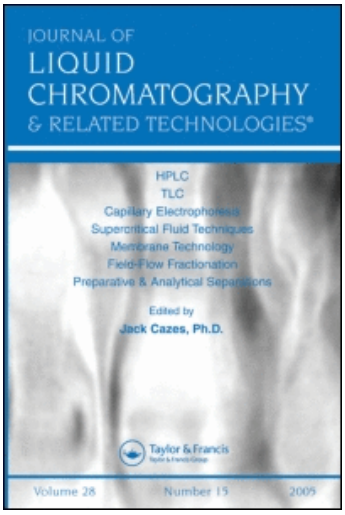

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Rapid Derivatization of Alcohols with Carboxylic-Sulphonic Mixed Anhydrides for HPLC-UV/Fluorescence Analysis: Application to the Detection of Dihydroqinghaosu (DQHS) and its Metabolites in Biological Samples

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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-hydroxy-testosterone.

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).

Catalyst

4-Dimethylaminopyridine (DMAP).

Hydroxy Compounds

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

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 μ) 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 μL 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 μL aliquots of the reaction mixture were taken and evaporated under a stream of nitrogen, and the residue redissolved in 200 μL of methanol, followed by HPLC analysis of 10 μL 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 (0.91 mmol; equivalent to about 3.3 % molar excess relative to DQHS) of the acid in 2 mL of dichloromethane (or chloroform) and adding 200 μL of triethylamine. The solution of the acid was then mixed with a solution of 250 mg (0.88 mmol) of DQHS in 2 mL of dichloromethane.

A solution of 300 mg (0.99 mmol, equiv. 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 (1.23 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 μL of triethylamine, was added 250 mg (0.88 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°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 for 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 μL of triethylamine. The solution of the acid was mixed with a solution of 250 mg (0.88 mmol) of DQHS in 2 mL of dichloromethane and a solution of 170 μL (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 μg of DQHS (20 μL 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 μL 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 μL 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 μL 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 μg/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 μg 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 μM of DQHS. After incubation for 90 or 180 min, the reaction was terminated by the addition of 100 μL 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 μg) and 6β-hydroxytestosterone (500 μg) 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 procedure. Esterification of 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.

