism to use in order to closely mimic the mammalian metabolism of the compound. Furthermore reports so far available have also shown that fungi

metabolism of these compounds do not correlate well with the mammalian metabolism, with a particular fungi producing only one or two metabolites which are also mammalian metabolites. This has meant that different fungi had to be sought to produce different mammaliam metabolites. Finding the right microorganism, therefore, requires some trial and error effort. For example, in study of the microbial metabolism of artemisinin itself, a total of 33 microorganisms had to be screened to be able to select two that would produce two mammalian metabolites [20]
The low yields of metabolites obtained is another drawback of this approach. Of the artemisinin analogues, only the fungi metabolism of arteether has been studied [21, 22, 23].

We have recently sought a more efficient and less time-consuming method of making the metabolites of the artemisinin compounds through the reaction of the compounds with chemical models of cytochrome P-450.
The catalytic mechanism of the cytochrome p-450 enzymes has been extensively studied and many chemical models of p-450 have been developed to elucidate the functions of these enzymes. In these studies, however, only very simple compounds such as cyclohexane, styrene, adamantane, aniline, toluidine, etc have been used as substrates. There have been very few reports on the application of chemical models of the p-450 enzymes to study the metabolism of actual drugs. Application of model systems to study drug metabolism has many attractive advantages. The most important of these are the simplicity of procedure and the formation and isolation of metabolite candidates in sufficient amounts to be used to identify the real *in vivo* metabolites. Application of model systems to study drug metabolism may also cut down on the use of experimental animals.

In the last few weeks, we have studied the reaction of DQHS and artelinic acid, respectively, with a number of P-450 model systems and we are in the process of isolating metabolites of these compounds from these systems, to be used for unambiguous identification of the microsomal metabolites of these compounds. Apart from our current work on DQHS and artelinic acid, this new approach will be useful in the overall work going on at WRAIR on the development of future antimalarials based on artemisinin.

5. SUMMARY AND CONCLUSIONS

1. The metabolites of DQHS have been tentatively identified on the basis of information provided by their thermospray mass spectra. Interpretation of the mass spectrometry data has been greatly aided by the use of deuterium-labeled DQHS in the metabolism studies.

2. DQHS undergoes metabolic transformation involving the hydroxylation of the A- and C-rings of the molecule together with deoxygenation of the endoperoxide group to give hydroxy DQHS isomers and hydroxy deoxy-DQHS isomers. Hydroxylation apparently occurs in the 2-, 3-, 9- or 14-positions. Deoxy-DQHS itself was found only in bile from the IPRL experiments.

3. Hydroxylated deoxy-qinghaosu and related compounds were also detected as metabolites of DQHS in the *in vitro* rat liver systems.

4. Metabolism of DQHS in the *in vitro* rat liver systems also involves the rearrangement of the B and C rings of the compounds.

5. Derivatisation of DQHS (and metabolites) for the purpose of analysis by HPLC with UV or fluorescence detection has been found to be possible by reaction of the compounds with mixed carboxylic-sulfonic anhyhdrides

6. Recently we achieved a minor breakthrough in the application of cytochrome P-450 chemical model systems to obtain sufficient amounts of metabolites of DQHS and artelinic acid to be used in unambiguous identification of the *in vitro* microsomal metabolites of these compounds

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Identification of *in vitro* Metabolites of Dihydroqinghaosu (Dihydroartemisinin) by High Performance Liquid Chromatography/Mass Spectrometry

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Abstract

The metabolism of dihydroqinghaosu (DQHS) (dihydroartemisinin) in rat liver microsomes and the isolated perfused rat liver was studied. The metabolites were identified by high performance liquid chromatography/thermospray- (TSP) mass spectrometry. The metabolism of DQHS was found to involve C-hydroxylation at the 2-, 3-, 9-, or 14-position. Deoxygenation of the endoperoxide function together with C-hydroxylation gave rise to different isomers of hydroxy deoxydihydroqinghaosu (hydroxy deoxyDQHS), the prevalent metabolites of DQHS. DQHS also apparently underwent enzymatic oxidation to isomers of both hydroxy deoxyqinghaosu and dihydroxy deoxyQHS. Metabolites apparently resulting from a rearrangement of the A- and B-rings of the molecule have also been tentatively identified.

1. Introduction

Qinghaosu (QHS) (also known as artemisinin), a sesquiterpene lactone with an unusual endoperoxide linkage, is the clinically active antimalarial principle isolated from the Chinese medicinal herb, Qinghao or *Artemisia annua* L [1,2]. For the past ten years the Steering Committee of the Scientific Working Group on Malaria of the World Health Organization (SWG-CHEMAL) and the Walter Reed Army Institute of Research, have actively pursued the development of new anti-malarial agents based on the lead provided by qinghaosu (QHS).

The development of more potent semi-synthetic derivatives of QHS with improved physico-chemical and/or pharmacological properties has focused on the reduction of QHS to the lactol, dihydroqinghaosu (DQHS), and subsequent preparation of the ether (or ester) derivatives of DQHS which are soluble either in water or oils and may be administered parenterally in the treatment of cerebral malaria The water-soluble derivatives of DQHS of clinical interest are artesunate the hemisuccinate ester of DQHS and artelinic acid, the 4-carboxybenzyl ether of DQHS. The oil-soluble derivatives of DQHS of interest are artemether and arteether, which are the methyl and ethyl ethers of DQHS respectively. DQHS itself has potent anti-malarial activity and was reported to be more active than QHS. The ether and ester derivatives of DQHS have also been shown to produce DQHS as a major primary metabolite and these too seem to serve as pro-drugs of DQHS[3-7]. Because of the apparent central role of DQHS in the metabolism and *in vivo* activity of its ether and ester derivatives, the major objective of the present study was to identify the metabolites of DQHS formed in rat liver preparations. After separation by gradient reversed phase liquid chromatography, the metabolites of DQHS were identified by thermospray mass spectrometry. Interpretation of the mass spectra of DQHS and its metabolites was aided utilizing specifically labeled trideutero-DQHS obtained by total synthesis(Figure 1)[8].

2. Experimental

2.1 Materials

2.1.1 Labeled Dihydroqinghaosu:

^{14}C-Labeled DQHS (specific activity 12.59 mCi/mmole or 44.33 uCi/ıng) with the label in the 13-position . Deuterium-labeled DQHS with the label on the 13-position [(13-$^{2}H_3$)DQHS]

2.1.2 Non-labeled Compounds:

DQHS ,QHS, deoxyqinghaosu (deoxyQHS), deoxydihydroqinghaosu (deoxyDQHS), 3-hydroxy deoxyqinghaosu (3-hydroxy deoxyQHS), 3-hydroxy deoxydihydroqinghaosu (3-hydroxy deoxyDQHS), 2α -hydroxy arteether, 9α-hydroxy arteether, 9β-hydroxy arteether

Other reagents and chemicals were obtained from regular sources.

2.1.3 Animals:

Male Sprague-Dawley rats (250-300 g), were housed at room temperature of 24°C in well-ventilated cages. They had access to pelleted feed and tap water *ad libitum.* Animals were cared for in accordance with the principles of the Guide for the Care and Use of laboratory Animals (Department of Health, Education and Welfare No NIH 85-23.). Animals were fasted overnight prior to microsome isolation.

2.1.4. HPLC-Mass Spectrometry (HPLC-MS)

Analytical HPLC separation and mass spectrometric identification of DQHS and its metabolites were performed using a Hewlett Packard 1090 Liquid Chromatograph linked with a Raytest Ramona 5LS radiodetector and interfaced with a Hewlett Packard HP 5989A mass spectrometer equipped with a Hewlett Packard thermospray interface.

2.2. Methods

2.2.1 Preparation of Rat Liver Microsomes

Microsomes were prepared from homogenised rat liver in KCl (0.15M)-phosphate (0.01M) buffer (pH 7.4.) by differential centrifugation, first at 9000g for 30 min and then at 105,000g for 75 min.

2.2.2 Incubation of DQHS with Rat Liver Microsomes

A mixture of ^{14}C-labeled DQHS (3.4 uCi) and unlabeled DQHS (1 mg). was incubated with microsomal protein (3-8 mg of protein) and co-factors at 38°C for either 30, 60 or 90 min. The incubation mixture was then extracted with ethyl acetate. Appropriate blanks with or without microsomes or cofactors were prepared.

2.2.3 Incubation of DQHS with Isolated Perfused Rat Liver (IPRL):

The isolated perfused rat liver was set up as previously described[9]. A solution of DQHS prepared from 2.5 mg each of unlabeled DQHS and the deuterium-labeled DQHS, together with 2.55 uCi of ^{14}C-labeled DQHS in Cremophore EL^{R} was administered as a bolus dose (10 mg/kg body weight) into the perfusate reservoir. DQHS and

its metabolites present in bile were analysed by direct thermospray HPLC/MS of bile and after hydrolysis of the bile sample with β -glucuronidase.

2.2.4 Thermospray HPLC/MS

After incubation with DQHS, extracts of liver microsomes were analysed on a μ Bondapak CN column (3.9 mm x 300 mm; 5 m) maintained at 45°C. Elution was done with a linear gradient of 95:5 (v/v) ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 50:50 ratio at 40 min and then to a ratio of 40:60 in the next 20 min (flow rate 1.0 ml/min). Bile samples were directly analysed on a m Bondapak C_{18} column (3.9 mm x 300 mm; 5 m) maintained at 35°C or room temperature for hydrolysed bile.

The thermospray interface was operated in the "fragmenter on" mode at a vaporizer temperature of 85-96°C (or 87-105°C) and a source temperature of 220°C.

