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ANTHRACENE-9-CARBONYL CHLORIDE AS A FLUORESCENCE AND ULTRAVIOLET DERIVATISING REAGENT FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF HYDROXY COMPOUNDS

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SUMMARY

The synthesis of anthracene-9-carbonyl chloride and its application as a derivatising reagent for the detection of hydroxy compounds for high-performance liquid chromatographic analysis are described. The preparation and chromatographic properties of esters of short-chain alcohols, diols, trichothecene mycotoxins and sterols are discussed.

INTRODUCTION

The detection in liquid chromatography of compounds which do not possess a chromophore presents problems. Alternative detection principles, such as refractometry, flame ionisation, photoreduction-fluorescence[1] or displacement chromatography[2] with UV–VIS absorbers, impose severe limitations on sensitivity often together with unacceptable constraints on mobile phase selection.

For trace analysis, there is a balance to be attained between rigour of sample cleanup and selectivity of detection. Derivatisation of target compounds offers significant advantages, improving selectivity and thus reducing the complexity of the cleanup required. UV and fluorescence spectrophotometers are widely available and fluorescence derivatisation in particular permits high sensitivity, often with good selectivity. Many methods are available for the visualisation of amines and phenols, but hydroxy derivatising reagents have been less well studied.

A number of hydroxyl derivatisation reagents have been listed in a recent review of fluorescence detection in liquid chromatography[3] and others have been proposed[4,5]. However, the only commercially-available reagent specifically developed for these compounds is 1-ethoxy-4-(dichloro-1,3,5-triazinyl)naphthalene (EDTN)[6], which is of low reactivity. Fluorene-9-methyl chloroformate, which was

developed for amines, has also been applied to the derivatisation of hydroxyls, but the quantum yield is low and with an excitation wavelength of 260 nm, the potential for interferences is high.

Three aspects of the structure of a derivatising reagent are of interest; the fluorophore, the reactive group for coupling and (possibly) the bridge between them. A bridging group may be necessary either to isolate the fluorophore from the reactive site in order to prevent electronic interactions from quenching the fluorescence or to mitigate steric hinderance between reagent and target.

The most widely used fluorophores are polycyclic aromatics or coumarins as these are accessible and possess high molar extinction coefficients and quantum yields. The choice between coumarins and polycyclic aromatics is not easy to make. Coumarins are the smaller molecules and are more polar and thus might be more readily soluble in eluents for reversed-phase high-performance liquid chromatography (HPLC) and less likely to dominate the chromatographic characteristics of their derivatives. However their starting materials are more costly and the synthetic routes more complex. The anthryl fluorophore has the most favourable combination of properties of the polycyclic hydrocarbons. Wintersteiger[7] has reported the synthesis of anthracene-2-isocyanate and its limited application to the derivatisation of pharmaceuticals. Interfering byproducts restricted the usefulness of this reagent.

Many different reaction types are available for derivatising hydroxyls. Most reagents are either inconvenient or hazardous to prepare and use. For example, anthracene-9-carbonyl cyanide was synthesised by Goto *et al.*[8] and shown to be selective for primary hydroxyls, probably because of its reduced reactivity compared to the acid chloride, but it is highly toxic. Acylation is commonly used for labelling hydroxy compounds and permits rapid and quantitative derivatisation. Anthracene-9-carboxylic acid is commercially available and inexpensive, unlike its isomers. Its esters have high molar absorptivities and quantum yields of fluorescence. They also have characteristic absorption spectra and excitation–emission profiles which are convenient for confirming the presence of the anthracene-9 group[9]. The carboxyl group does not significantly reduce the anthryl quantum yield in polar solvents and increases it in non-polar solvents[10]. The anthracene-1 and -2 isomers have more favourable quantum yields in polar solvents[11] but this property is likely to be more structure-dependent than with anthracene-9 because of the geometry of the acids[11].

Thus anthracene-9-carboxylic acid chloride (ACC) was selected for study. The reactivity of this compound was found to be typical of an aliphatic, rather than an aromatic, acid chloride. However, use of standard nucleophilic catalysts in fact decreased the reaction rate, a phenomenon for which an explanation is proposed. ACC has been applied to the determination of diethylene glycol (DEG) in white wine[12].