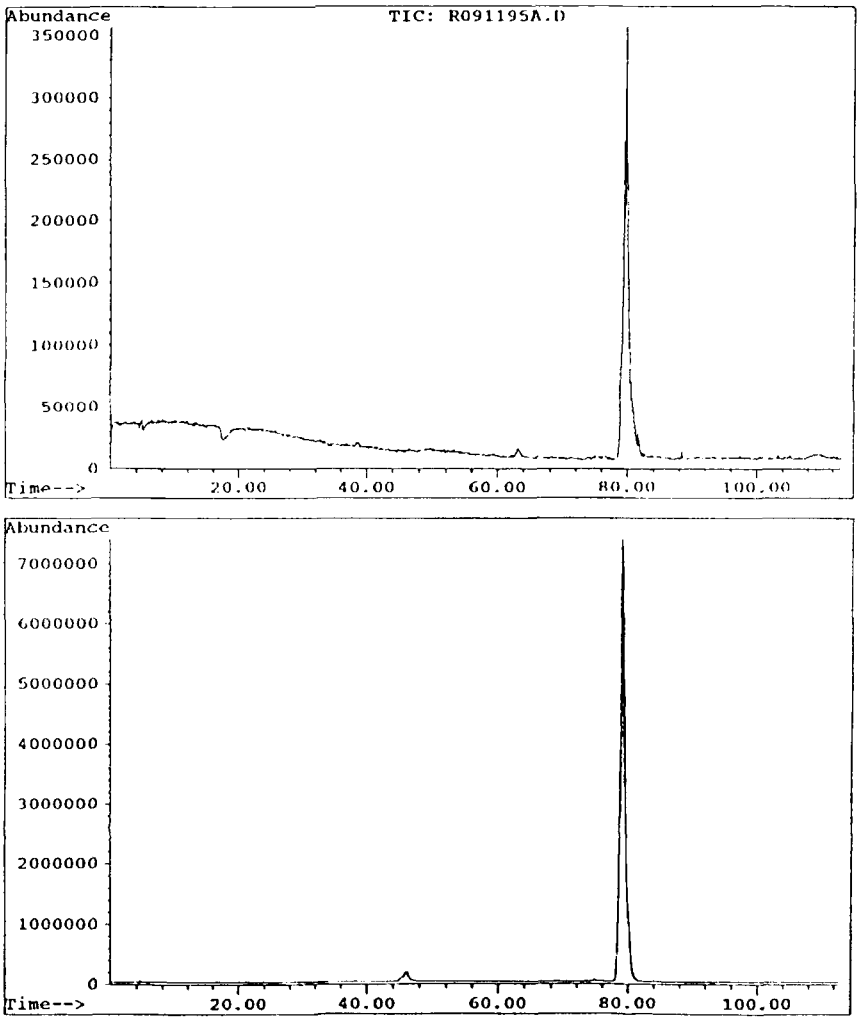


Figure 1. Total ion- and corresponding UV-chromatograms of the ester obtained from the semi-preparative reaction of DQHS with 