3. Results

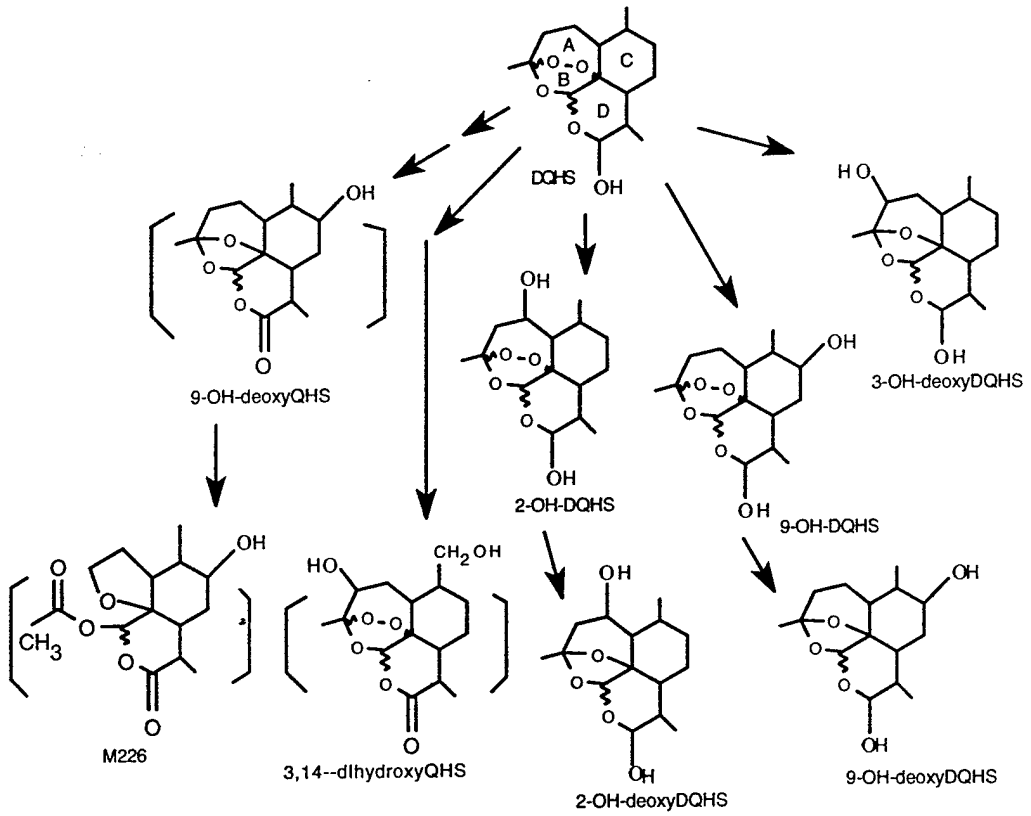
Eight metabolites were detected after incubation of DQHS with rat liver microsomes. In order of increasing retention time, these are 2-hydroxy DQHS, 9-hydroxy deoxyDQHS, 3-hydroxy deoxyDQHS, 2-hydroxy deoxyDQHS, 9-hydroxy DQHS, a dihydroxy QHS (possibly 3,14-dihydroxyQHS), M226: (an unkown compound tentatively suggested to be a rearrangement compound of hydroxyQHS analogous to a published rearrangement metabolite of arteether (AEM1))[3] and a hydroxy deoxyQHS (possibly 9 hydroxy deoxyQHS).

Metabolites detected in bile are 9-hydroxy deoxyDQHS, 3-hydroxy DQHS, 14-hydroxy deoxyDQHS, M224: An unknown compound tentatively suggested to be rearranged QHS analogous to the rearranged metabolite of arteether, (AEM1)[3], deoxyDQHS, 3-hydroxy deoxyDQHS, 3-hydroxy deoxyQHS, 9-hydroxy deoxyQHS and M222 - an unknown compound also tentatively suggested to be rearranged QHS.

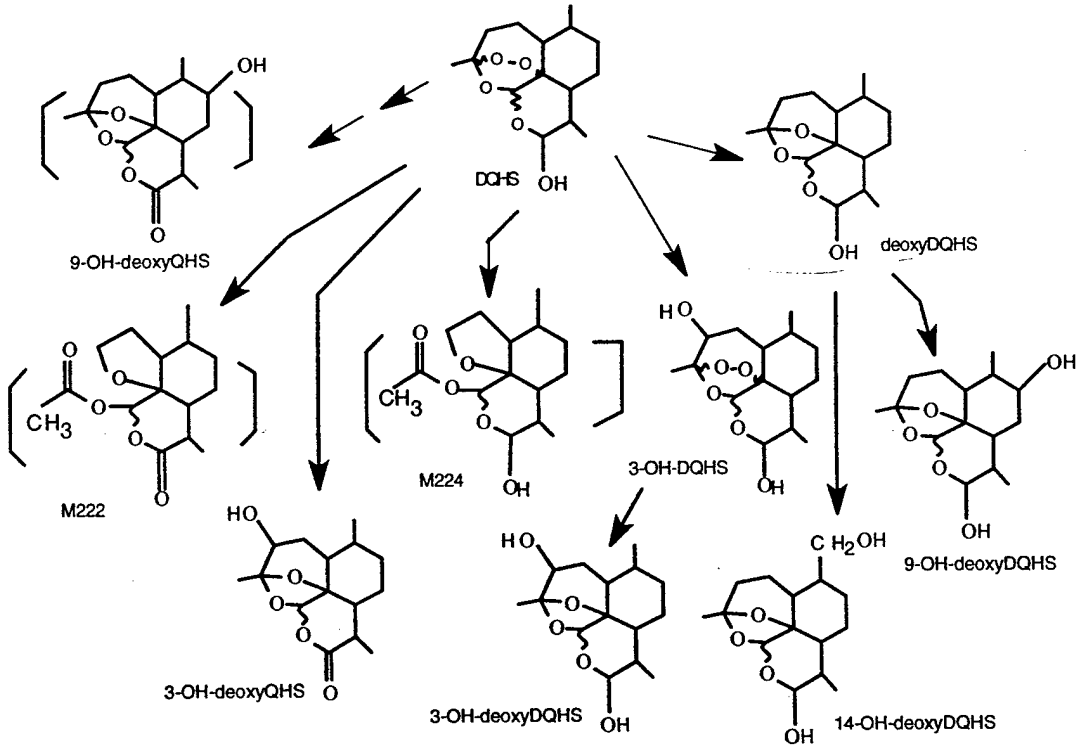
A series of N+3 doublets was observed in the spectra of a mixture of DQHS and trideutero-DQHS and the metabolites formed from it in the IPRL experiment, thus demonstrating that the deuterated 13-methyl group was unaffected by metabolism. The series of N+3 doublets also show that the deuterium label was retained in the fragment ions in the mass spectrum of DQHS and its metabolites.

4. Discussion

The metabolism of of qinghaosu (QHS) analogues has been reviewed[4]. Metabolism of these compounds generally involves hydroxylation of the A- and C-rings and/or deoxygenation of the endoperoxide group leading to isomeric hydroxy and hydroxy-deoxy compounds. The main problem in the study of the metabolism of these compounds is the paucity of authentic metabolites for comparison with the unknowns, a problem arising from the difficulty of synthezising the artemisinin skeleton. In the present study we have found that the mass spectra of the isomeric hydroxylated deoxy metabolites of DQHS exhibit subtle, but predictable, differences and we have shown that these isomeric hydroxylated metabolites of DQHS may be differentiated on the basis of a detailed interpretation of the fragmentation patterns apparent in each thermospray spectrum. The spectra of DQHS and arteether are virtually identical, the only difference being in the mass of the $[M + NH_4]^+$ adduct ions, which is m/z 302 for DQHS and m/z 330 for arteether. Because DQHS and arteether have nearly identical TSP spectra, it would also be expected that the isomers of hydroxy arteether would have TSP spectra similar to the spectra of the analogous isomers of hydroxy DQHS formed by the metabolism of DQHS. We have, accordingly, found the authentic spectra of the isomers of hydroxy arteether to be useful in identifying the isomers of hydroxy DQHS in the absence of authentic samples of the latter.



Scheme 1.: Proposed metabolic pathways of DQHS in rat liver microsomes



Scheme 2.: Proposed metabolic pathways of DQHS in the isolated perfused rat liver (IPRL).

Most of the metabolites of DQHS identified in the present study had TSP mass spectra which, like that of DQHS, exhibited a $[M + NH_4]^+$ ion at m/z 302 and a $[(M + NH_4) - H_2O]^+$ ion at m/z 284, thus indicating that one oxygen has been removed from the endoperoxide moiety of DQHS and one hydroxy group has been added elsewhere on the ring system to give isomers of hydroxy deoxyDQHS . Most of the other ions in the spectra of the hydroxy deoxyDQHS isomers apparently result from fragmentation of the A- and C-rings of the molecular (or pseudo-molecular) ions by loss of neutral entities and it is possible to distinguish between the mass spectrum of an A-ring hydroxy deoxyDQHS isomer and the spectrum of a C-ring hydroxylated isomer.

Three compounds (designated M222, M224, M226, respectively) were detected which have been tentatively deduced to be metabolites of DQHS formed by a rearrangement of the A- and B-rings of the DQHS molecule.

Based on the above results, the metabolic pathways of DQHS in rat liver microsomes and in the IPRL are presented in Schemes 1 and 2. Briefly, the metabolism of DQHS involved deoxygenation of the endoperoxide group and/or hydroxylation of the A- and C-rings of the molecule, oxidation of the hemiacetal hydroxy group of DQHS back to the lactone group of QHS. Another notable observation is the formation of metabolites by, presumably, the rearrangement of the A- and B-rings of DQHS.

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APPENDIX 2

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RAPID DERIVATIZATION OF ALCOHOLS WITH CARBOXYLIC-SULPHONIC MIXED ANHYDRIDES FOR HPLC-UV/FLUORESCENCE ANALYSIS. APPLICATION TO THE DETECTION OF DIHYDROQINGHAOSU (DQHS) AND ITS METABOLITES IN BIOLOGICAL SAMPLES.

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ABSTRACT

The formation of ester derivatives of alcohols for the purpose of HPLC-ultraviolet/fluorescence analysis is achieved rapidly when a mixture of the alcohol and the triethylamine salt of the required acid in chloroform or dichloromethane is treated with a solution of either 2,4,6-triisopropylbenzenesulphonyl chloride or 2,4,6-trimethylbenzenesulphonyl chloride (mesitylenesulphonyl chloride) followed by 4-dimethylaminopyridine in the same solvent. Aromatic carboxylic acids give a quantitative yield of the esters while the yield is reduced for arylacetic acids.

The procedure has been applied to the detection of dihydroqinghaosu (DQHS) and its metabolites in different types of biological samples.

The general applicability of the method is also demonstrated by the ready esterification of the sterically hindered hydroxy groups of testosterone and 6β-hydroxytestosterone.

This approach to analytical esterification of alcohols is more convenient and more efficient than previous methods which require the prior conversion of the carboxylic acids to the acyl chlorides, acyl nitriles, acyl azides or the symmetric anhydrides.

INTRODUCTION

Labeling of alcohols with UV-absorbing or fluorescent tags for the purpose of high performance liquid chromatography is usually based on acylation or esterification of the hydroxyl group of alcohols with UV-absorbing or fluorescent carboxylic acids. Because of the poor acylating ability of carboxylic acids, it is often necessary to activate a carboxylic acid for acylations. This is usually done by conversion of the carboxylic acid to the anhydride or acid chloride or pseudohalide such as the acyl nitrile [1-3].