EXPERIMENTAL

Materials

Anthracene-9-carboxylic acid, thionyl chloride (>99%), diol model compounds and all other chemicals (except trichothecenes and solvents) were obtained from Aldrich (Gillingham, U.K.). T-2 toxin and deoxynivalenol were purchased from Sigma (Poole, U.K.). Silica gel 60 for column liquid chromatography was purchased from BDH (Poole, U.K.). Water was purified using a Millipore Milli-Q system. All

other solvents were glass distilled or HPLC grade from Rathburn (Walkerburn, U.K.). Solvents for preparation of the reagent were purified before use. Heptane was dried with sodium wire. Chloroform was shaken with concentrated sulphuric acid, washed with water, dried with sodium sulphate, distilled from phosphorus pentoxide and then stored over type 4A molecular sieve under nitrogen. Dichloromethane was purified by passage through columns containing neutral alumina and anhydrous sodium carbonate. For derivatisation reactions, HPLC grade acetonitrile was used as received; chlorinated solvents were purified as above to remove alcohol stabilisers.

Apparatus

The HPLC gradient apparatus consisted of two 6000A pumps and a 660 controller (Waters), a Rheodyne injector with a 20-μl loop, a Pye UV detector and either Perkin-Elmer LS-4 or Model 3000 fluorimeters. Spherisorb 5-μm silica and ODS1 and Hypersil 5-μm ODS columns (250 × 4.9 mm I.D.) were supplied by Hichrom (Reading, U.K.). An Upchurch (Oak Harbor, WA, U.S.A.) precolumn filter was used, fitted with a 2-μm frit. Samples were passed through Acro LC13 0.2-μm filters (Gelman, Northampton, U.K.) before injection. Mobile phases were chloroform–hexane, acetonitrile–water or tetrahydrofuran (THF)–acetonitrile (compositions as given in text) at 1.0 ml/min, thermostatted at 35°C. Vials and PTFE-faced septa were from Chromacol (London, U.K.). UV detection was at 250 nm and fluorescence conditions were excitation at 360 nm and emission at 460 nm, both with 10-nm slit widths.

UV spectra were determined on a Pye SP8-200 spectrophotometer using 10-mm quartz cells. Uncorrected fluorescence spectra were recorded on a Perkin-Elmer LS-5 spectrofluorimeter with a 3600 data station using 10 mm × 10 mm quartz cells. Quantum yields were obtained using quinine sulphate in 0.05 M sulphuric acid as a standard[13,14].

Infrared spectra were recorded on a Perkin-Elmer 684 spectrophotometer using potassium bromide discs or in chloroform solution using a potassium bromide liquid cell. NMR spectra were obtained in deuterochloroform with tetramethylsilane as an internal standard using a Jeol PMX60SI spectrometer at 60 MHz for ^{1}H NMR and a Jeol FX-100 spectrometer at 25 MHz for ^{13}C NMR. Electron impact mass spectra were obtained with Kratos MS25 or VG 15-250 spectrometers set at 70 eV.

Methods

Preparation of reagent. Anthracene-9-carboxylic acid (5 g) was suspended in a mixture of dry chloroform (20 ml) and thionyl chloride (5 ml) and refluxed for about 1 h. A drying tube packed with calcium chloride was placed in the outlet of the condenser. On completion of the reaction, a clear golden-yellow solution was obtained. Solvent was removed under vacuum at 50°C and all subsequent manipulations were carried out under a dry nitrogen atmosphere.

Heptane (20 ml) was added to the solid residue and sufficient dry chloroform added to dissolve the product; *ca.* 10 ml were added in excess. The solution was filtered under vacuum and the chloroform slowly evaporated (also under vacuum) at room temperature. Three fractions of golden-yellow needles were collected. Traces of thionyl chloride were removed using a rotary vacuum pump. Total yield 75.1%. The melting point with the reagent under a dry nitrogen atmosphere was 95°C (uncorrected; lit.[15], 94°C).