4-biphenylcarboxylic acid /METS-chloride.

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]

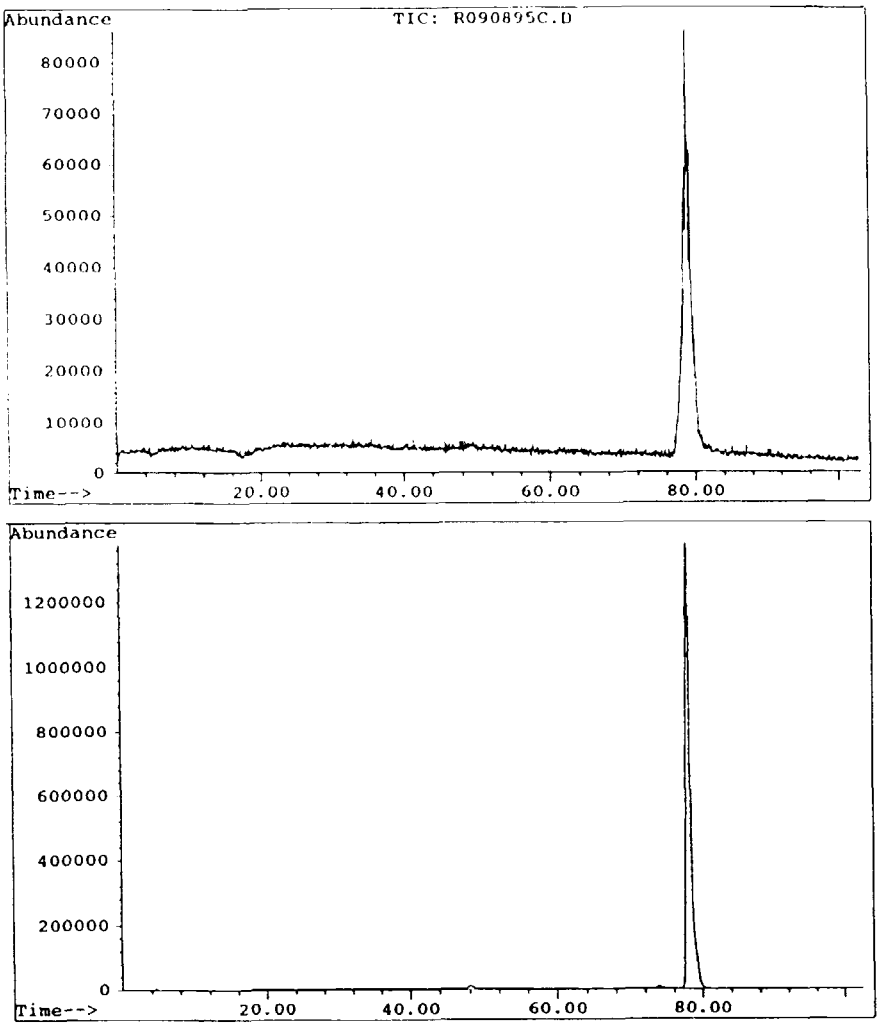
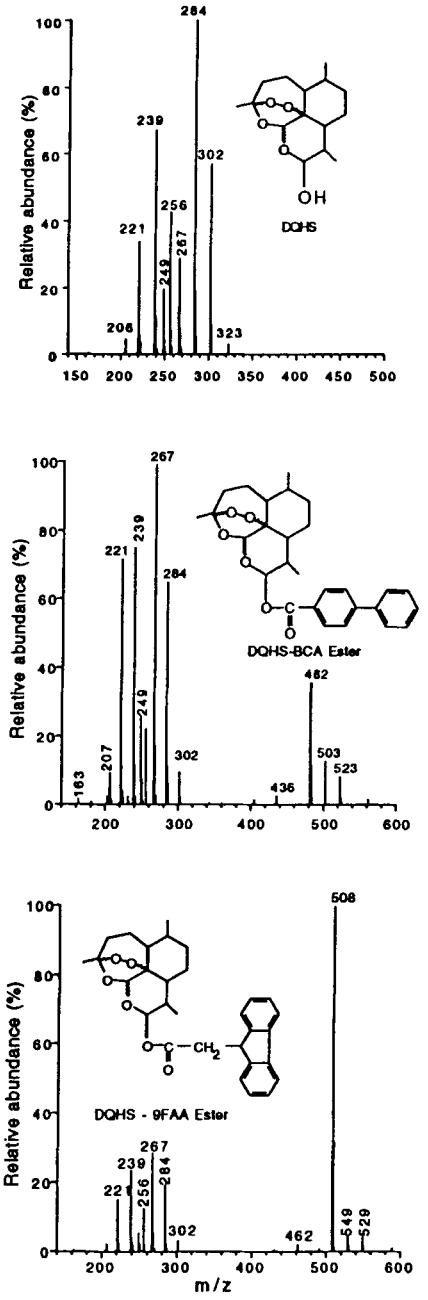