Analytical esterification with acyl chlorides, acyl nitriles or anhydrides is neither a satisfactory nor a convenient procedure because of the instability of these reagents towards moisture.

The derivatization of alcohols with fluorescent carbonyl azides has also been reported [4]. Although the carbonyl azides may be more stable than the acid chlorides they too are prepared initially from the acid chlorides. Furthermore, reaction of carbonyl azides with alcohols require extreme conditions for the thermal decomposition of the carbonyl azide to the isocyanate which is the acylating species. Thermal decomposition of the carbonyl azide also gives rise to many other unknown compounds which may interfere in the chromatography of the desired carbamate derivative. There is, therefore, still a great need for versatile and facile procedures for the analytical esterification of alcohols based on stable, readily available reagents which may be used under mild conditions.

During our study of the metabolism of dihydroqinghaosu (DQHS), a new antimalarial, there arose the need to derivatize the compound and its metabolites for the purpose of HPLC with UV or fluorescence detection. DQHS possesses a relatively unreactive, acid-sensitive hemiacetal hydroxy group. This prompted an investigation of the mixed anhydride method as a mild and facile approach to the analytical derivatization of alcohols, with DQHS as a model compound for the study.

Formation of peptide bonds which involves activation of the carboxylic group by conversion to a mixed anhydride was introduced over 40 years ago [5-7]. The following is a report on the preparation of UV/fluorescent derivatives of DQHS and its metabolites by a mixed carboxylic-sulphonic anhydride method. 2,4,6-Trimethylbenzenesulfonyl chloride (mesitylenesulfonyl chloride) and 2,4,6-triisopropylbenzene sulfonyl chloride were investigated as the activating agents.

MATERIALS

Acids

Aromatic carboxylic acids:
9-Anthracenecarboxylic acid (9ACA), 4-Biphenylcarboxylic acid (BCA),
2-Naphthoic acid, 9-Phenanthrenecarboxylic acid (9PCA),
1-Pyrenecarboxylic acid.
Arylalkanoic acids :
4-Biphenylacetic acid, 9-Fluoreneacetic acid (9FAA), 9-Fluorenecarboxylic acid,
1-Pyreneacetic acid, 1-Naphthylacetic acid, 1-Pyrenebutyric acid .

Coumarin acids:
Coumarin-3-carboxylic acid, 7- (Carboxymethoxy)-4-methylcoumarin

Condensing Agents
2,4,6-Trimethylbenzenesulphonyl chloride (mesitylenesulphonyl chloride) (METS-chloride), 2,4,6-Triisopropylbenzenesulphonyl chloride (TIPS- chloride) 2,4,6-Trichorobenzoyl chloride (TCB-chloride)

Hydroxy Compounds

Dihydroqinghaosu (DQHS), testosterone, 6β-hydroxytestosterone, octanol, 3,4-dimethyl-2-hexanol

Catalyst: 4-Dimethylaminopyridine (DMAP)

All reagents and chemicals were obtained from Aldrich Chemical Co (Milwakee, USA) except testosterone and hydroxytestosterone (Sigma Chemical Co, St. Loius, USA), and DQHS (Walter Reed Inventory).
9-Phenanthrenecarboxylic acid was obtained by the alkaline hydrolysis (10 M NaOH; reflux for 28 h) of 9-cyanophenanthrene obtained from Aldrich.

Instrumentation

HPLC was performed on a Waters liquid chromatography system consisting of a Waters model 510 solvent delivery unit, a U6K injector and a Waters model 440 UV detector set at 254 nm. A Beckman Ultrasphere C8 column (4.6 mm x 15 cm) was used with a mobile phase of acetonitrile:water (80:20 v/v) at a flow rate of 3 ml/min.

Mass spectrometric identification of the derivatives was performed using a HPLC-MS system consisting of a Hewlett Packard 1090 Liquid Chromatograph System linked with a Hewlett Packard HP 5989A Mass Spectrometer via a Hewlett Packard thermospray interface. A μBondapak C_{18} column (2.1 mm x 100 mm; 5 m) was used, with a mobile phase consisting of 0.1M ammonium acetate (pH 4.5) and acetonitrile. Elution was done with a linear gradient of 95:5 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 30:70 ratio at 60 min, and held at this ratio for a further 20 min (flow rate 0.4 ml/min). The thermospray interface was operated in the "fragmenter on" mode at a vaporiser temperature of 85-96°C (or 87-105°C) and a source temperature of 220°C.

METHODS

Esterification of DQHS using Mesitylenesulphonyl chloride (METS-chloride) or Triisopropylbenzenesulphonyl chloride (TIPS-chloride) or Trichorobenzoyl chloride (TCB-chloride) as Condensing Agent and Prior Preparation of the Mixed Anhydrides

Esterification of DQHS involving prior formation of the mixed anhydride is illustrated by the reaction with 9-fluoreneacetic acid:

To a solution containing 40 mg (0.178 mmol) of 9-fluoreneacetic acid and 30 ul of triethylamine in 2 ml of dichloromethane (or chloroform) was added 2 ml of a solution of 39 mg (0.18 mmol) of mesitylene sulphonyl chloride in 2 ml of dichloromethane. The mixture was kept at room temperature for 30 min to allow formation of the carboxylic-sulphonic mixed anhydride reagent

A 2 ml portion of the reagent was then added to a solution of 25 mg (0.088 mmol) of DQHS and 12 mg (0.098 mmol) of DMAP in 2.5 ml of dichloromethane. The remaining 2 ml of the reagent solution served as a blank.

At 30 min intervals, 20 ul aliquots of the reaction mixture were taken and evaporated under a stream of nitrogen, and the residue redissolved in 200 ul of methanol, followed by HPLC analysis of 10 ul of the solution. The blank reagent mixture was treated similarly.

After keeping at room temperature for 2.5 h, the reaction mixture was shaken successively with 2 ml each of 2M hydrochloric acid, water, 2M sodium hydroxide and water. The chloroform solution was then dried with anhydrous sodium sulphate and evaporated and the white solid obtained examined by HPLC-MS.

Semi-Preparative Esterification of DQHS using Mesitylenesulphonyl chloride (METS-chloride) or Triisopropylbenzenesulphonyl chloride (TIPS-chloride) or Trichorobenzoyl chloride (TCB-chloride) as Condensing Agent and *in situ* Preparation of the Mixed Anhydrides

The general procedure involves the reaction of DQHS with an excess of the acid (relative to DQHS) together with an excess of the condensing agent (relative to the acid) and an excess of DMAP (relative to the condensing agent). This is illustrated by the following preparations:

Esterification of DQHS with 9-Fluoreneacetic Acid using TIPS-chloride as Condensing Agent

A solution of the triethylamine salt of 9-fluoreneacetic acid was prepared by dissolving 204 mg (0.9097 mmol; equivalent to about 3.3 % molar excess relative to DQHS) of the acid in 2 ml of dichloromethane (or chloroform) and adding 200 ul of triethylamine. The solution of the acid was then mixed with a solution of 250 mg (0.8803 mmol) of DQHS in 2 ml of dichloromethane. A solution of 300 mg (0.9906 mmol;equivalent to about 12.5 % molar excess relative to DQHS) of TIPS-chloride in 5 ml of chloroform was then added, followed immediately by a solution of 150 mg (1.2278 mmol; equivalent to about 40 % molar excess relative to DQHS) of DMAP in 2 ml of dichloromethane.

After keeping the reaction mixture at room temperature for 3h, the solvent was evaporated under a steam of nitrogen. The residue was mixed with 5 ml of sodium carbonate buffer (pH 11) and allowed to stand at room temperature for 30 min. The mixture was then extracted with 15 ml of methyl t-butyl ether by shaking on a vortex mixer for 3 min. After removal of the aqueous layer, the organic extract was washed successively with 5 ml each of distilled water,

hydrochloric acid (2M) and distilled water. The extract was then dried with anhydrous sodium sulfate and evaporated under a stream of nitrogen to obtain an oily residue which on warming briefly with 3 ml of methanol turned to a pure white powder, which was filtered and dried by suction. HPLC analysis of the white solid showed only one peak which was not that of 9-fluoreneacetic acid. Similarly, HPLC-MS showed that the product was pure and contained no unreacted DQHS.

Esterification of DQHS with 9-Anthracenecarboxylic Acid using METS-chloride as Condensing Agent

To a solution of the triethylamine salt of 9-anthracenecarboxylic acid, prepared by dissolving 400 mg (1.8 mmole) of the acid in 10 ml of dichloromethane and 300 ul of triethylamine, was added 250 mg (0.8803 mmole) of DQHS and the mixture shaken well to dissolve the DQHS. A solution of METS-chloride was prepared by dissolving 400 mg (1.83 mmole) in 5 ml of dichloromethane with brief warming in a water bath (60 ^{o}C). The solution of METS-chloride was added to the mixture of anthracenecarboxylic acid and DQHS, followed immediately by 250 mg (2.04 mmole) of DMAP. The intense yellow mixture was kept at room temperature overnight (15 h).

The mixture was then evaporated under a stream of nitrogen to obtain an oily residue which, on shaking with 20 ml sodium carbonate buffer, turned into a yellow powder. After allowing the yellow powder to settle, the aqueous layer was removed and the powder washed again with 2 x 20 ml of sodium carbonate buffer. The yellow powder was then washed with 3 x 20 ml of distilled water, filtered by suction, and washed successively on the filter paper with 50 ml of 2M hydrochloric acid and 100 ml of distlled water. On drying, a bright yellow powder was obtained. The excessive yield of 560 mg and the intensely yellow filtrate obtained when the powder was being washed with either alkaline buffer or acid indicated the presence of a substantial amount of impurity, probably the anhydride of 9-anthracenecarboxylic acid, in the product. Heating with water in a boiling water bath was found to be only partially effective in removing this side-product. The yellow powder was, therefore, mixed with carbonate buffer and heated in a boiling water bath 30 min to obtain a pale yellow solid and a dark yellow solution. The mixture was filtered and the solid washed with distilled water until the washing was colorless. On drying, a pale yellow powder (205 mg) was obtained, which was found to be pure by HPLC-MS.