Preparation of reference compounds. Anthracene-9-carboxylic acid anhydride was prepared by the reaction overnight of equimolar amounts of the acid and the acid chloride in acetonitrile. The identity of the product, which precipitated out of solution, was confirmed by IR spectroscopy.

Standard anthracene-9-carboxylic esters of alcohols were prepared either by leaving a dichloromethane solution of the alcohol (in excess) and reagent at room temperature overnight or by refluxing a mixture in chloroform for 30 min. Solvent was removed and the residue dissolved in dichloromethane and washed sequentially with saturated sodium bicarbonate and water. The organic layer was dried over sodium sulphate, the solvent evaporated and the residue twice recrystallised from aqueous acetone (simple alkyl monoesters only) or purified by column chromatography on silica gel in chloroform–hexane followed if necessary by recrystallisation from chloroform–methanol. DEG monoester was synthesised using a ten-fold excess of the diol and DEG diester prepared with a four-fold excess of reagent; both were purified by column chromatography. All crystallised esters were characterised by elemental analysis, IR, mass spectrometry (MS) and NMR.

For evaluation of solute reactivity, analytes (typically 200 μg, *ca.* 1 μmol) were dissolved in acetonitrile or dichloromethane (1 ml), depending upon their solubility characteristics. Reagent (200 μl, 0.1–0.4 *M*, in the same solvent) was added and the mixture shaken and left at room temperature for 1 h (12 h for 17-α-methyltestosterone). An aliquot (10 μl) of the reaction mixture was diluted with acetonitrile–water (80:20) (1 ml) prior to analysis by reversed-phase HPLC. Excess reagent was sometimes eliminated by reaction with methanol before addition of the aqueous diluent, but this was not generally necessary as reaction with water rapidly gave the free acid. Peaks assigned to derivatives were trapped and examined by MS to confirm their identity.

RESULTS AND DISCUSSION

Fluorescence reagents capable of derivatising hydroxy compounds are inherently non-selective, typically being more reactive towards other nucleophiles such as amino groups, thiols and phenols. Reagents which react readily with hydroxyls will also be susceptible to hydrolysis, which can be a problem in their preparation and storage. The synthesis of ACC is straightforward but requires care to maintain anhydrous conditions. It was found convenient to handle the reagent inside a glove bag purged with dry nitrogen to provide a positive pressure and eliminate diffusion of moist air into the bag.

Derivatisation of alcohols was carried out in acetonitrile because it was found that the HPLC grade as supplied was dry and sufficiently free of active hydrogen compounds such as the alcohol stabilisers or amine degradation products commonly found in other solvents. Stock solutions of the reagent in acetonitrile are stable for at least three weeks if kept in a sealed vial in a desiccator over phosphorus pentoxide. No other precautions are required in routine use.

Derivatisation rates were followed by reversed-phase HPLC, with injection of suitably diluted reaction mixtures. The reaction was typically complete at room temperature within 10 min (saturated reagent, *ca.* 0.25 *M*) or 30 min (0.1 *M* reagent), as shown in Fig. 1. Some acid anhydride (insoluble in acetonitrile) was seen to precip-