Figure 2. Total ion- and corresponding UV-chromatograms of the ester obtained from the semi-preparative reaction of DQHS with 9-fluoreneacetic acid/TIPS-chloride.

Figure 3. (right) Thermospray mass spectra of DQHS and its respective biphenylcarboxylic and 9-fluoreneacetic acid esters.



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 Figures 1 and 2 respectively.

The thermospray (TSP) mass spectra of the DQHS esters of the two acids are also shown in Figure 3 while the proposed fragmentation pattern is shown in Figure 4. 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 3, 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 5. 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.

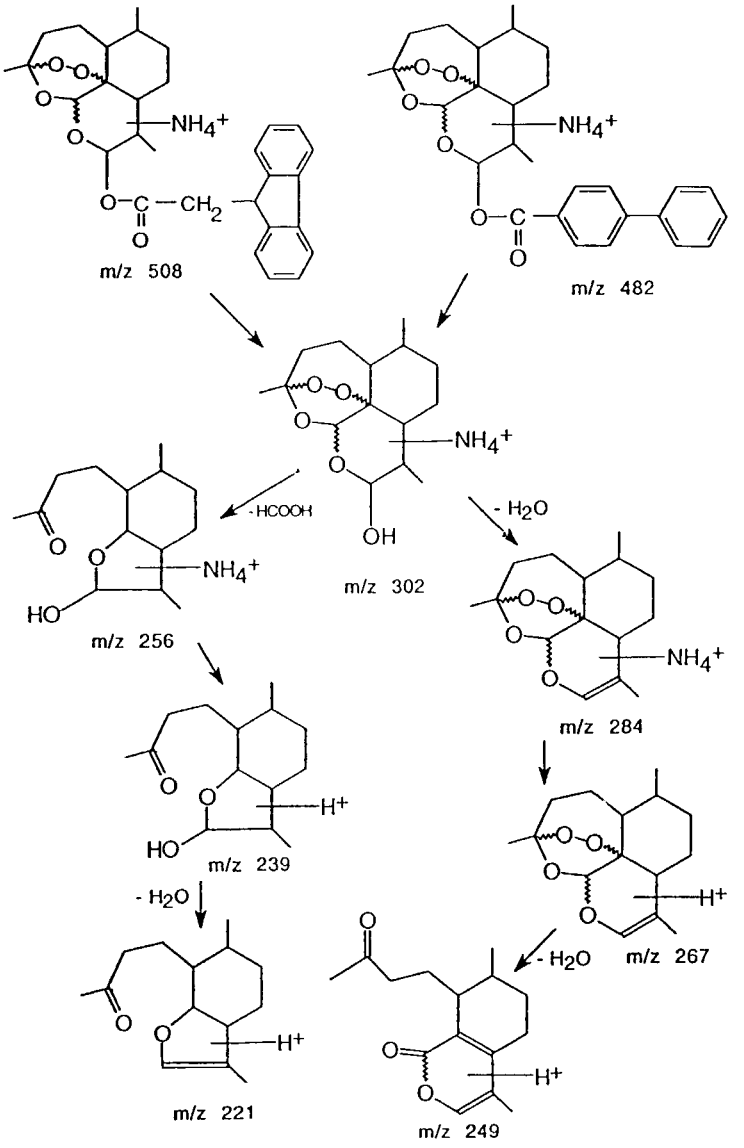


Figure 4. Thermospray mass spectra fragmentation pattern of DQHS and its respective biphenylcarboxylic and 9-fluoreneacetic acid esters.

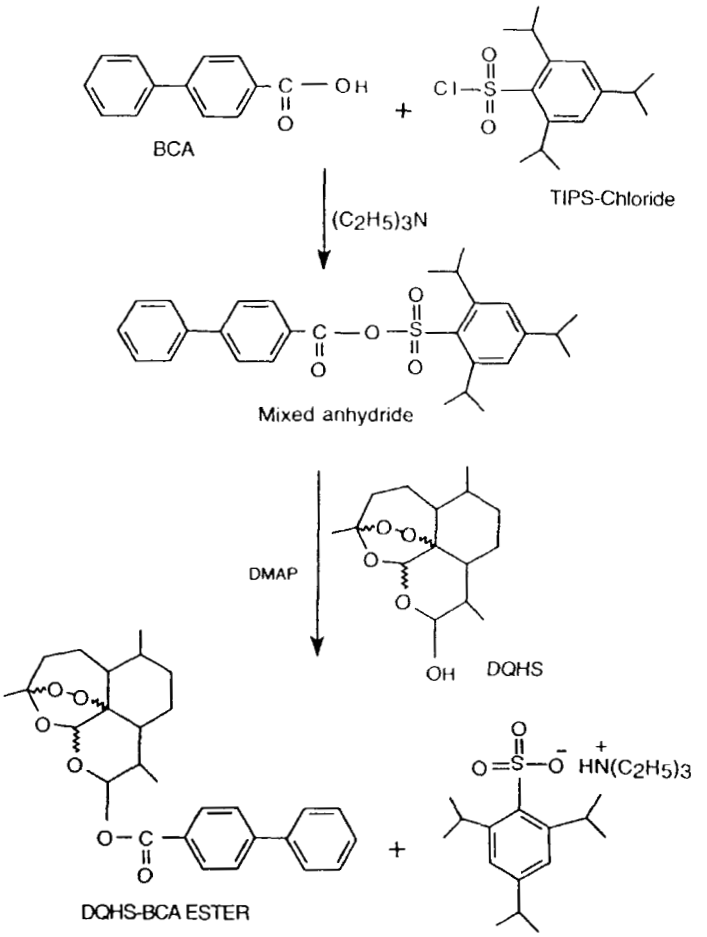


Figure 5. Illustration of the esterification of DQHS by the mixed anhydride method.