Esterification of DQHS with 4-Biphenylcarboxylic Acid (BCA) using TCB-chloride as Condensing Agent

A solution of the triethylamine salt of 4-biphenyl- carboxylic acid was prepared by dissolving 202 mg (1.01 mmol) of the acid in 2 ml of dichloromethane and adding 200 ul of triethylamine. The solution of the acid was mixed with a solution of 250 mg (0.8803 mmol) of DQHS in 2 ml of dichloromethane. and a

solution of 170 ul (1.1 mmole) of TCB-chloride in 5 ml of chloroform was then added, followed immediately by a solution of 150 mg (1.2278 mmol) of DMAP in 2 ml of dichloromethane.

After keeping the reaction mixture at room temperature for 3h, it was processed as described previously for the reaction of DQHS with 9-fluoreneacetic acid and TIPS-chloride.

Derivatization of Microgram Quantities of DQHS with Carboxylic Acids using TIPS- or METS-chloride as Condensing Agent.

To 100 - 500 ug of DQHS (20 ul of 25 mg/ml solution in dichloromethane) was added 0.2 ml of a solution of the triethylamine salt of 9-fluoreneacetic acid prepared by dissolving 20 mg of the acid in 2 ml of dichloromethane and 20 ul of triethylamine. To the mixture of DQHS and 9-fluoreneacetic acid was added 2 ml of a 3 mg/ml solution of TIPS-chloride (or METS-chloride) followed by 2 ml of a 2.5 mg/ml solution of DMAP in dichloromethane.

After keeping the mixture at room temperature for 2 h, the solvent was evaporated under a stream of nitrogen. The residue was shaken with 2.5 ml of carbonate buffer and the mixture kept at room temperature for 15 min. The mixture was extracted with 4 ml of methyl tert-butyl ether by shaking on a vortex mixer for 2 min . The aqueous layer was removed and the ether extract washed successively with distilled water (4 ml), 2M hydrochloric acid (2.5 ml) and distilled water (4 ml). After removing the last aqueous layer, the ether extract was dried with anhydrous sodium sulfate, transferred to a clean test tube and evaporated with a stream of nitrogen. The residue was redissolved in 1 mL of methanol and 30 uL of the solution was analyzed.

The above procedure was also carried out using 2 ml of a solution of benzenesulphonyl chloride (prepared by mixing 15 ul of benzenesulphonyl chloride with 10 ml of dichloromethane) in place of TIPS-chloride.

Derivatization of Microgram Quantities of DQHS following Extraction from Blood Plasma and Reaction with Carboxylic Acids, using TIPS- or METS-chloride as Condensing Agent.

Solutions of DQHS (100 - 500 ug/ml) were prepared in sheep plasma and 1 ml aliquots were extracted with 5 ml of methyl tert-butyl ether by shaking on a vortex mixer for 3 min. After centrifuging at 2500 rpm for 15 min, the ether layer was removed and evaporated under a stream of nitrogen. The residue was reacted with the acid and the reaction mixture treated as described in the preceeding section for solutions of DQHS in dichloromethane.

Derivatization of DQHS and its Metabolites Extracted from Rat Liver Microsomes

Rat liver microsomes were prepared from homogenized liver tissue by differential centrifugation as previously described [8], and the microsomal suspensions were stored at -80° C in 0.10 M potassium phosphate buffer (pH 7.4) containing 20 % glycerol until needed.

Rat liver microsomes (980 ug of protein) were pre-incubated at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) containing a NADPH regenerating system ($NADP^+$: 0.5 mM, glucose-6-phosphate: 10mM, glucose-6-phosphate dehydrogenase: 1.0 I.U./ml, $MgCl_2$: 5 mM). The final volume of incubation was 1 ml. The reaction was initiated by the addition of 353 uM of DQHS. After incubation for 90 or 180 min, the reaction was terminated by the addition of 100 ul of 0.05M sodium hydroxide and extracted with 5 ml of methyl tert-butyl ether as described above for plasma samples. The residue obtained after evaporating the ether was reacted with the chosen acid using TIPS- or METS- or TCB-chloride as condensing agent as described previously for solutions of microgram levels DQHS in dichloromethane.

Derivatization of DQHS and its Metabolites Extracted from Rat Bile after Incubation of Arteether with the Isolated Perfused Rat Liver (IPRL)

The livers were isolated using standard techniques and perfused in a constant flow (15 ml/min) recirculating system at a controlled temperature of 37°C as previously described [9]. The perfusate (100 ml) contained 20% washed sheep red blood cells, 1% (w:v) bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and 0.1% glucose in a standard Krebs Henseleit buffer. A 5 mg/kg bolus injection of arteether (n=4) was added directly into the perfusate reservoir as a 5 mg/ml solution in ethanol/H_2O (50/50). Bile was continuously collected into preweighed vials at the time intervals 0-30, 30-60, 60-90, 90-120, 120-180, and 180-240 min after dosing. Bile was extracted with methyl t-butyl ether and the extract derivatized with 9-fluoreneacetic acid or 9-phenanthrenecarboxylic acid as described previously for plasma extracts.

Esterification of Testosterone, 6β-Hydroxytestosterone and other Hydroxy Compounds

Testosterone (500 ug) and 6β-hydroxytestosterone (500 ug) were respectively reacted with 9-fluoreneacetic acid using either METS-chloride or TIPS-chloride as the condensing agent, following the procedure described for derivatization of microgram quantities of DQHS with carboxylic acids using these sulfonyl chlorides as condensing agents,

Similarly, octanol and 3,4-dimethyl-2-hexanol were each esterified by the same procdure.

Esterification of the these compounds were also carried out using TCB-chloride in place of the sulphonyl chlorides.

RESULTS AND DISCUSSION

The acylation reaction resulting in the formation of esters and amides is one of the most important reactions in organic synthesis and analysis. In particular, trace analysis of alcohols is based almost exclusively on the acylation of alcohols and subsequent chromatographic analysis of the ester derivatives. Because of the fundamental importance of the acylation reaction, new reagents and approaches are constantly being developed for the activation of carboxylic acids under mild conditions. However, most of the reagents which have been introduced for the activation of the carboxyl group are costly and/or not readily accessible and have found no application in the analytical derivatization of alcohols. The esterification of alcohols for the purpose of chromatographic trace analysis is, therefore, still based on reaction with acyl chlorides or acyl nitriles or acyl anhydrides, which are generally not satisfactory as analytical reagents.

Of the principles that may be adopted for the activation of carboxylic acids, conversion to the mixed carboxylic-sulphonic anhydride is one of the most attractive for analytical esterification, because the reagents are readily available and easy to handle.

The reagents we investigated for the formation of the mixed carboxylic-sulphonic anhydrides are the hindered benzenesulphonyl chlorides 2,4,6-trimethylbenzenesulphonyl chloride (mesitylenesulphonyl chloride) and 2,4,6-triisopropylbenzenesulphonyl chloride. These hindered sulphonyl chlorides have been widely used only as condensing agents in the formatiom of internucleotide bonds [10, 11], although there are two reports on the use of mesitylenesulphonyl chloride in the cyclisation of amino acids to macrocyclic lactams [12, 13]. For comparison, 2,4,6-trichlorobenzoyl chloride (TCB-chloride) was also investigated as a condensing agent in the esterification of DQHS. TCB-chloride, a hindered benzoyl chloride which may be considered analogous to the hindered benzenesulphonyl chlorides, has been applied to the synthesis of macrocyclic lactones [14]. TCB-chloride was also reported to be useful in the preparation of esters, but it has the disadvantage of reacting with some alcohols to form the trichlorobenzoyl ester as a side-product.

Esterification of DQHS using Mesitylenesulphonyl chloride (METS-chloride) or Triisopropylbenzenesulphonyl chloride (TIPS-chloride) as Condensing Agent in presence of 4-Dimethylaminopyridine (DMAP)

It was found that the relatively unreactive hydroxy group of DQHS was readily acylated when a mixture of the triethylamine salt of the acid and DQHS were treated with METS-chloride (or TIPS-chloride) followed by the powerful acylation catalyst 4-dimethylaminopyridine (DMAP).

2,4,6-Trichlorobenzoyl chloride (TCB-chloride) also served well in place of the benzenesulphonyl chlorides. However, with alcohols which are more reactive than DQHS, TCB-chloride was less satisfactory because it reacted with some of the alcohol to form the trichlorobenzoyl ester. Furthermore, being a moisture sensitive liquid, TCB-chloride is a little less convenient to use than the solid and more moisture-resistant benzenesulphonyl chlorides.

Benzenesulphonyl chloride itself failed in this reaction, the symmetrical anhydride of the acid being the major product in most cases. Formation of the carboxylic anhydride instead of the ester was observed in some cases in an early report on the use of benzenesulphonyl chloride as the activating reagent in esterifications [15].

With the exception of 9-anthracenecarboxylic acid, the aromatic carboxylic acids (4-biphenylcarboxylic acid, 2-naphthoic acid, 9-phenanthrenecarboxylic acid and 1-pyrenecarboxylic acid) reacted with DQHS to give isolated yields of the pure DQHS ester of 70 % or above. The reaction was found to take place rapidly and there was no evidence of a time-dependent yield of the DQHS ester. HPLC-MS of the reaction products showed there was no unreacted DQHS, suggesting that the yields were quantitative. This is demonstrated, for example, by the total ion- and UV chromatograms of the DQHS esters of 4-biphenylcarboxylic acid and 9-fluoreneacetic acid shown in Figure 1.

The thermospray (TSP) mass spectra of the DQHS esters of the two acids are also shown in Figure 2. The TSP mass spectra of the esters of DQHS are characterised by an abundance of the quasi-molecular, ammonium adduct ion ($[M + NH_4]^+$; M + 18). The spectra also exhibit M+39 and M+59 ions. The former is of obscure origin and has been observed only with these aromatic esters of DQHS, while the M+59 line is a known feature of the TSP mass spectra of DQHS and its ester and ether derivatives and is probably due to the $[M + NH_4 + CH_3CN]^+$ ion. The primary fragmentation of the [M+18] ion results in the loss of acyl group of the ester, and the rest of the spectrum is accounted for by the fragmentation pathways of the DQHS portion of the molecule. As illustrated in the spectra of the DQHS esters shown in Figure 2, the spectra of the different esters differ only in the molecular ion region while the rest of the spectra are identical to that of DQHS itself.

Using 4-biphenylcarboxylic acid as an example, the acylation of DQHS and other alcohol hydroxy groups with METS-chloride or TIPS-chloride as the carboxylic acid activating agent is illustrated in Figure 3. It is expected that when the hindered benzenesulphonyl chloride is added to a mixture of the alcohol and the triethylamine salt of the acid, the hindered benzenesulphonyl chloride would react preferentially with the carboxylic anion to form the mixed anhydride which then acylates the alcohol. The rapidity of the reaction is an indication that the mixed carboxylic-sulphonic anhydride generated *in situ* is the acylating species rather than the symmetric anhydride of the carboxylic acid. In contrast, when the acid was first allowed to react with the hindered benzenesulphonyl chloride for about 1 h before the addition of DQHS and catalyst, there was formation of the acid anhydride and a reduced yield of the DQHS ester.