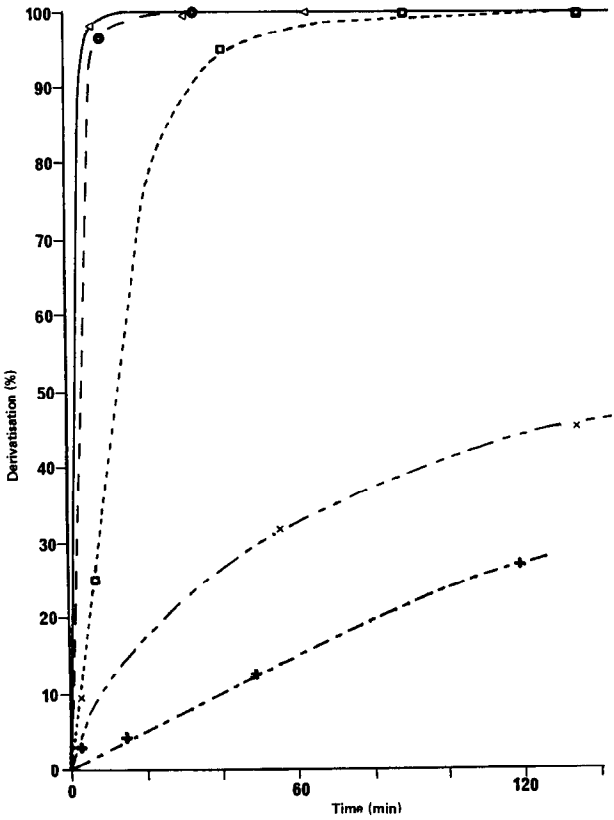


Fig. 1. Effect of bases on reaction between DEG and ACC. All plots were obtained with DEG at 1 m*M* and ACC at 0.25 *M*, unless otherwise indicated. Bases, if present, were at a concentration of 1 *M*. Reactions were carried out in acetonitrile at ambient temperature, and yields were determined chromatographically by comparison with standards prepared from a crystalline sample of the product. (△) DEG monoester + ACC; (○) DEG + ACC; (□) DEG + ACC (0.1 *M*); (×) DEG + ACC + triethylamine; (+) DEG + ACC + pyridine.

itate. Reaction with cholesterol, testosterone and the trichothecene T-2 toxin was quantitative at room temperature within 1 h. Formation of the esters of sterically hindered alcohols, such as *tert.*-butanol and 17α-methyltestosterone, could be achieved by reaction at room temperature overnight or by refluxing in chloroform for 1 h. All esters prepared were characterised by IR, NMR and MS. It is uncertain whether the deoxynivalenol product was the di- or triester, because of the instability of the molecule to electron impact; only one major peak was found on HPLC (Fig. 3c) but derivatisation of the 7-hydroxyl requires forcing conditions with other reagents[16].

The fluorescence characteristics of DEG diester (Table I) were markedly different from those of typical monoesters. In all solvents examined, the ratio of fluorescence to UV peak areas was approximately nine times lower for the diester. Results suggest that this is due to partial formation of a non-fluorescing intramolecular ex-

TABLE I

QUANTUM YIELDS FOR ANTHRACENE-9-CARBOXYLIC ESTERS

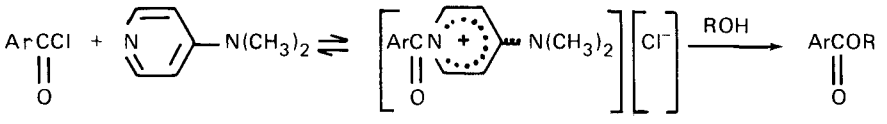
Solvents: A = acetonitrile; C = chloroform; CY = cyclohexane; E = ethanol; M = methanol; W = water.

Alcohol	*Solvent*	*Quantum yield (determined) aerated*	*Quantum yield (published)*		*Ref.*
			Degassed	*Aerated*	
Methanol	M	0.12	0.11	0.11	9
	A	0.32	0.41	0.33	9
	C	0.59			
	CY	–	0.85	0.65	9
	E:W, 50:50	–	0.08	–	11
	E:W, 10:90	–	0.02	–	11
DEG (diester)	C	0.08	–	–	
	M	0.02	–	–	

cimer, favoured by the conformational freedom afforded to the DEG diester by the etherial oxygen. The DEG diester is much less retained than the diderivative of 1,5-dihydroxypentane (Table II). These results will be described elsewhere.

Attempted catalysis of derivatisation of any of the solutes tested gave unexpected results, shown in Fig. 1. When the reaction in dichloromethane between ACC and cholesterol or testosterone was monitored by HPLC and NMR, only very slow esterification was observed in the presence of either triethylamine or pyridine. With 4-dimethylaminopyridine (DMAP) catalyst, essentially no ester was produced, whereas rapid formation of product occurred in the uncatalysed reaction. Formation of an intermediate complex was observed with the reaction mixture undergoing a marked colour change from yellow to dark red, but production of ester was retarded, even under rigorously dry conditions.