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 symmetric 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 symmetric 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 either 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

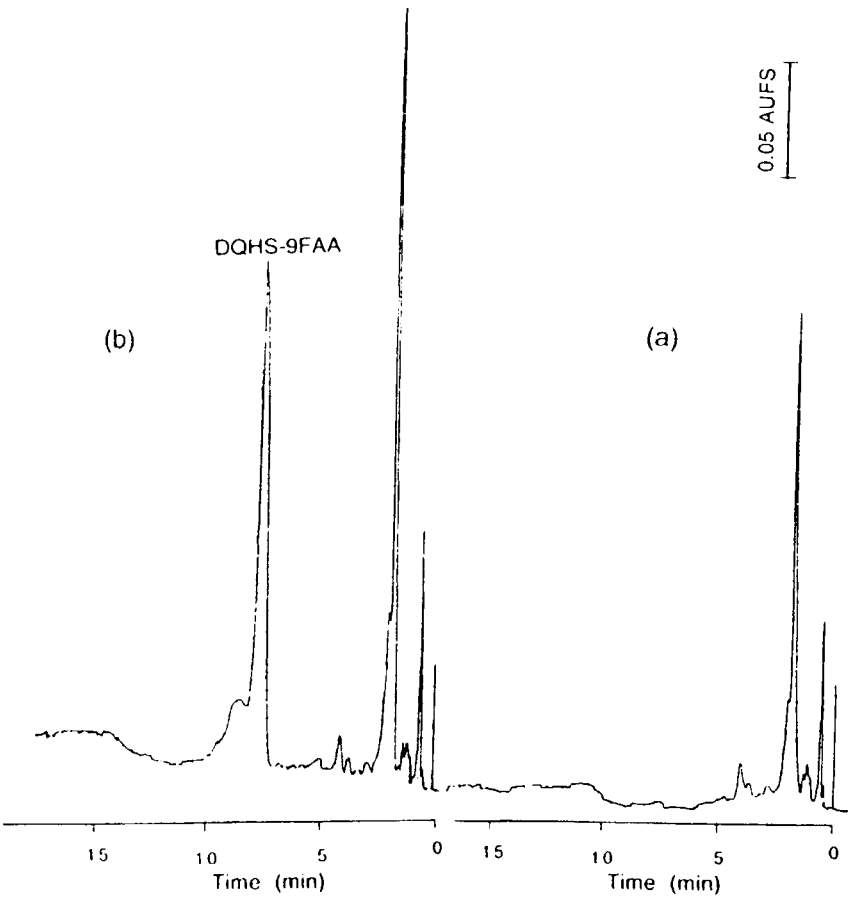


Figure 6. HPLC chromatograms of (a) blank plasma extract and (b) DQHS extracted from plasma and derivatized with 9-fluoreneacetic acid/TIPS-chloride.

is shown in Figure 6. Chromatograms of DQHS and its metabolites extracted from rat liver microsomes and then acylated by the mixed anhydride method are shown in Figures 7-9. Arteether, the ethyl ether analogue of DQHS, undergoes metabolic deethylation to DQHS. As shown in Figure 10, 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 liver (IPRL).