With 9-anthracenecarboxylic acid, the yield of the DQHS ester was poor in an initial attempt to react equivalent quantities of the acid with DQHS. It was

found that the acid was converted in good yield to the symmetrical anhydride, which in this case must have been the predominant acylating agent. When two equivalents of acid was used, the DQHS ester of 9-anthracenecarboxylic acid was obtained in an isolated yield of 48 %. Some of the ester must have been lost when the reaction product was warmed with alkali to destroy the unreacted 9-anthracenecarboxylic anhydride.

Similarly, the yields of the esters of DQHS with the arylacetic acids were poor. The poor performance of this class of acids in this reaction may have been due to the instability of their mixed carboxylic-sulphonic anhydrides, probably brought about by the lability of their benzyl group. In particular, the reaction failed completely with 9-fluorenecarboxylic acid, which has the even more labile fluorenyl group adjacent to the carboxylic function. The instability of the mixed anhydrides of these acids also shows in their tendency to form the less reactive symmetrical anhydrides which may be thought of as resulting from attack of the carboxylate anion released from the initial decomposition of the mixed anhydride with the intact mixed anhydride.

In contrast, 9-fluoreneacetic acid, in which the fluorenyl group is further removed from the carboxylic group, was found to be one of the best acids for the analytical esterification of DQHS by the present mixed anhydride approach.

7-(Carboxymethoxy)-4-methylcoumarin, which has been proposed as a fluorescent reagent for the precolumn derivatization of hydroxy compounds [16], was also found to react readily with DQHS, using the TIPS- or METS-chloride as the carboxylic acid activating agent rather than conversion of the acid to the acid chloride as originally reported.

Detection of DQHS and its Metabolites Extracted from Biological Samples.

To date, only the diacetyldihydrofluorescein (DADF) ester of DQHS has been proposed for the HPLC/UV analysis of the compound [17, 18]. The derivatization of DQHS with diacetyldihydrofluorescein was carried out using dicylohexylcarbodiimide (DCC) as the carboxylic acid activating agent. The DADF ester was obtained from reaction with pure DQHS, but no attempt was made to apply this reaction to the detection of DQHS extracted from biological samples. Our attempt to apply this reaction to the derivatization of DQHS extracted from biological samples was unsuccessful. Irrespective of the acid used, we found the corresponding N-acyl dicyclohexylurea was formed as a major side-product which interfered seriously in subsequent chromatography of the DQHS ester derivative. Besides, dicylohexylurea which is also another side product normally formed during DCC-catalysed acylations interfered with the recovery of the derivative because of its insolubility in common organic solvents.

In the present work, microgram to subnanogram levels of DQHS extracted from biological fluids were readily detected by HPLC-UV after acylation with aromatic carboxylic acids, using either TIPS- or METS-chloride as the condensing agent. A chromatogram of a derivatized plasma extract of DQHS is shown in Figure 4. Chromatograms of DQHS and its metabolites extracted from rat liver microsomes and then acylated by the mixed anhydride method are shown in

Figures 5a-c. Arteether, the ethyl ether analogue of DQHS, undergoes metabolic deethylation to DQHS. As shown in Figure 6, the present derivatization method has also been successfully applied to the HPLC-UV detection of DQHS and another hydroxylated metabolite (AEM) formed by the metabolism of arteether in the isolated perfused rat live (IPRL).

Esterification of Testosterone, 6β-Hydroxy- testosterone and other Hydroxy Compounds

To further demonstrate the general applicability of the present derivatization method, the acylation of testosterone, and 6β-hydroxytestosterone, by the mixed carboxylic-sulphonic anhydride method was investigated. The best available approach to the derivatization of the hydroxy group of hydroxysteroids is based on acylation with acyl nitriles [19, 20, 21]. A serious drawback to acylation with acyl nitriles is the pronounced sensitivity of the reaction to steric hindrance in the neighborhood of the hydroxy groups of hydroxysteroids [22]. For examples, testosterone, which possesses a quasi-equitorial hydroxy group reacts poorly with 4-dimethylamino-1-naphthoyl nitrile, while both the 11β and 17α hydroxy groups of cortisol are inert towards this reagent. Quantitative acylation with acyl nitriles is achieved only for primary hydroxy groups while only yields of 30 % or less are achieved with secondary hydroxy groups, and teriary hydroxy groups are unreactive. Esterification of testosterone and 6β-hydroxytestosterone by the mixed anhydride method was, therefore, investigated because of the reported poor reactivity of the 6β- and 17β–hydroxy groups of these compounds with the acyl nitriles such as benzoyl nitrile, pyrene-1-carbonitrile, anthracene-1- and 9-carbonitrile which have been proposed for the derivatization of hydroxysteroids. Both testosterone and 6β-hydroxytestosterone were quantitatively acylated by the present mixed anhydride method. A chromatogram of the ester derivatives of testosterone and 6β-hydroxytestosterone are shown in Figure 7. Both of the 6β– and 17β-hydroxy groups in 6β-hydroxytestosterone were quantitatively acylated.

Similarly, the acylation of 1-octanol and the secondary hydroxy group of 3,4-dimethyl-2-hexanol by the carboxylic-sulphonic mixed anhydride method was quantitative.

CONCLUSION

Rapid, direct derivatization of alcohols with UV/fluorescent carboxylic acids is readily achieved when the hindered benzenesulphonyl chlorides (TIPS- or METS-chloride) are used as condensing agent in combination with DMAP as catalyst. This is a very convenient procedure which is superior to previous approaches that require the prior conversion of the carboxylic acid to the acid chloride or acyl nitrile or acyl azide or acid anhydride. The obvious lack of

reaction between alcohols and the hindered benzenesulphonyl chlorides under the present conditions suggests that this procedure may also be adapted for the derivatization of carboxylic acids with UV/fluorescent alcohols for HPLC-UV/fluorescence analysis.

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Figure 1. Total ion- and corresponding UV-chromatograms of the esters obtained from the semi-preparative reaction of DQHS with 4-biphenylcarboxylic acid /METS-chloride and 9-fluoreneacetic acid/TIPS-chloride respectively.

Figure 2. Thermospray mass spectra and fragmentation pattern of DQHS and its respective biphenylcarboxylic and 9-fluoreneacetic acid esters.

Figure 3. Illustration of the esterification of DQHS by the mixed anhydride method.

Figure 4. HPLC chromatograms of (a) blank plasma extract and (b) DQHS extracted from plasma and derivatized with 9-fluoreneacetic acid/TIPS-chloride.

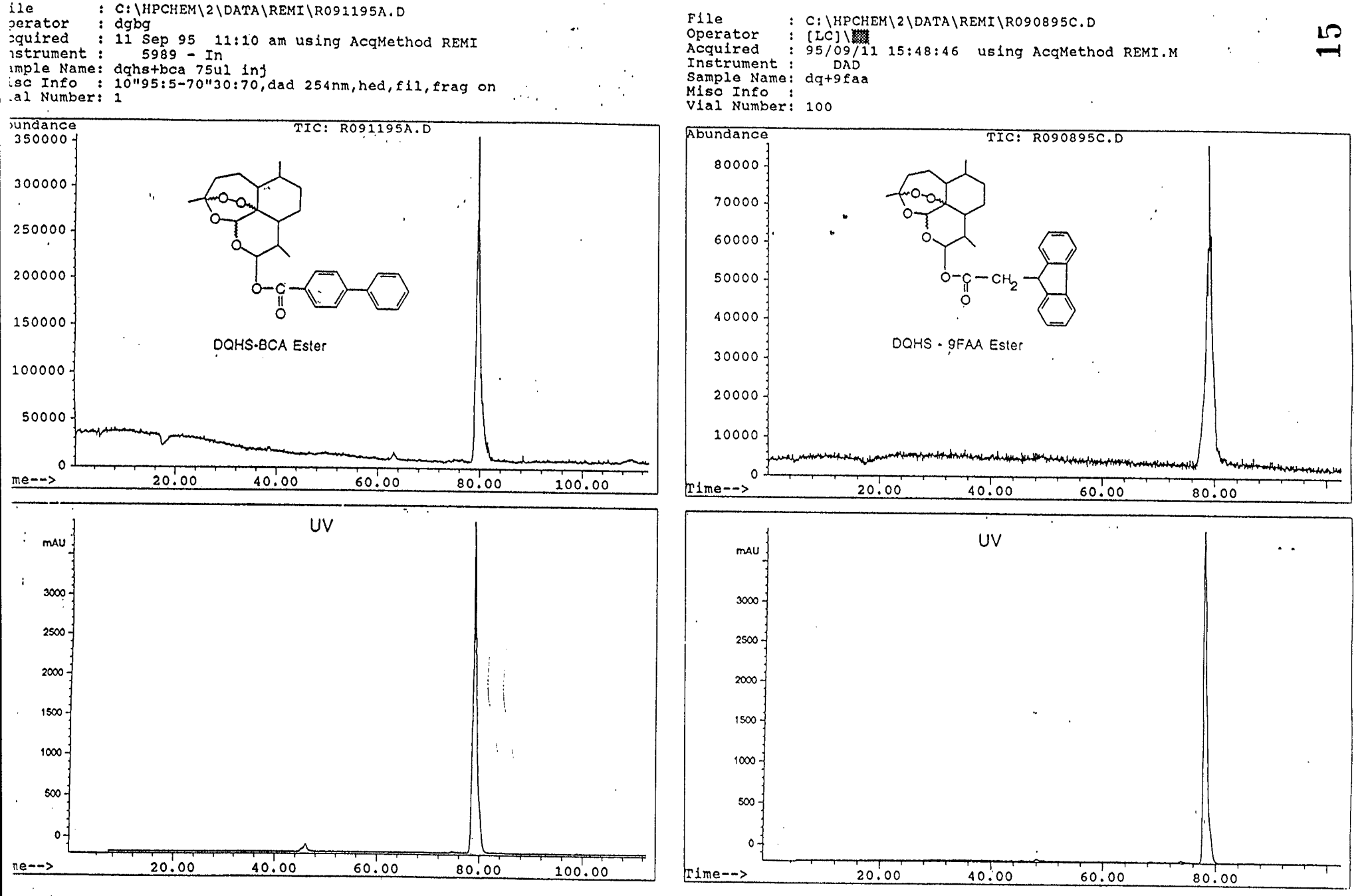
Figure 5a. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolites (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 180 min) and derivatized with 9-fluoreneacetic acid/METS-chloride.