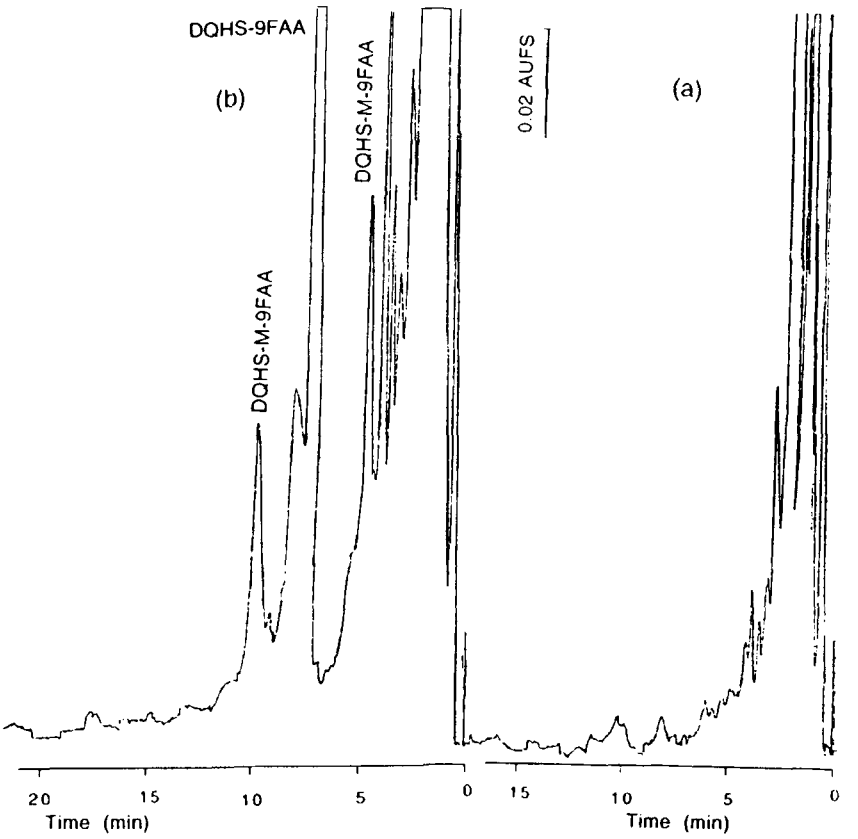


Figure 7. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolites (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 180 min) and derivatized with 9-fluoreneacetic acid/METS-chloride.

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-hydroxy-testosterone, 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

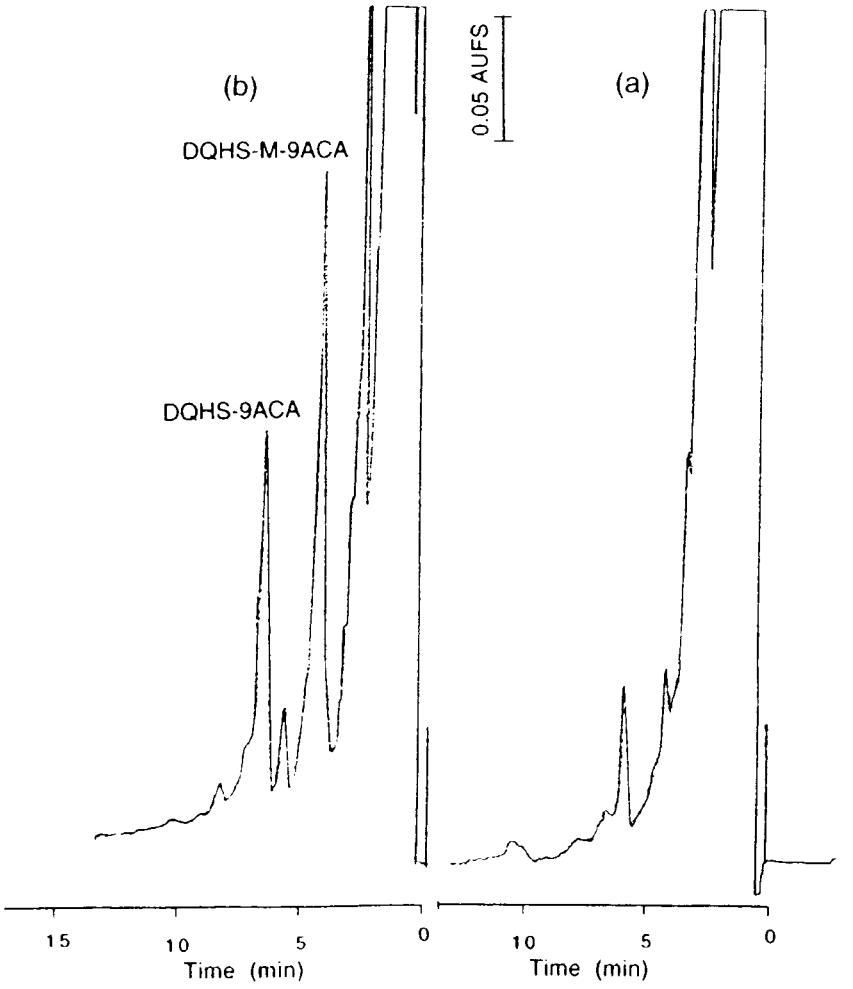


Figure 8. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolite (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 90 min) and derivatized with 9-anthracenecarboxylic acid/TIPS-chloride.

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.

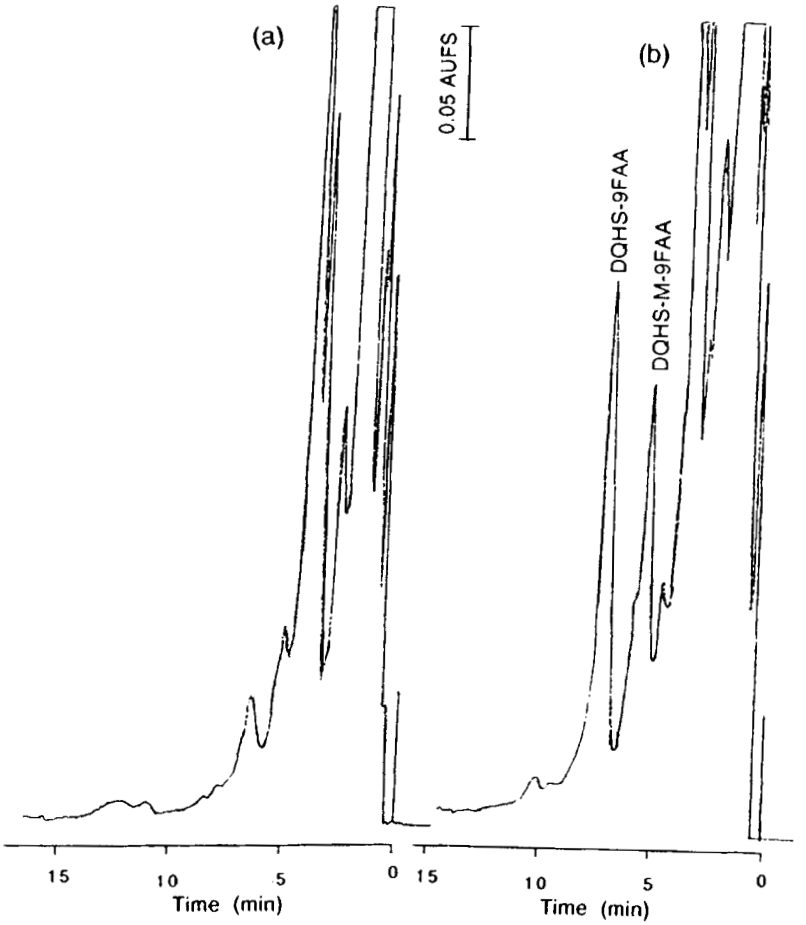


Figure 9. HPLC chromatograms: (a) extract of blank rat liver microsomes; (b) DQHS and its metabolite (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 90 min) and derivatized with 9-fluoreneacetic acid/TCB-chloride.