Figure 5b. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolite (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 90 min) and derivatized with 9-anthracenecarboxylic acid/TIPS-chloride.

Figure 5c. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolite (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 90 min) and derivatized with 9-fluoreneacetic acid/TCB-chloride.

Figure 6. HPLC chromatograms of (a) extract of blank rat liver bile and (b) metabolites of arteether (DQHS and AEM) extracted from rat liver bile and derivatized with 9-phenanthrenecarboxylic acid/TIPS-chloride.

Figure 7. HPLC chromatogram of the 9-fluoreneacetic acid esters of testosterone (TST-9FAA) and hydroxytestosterone (OH-TST-9FAA).



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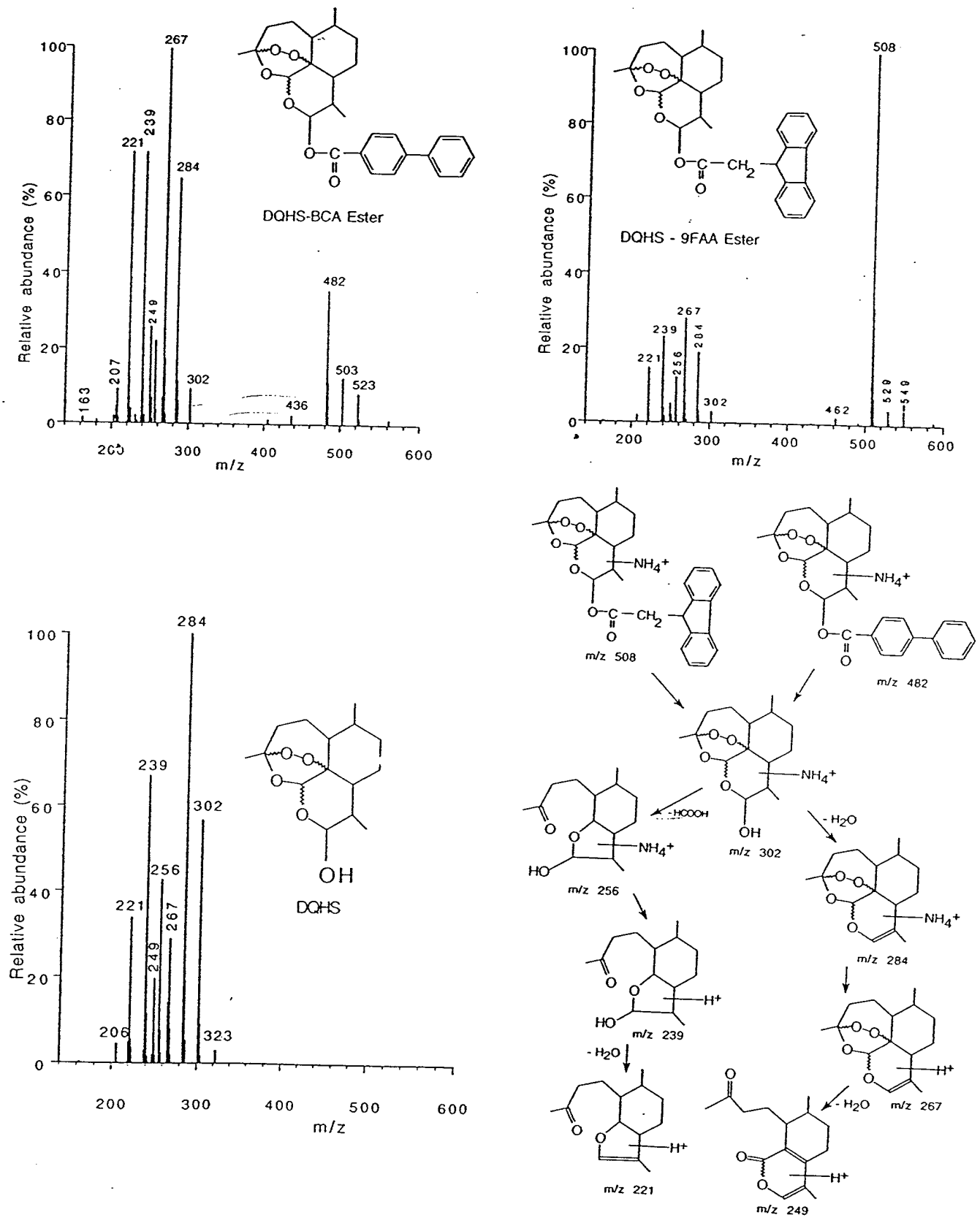

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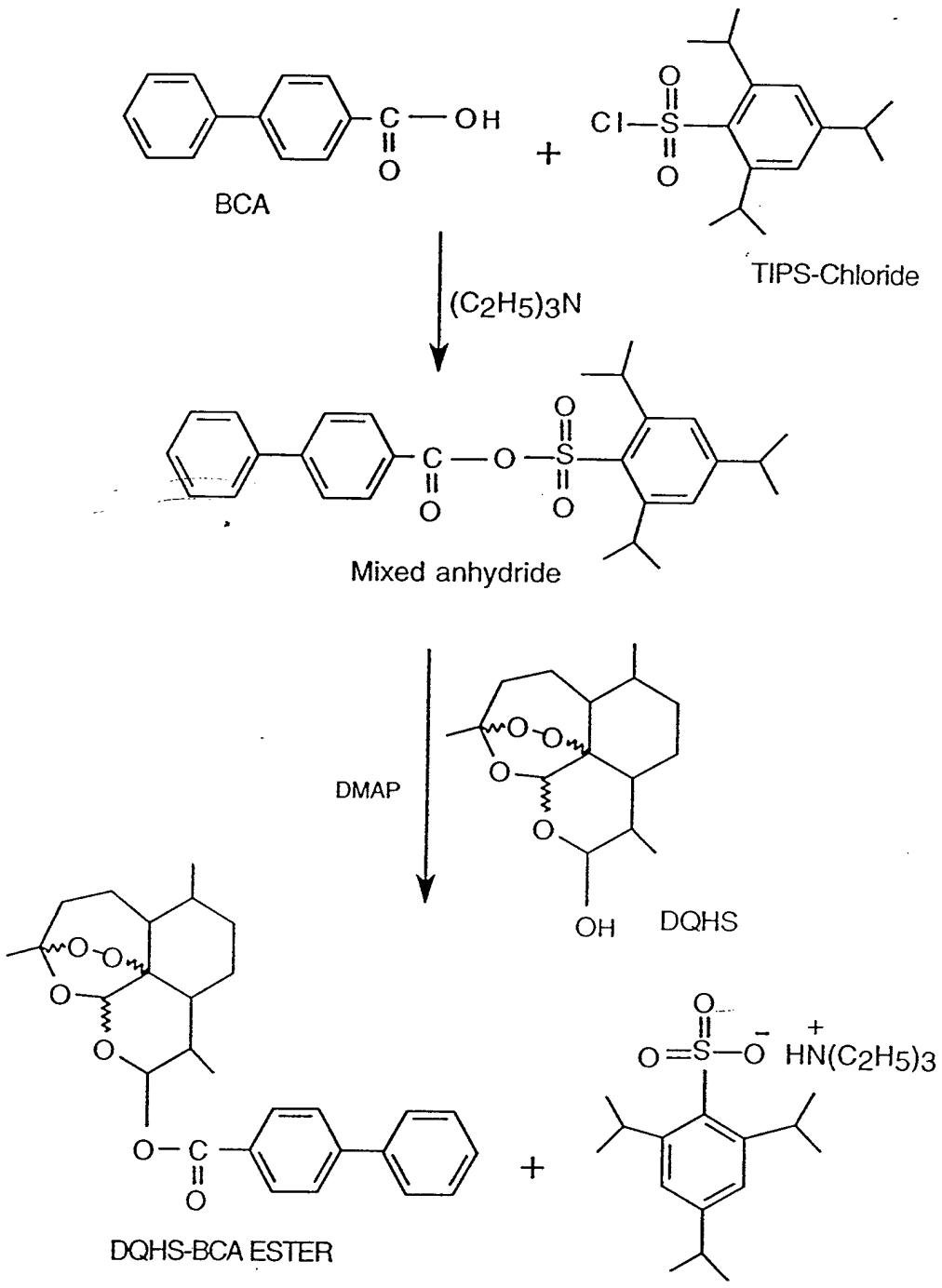

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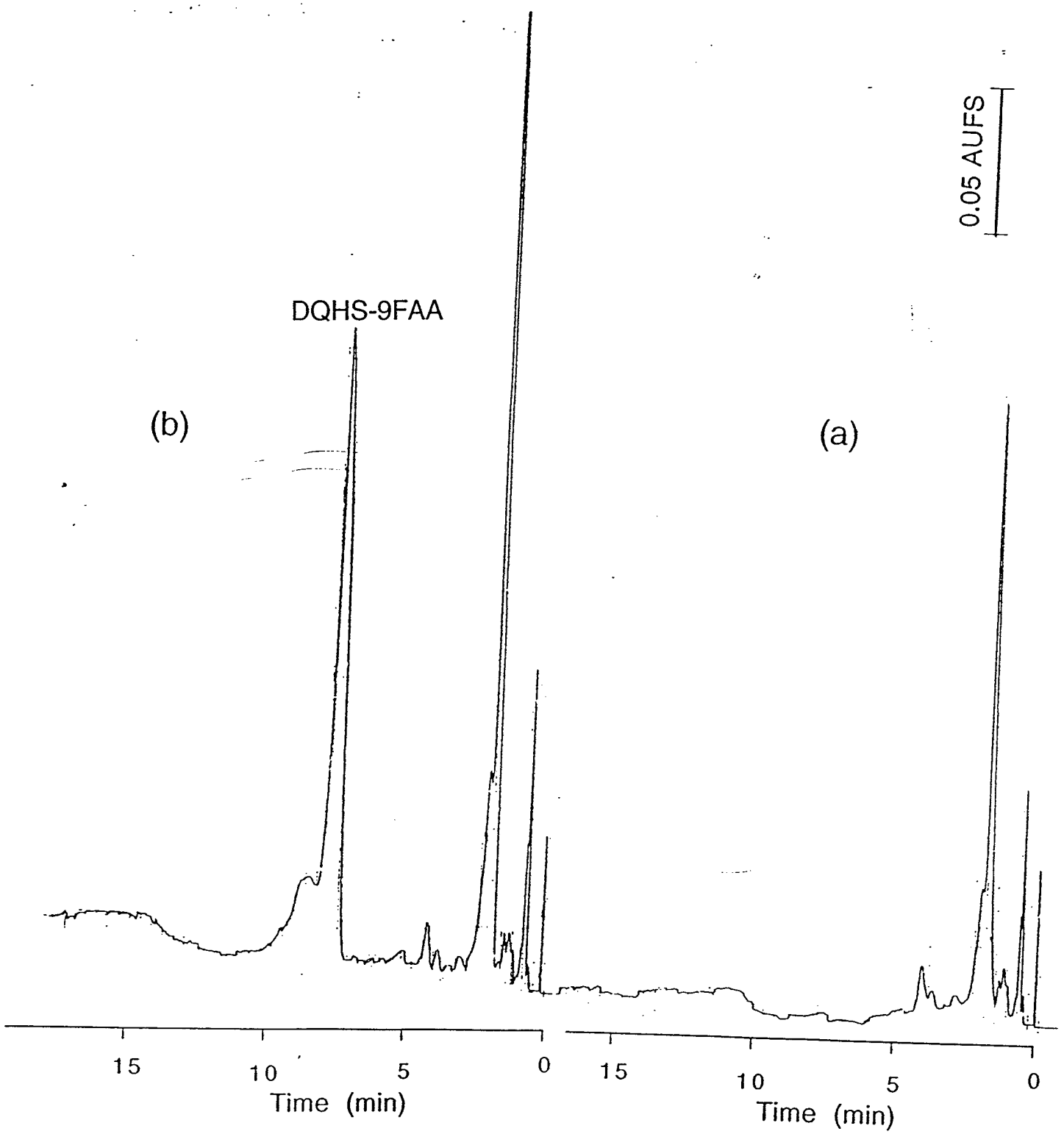