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

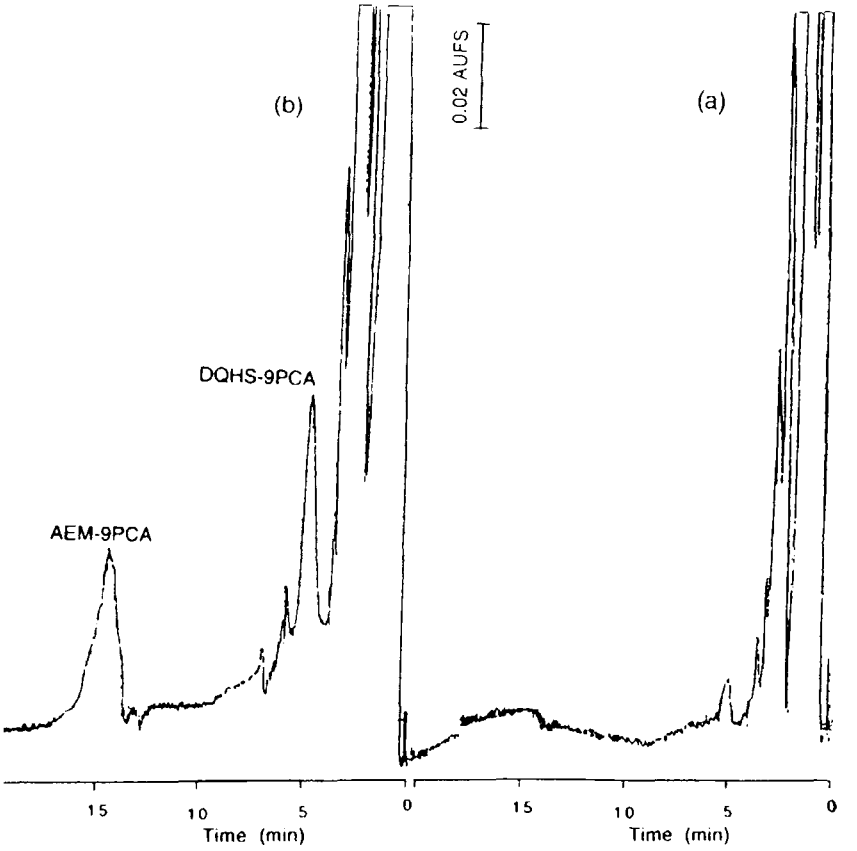


Figure 10. HPLC chromatograms of (a) extract of blank rat liver bile and (b) metabolites of arteether (DQHS and AEM) extracted from rat liver bile and derivatized with 9-phenanthrenecarboxylic acid/TIPS-chloride.

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 11. 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.

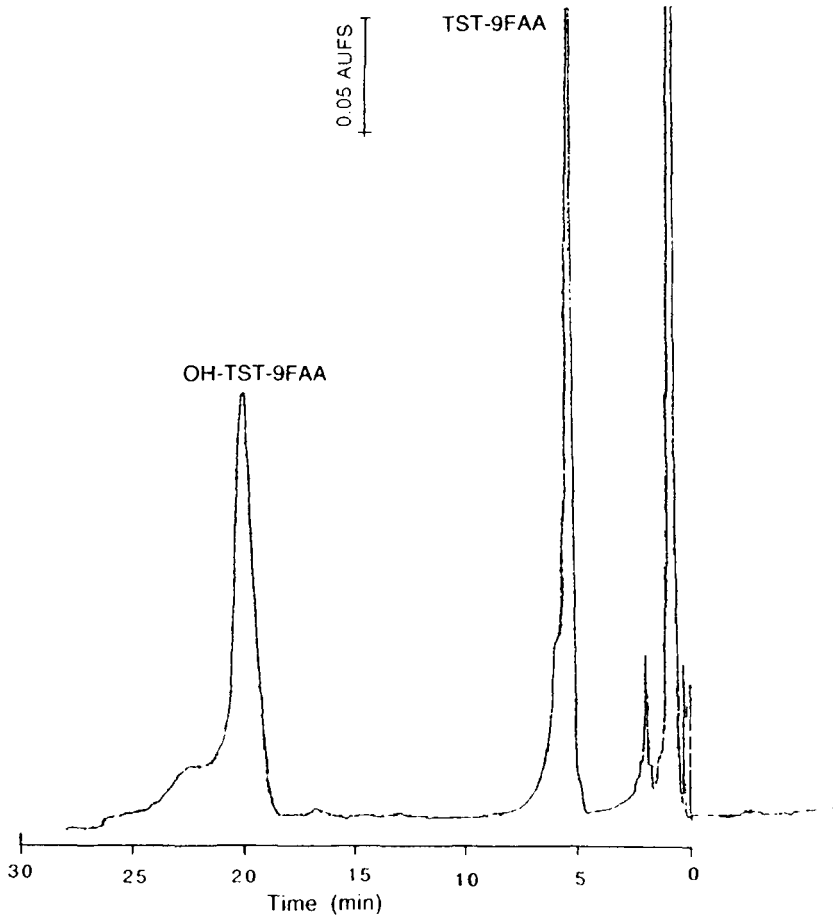


Figure 11. HPLC chromatogram of the 9-fluoreneacetic acid esters of testosterone (TST-9FAA) and hydroxytestosterone (OH-TST-9FAA).

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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