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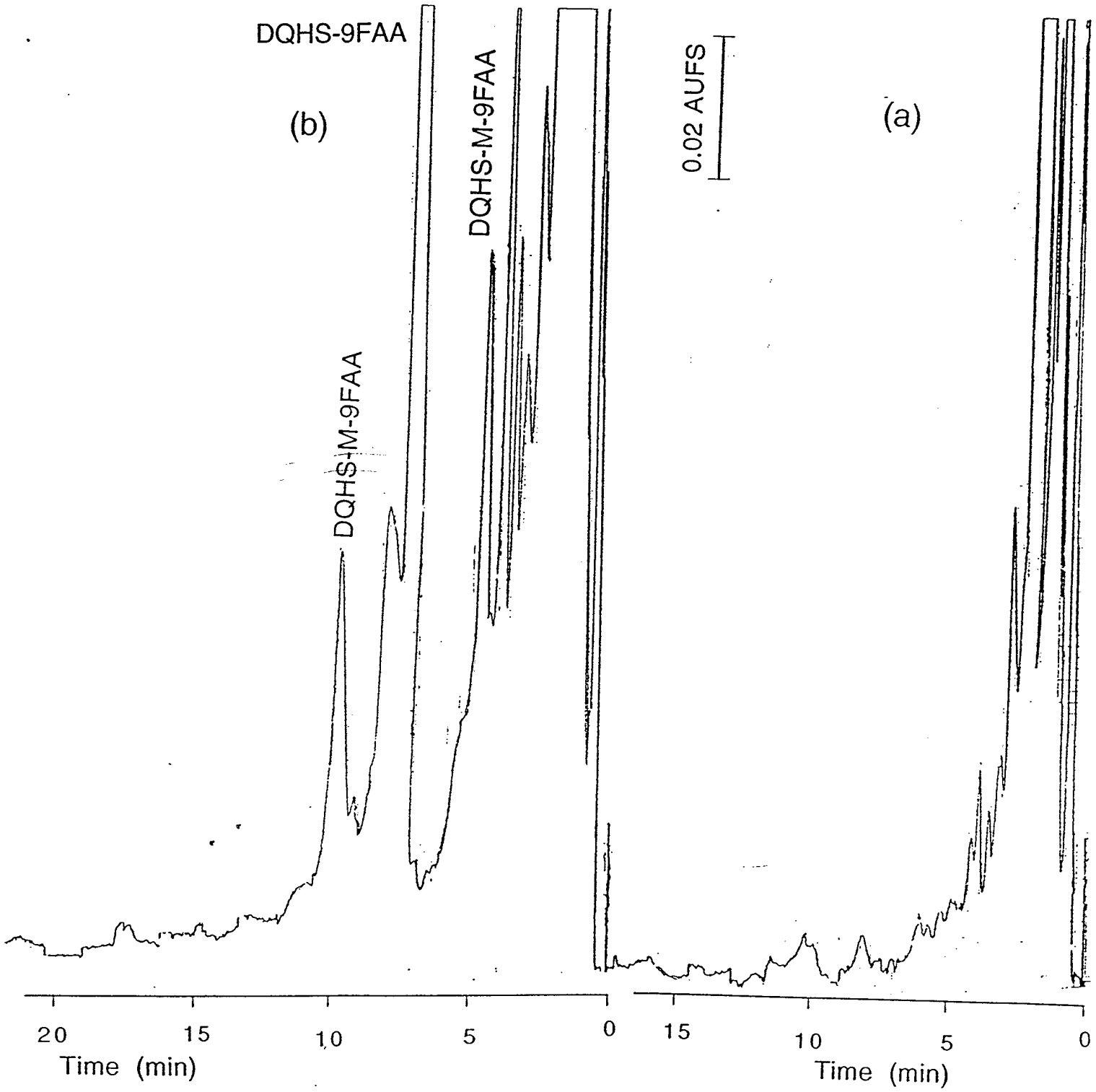

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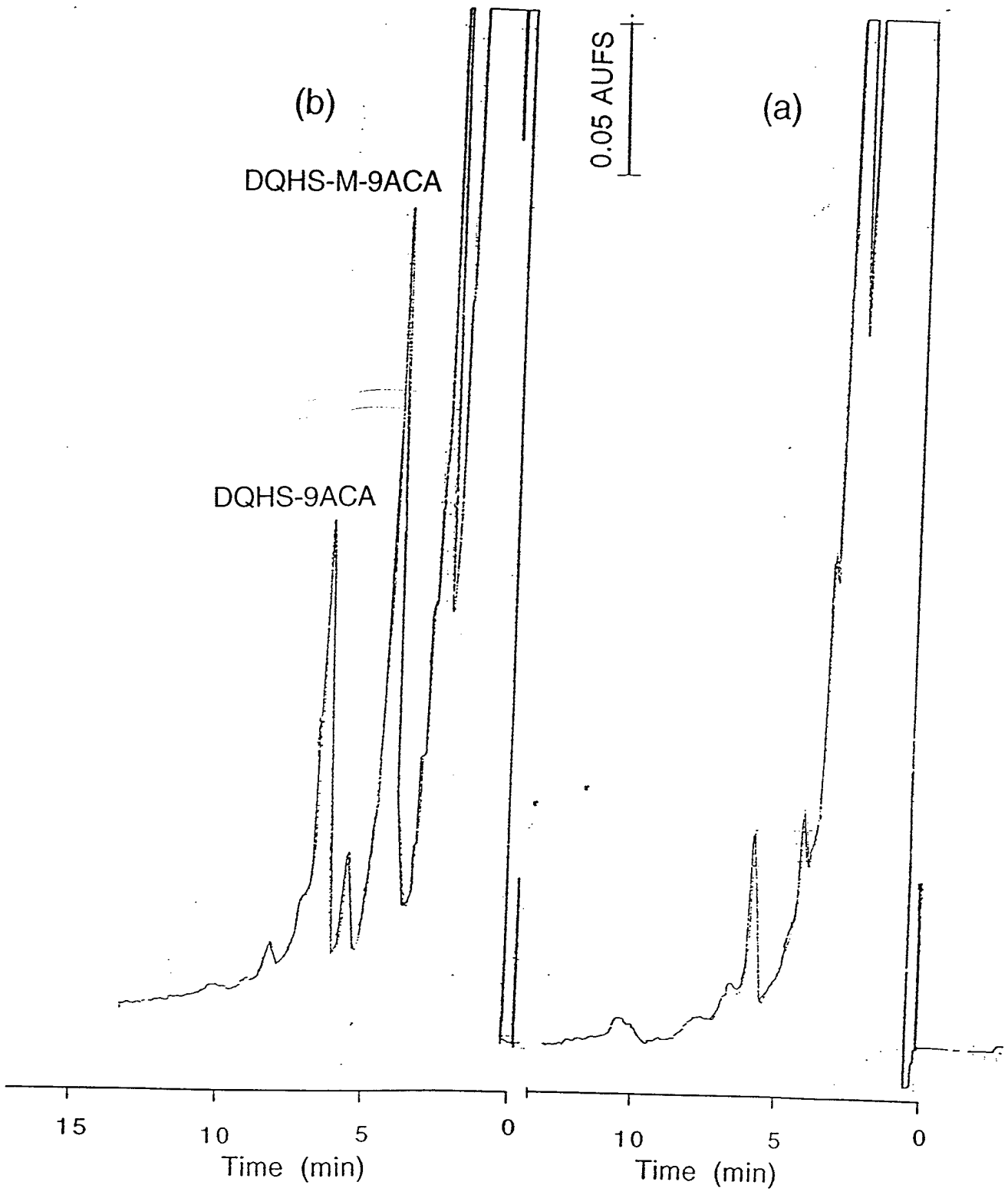
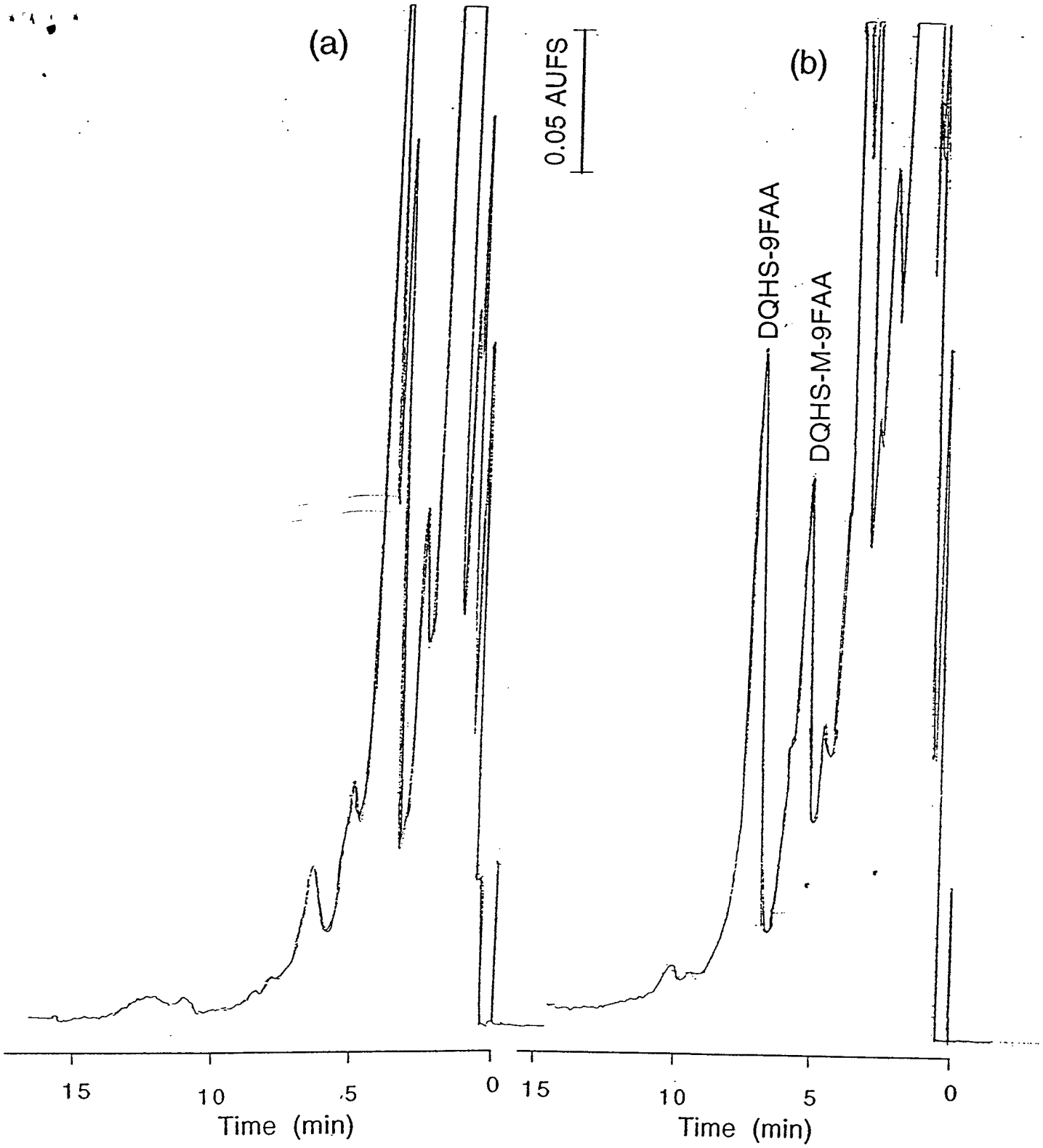


Figure 5b. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolite (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 90 min) and derivatized with 9-anthracenecarboxylic acid/TIPS-chloride.



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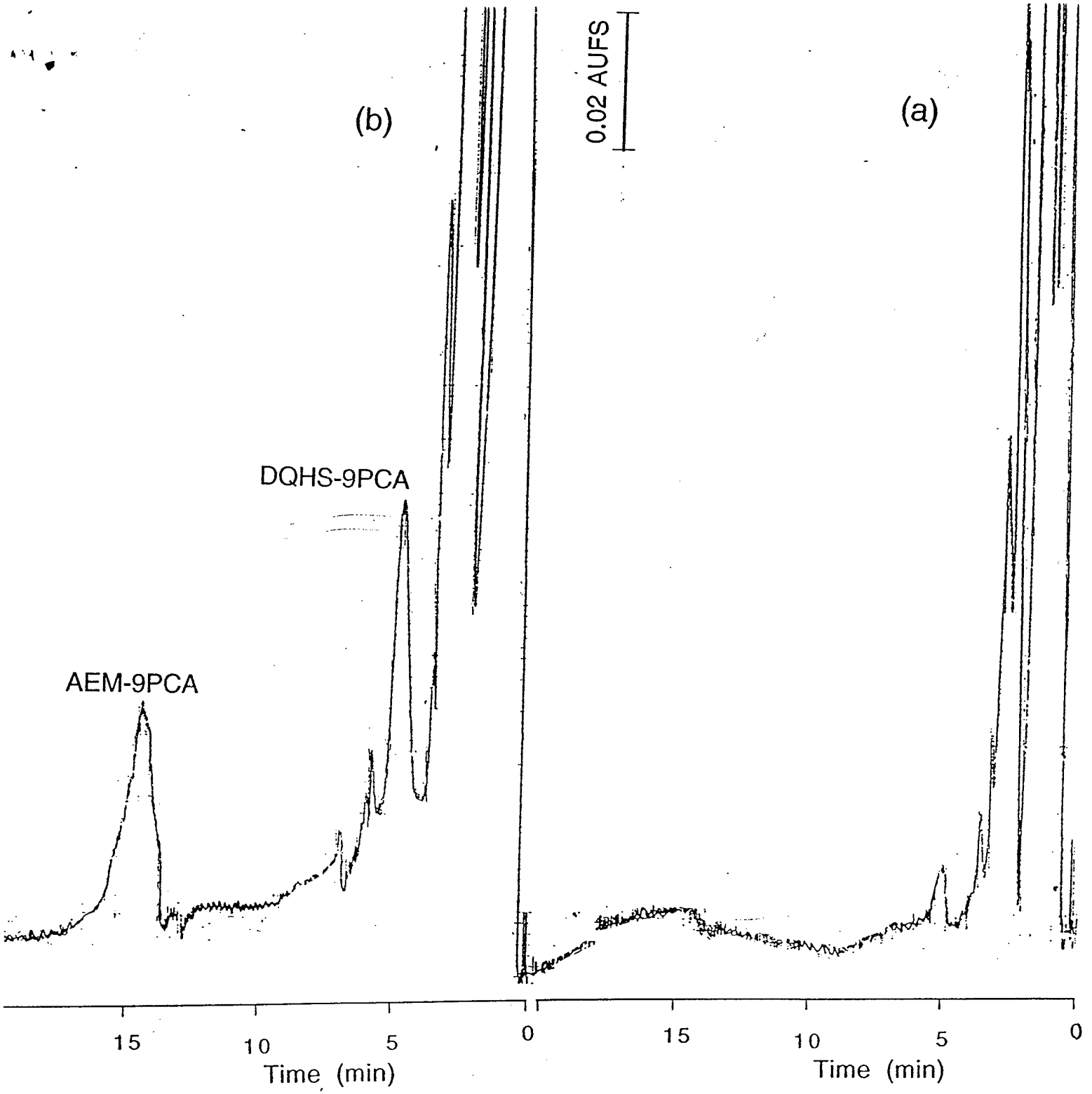

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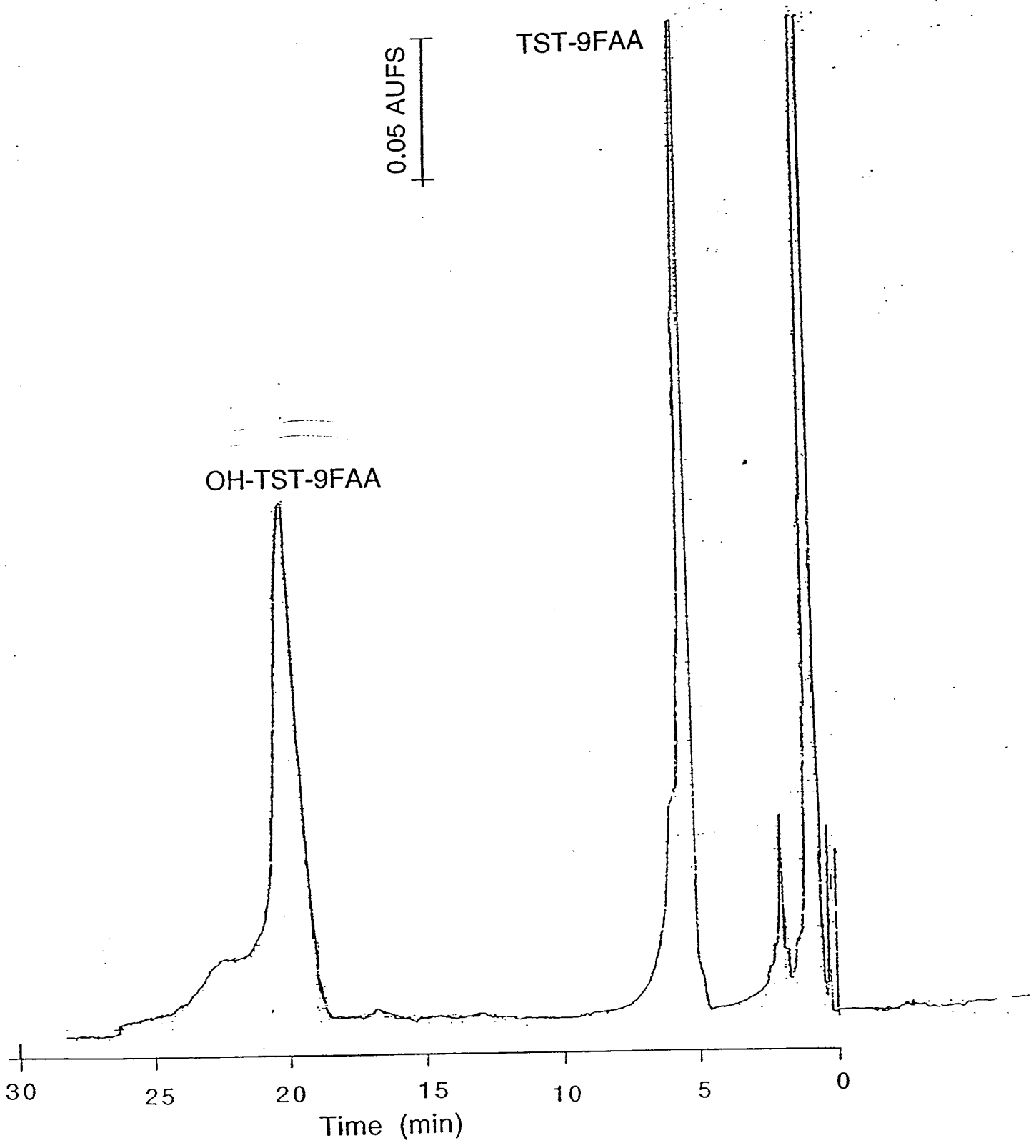

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