Quantitative formation of the postulated intermediate with DMAP:



was observed by IR spectroscopy and ^{1}H and ^{13}C NMR. The intermediate was exceptionally stable to hydrolysis, with a halflife in water of > 10 h, compared to the reported[17] halflife of 3.9 min for the acetyl chloride analogue. Esterification is considered usually to proceed via an S_N2 mechanism, involving a tetrahedral transition state or intermediate, but in this case an S_N1-type mechanism[18,19] is proposed. This means that nucleophilic catalysis cannot be used with this reaction. Catalysis via Lewis acids or hindered bases might be applicable but has not been evaluated here. This work will be described more fully in another publication.

The esters of the seven C_1–C_4 alkanols showed surprisingly good resolution in reversed phase, with only the ethyl and *tert.*-butyl esters coeluting. Thus the anthracene group fortunately does not dominate the separation to the extent antici-

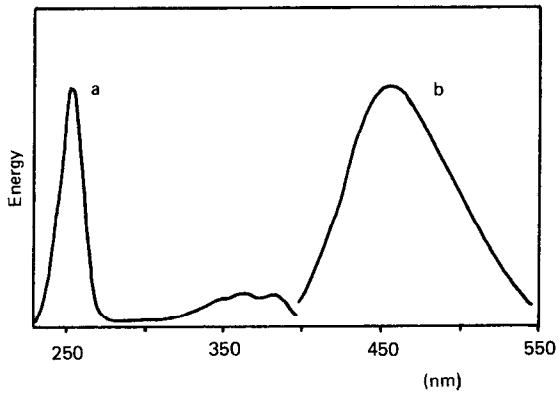


Fig. 2. Excitation and emission spectra of methyl ester of anthracene-9-carboxylic acid. Conditions: see Methods. Ester at $0.98 \cdot 10^{-7}$ *M* in chloroform. Slit widths 10 nm. (a) Excitation scan. Emission set to 460 nm. (b) Emission scan. Excitation set to 256 nm.

pated. Quantum yields of fluorescence of the methyl ester and DEG diester in various solvents are given in Table I and the excitation and emission spectra of the methyl ester in chloroform are shown in Fig. 2. Solvent dependance of the position of excitation and emission was minimal, with wavelength shifts of no more than 5 nm over the whole range of polar and non-polar solvents examined here. The absorption maximum at 250 nm is about ten-fold more intense than that at 360 nm, but for normal-phase solvents background fluorescence is considerably greater at the lower wavelength and there is no gain in sensitivity. Although for polar solvents there is some improvement in sensitivity with excitation at 250 nm, selectivity is enhanced by working at the higher excitation wavelength. Increasing the proportion of water in the mobile phase leads to a reduction in fluorescence intensities but sensitivity remains in the femtomole range. The fine structure on the anthracene absorption spectrum at 360 nm is observable only when using a narrow (2.5 nm) excitation slit width.

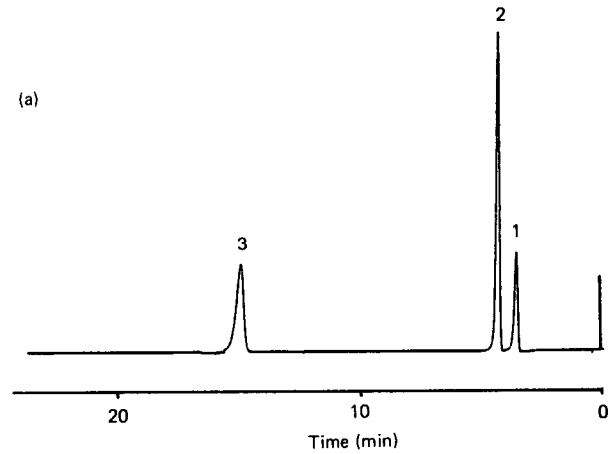


Fig. 3.

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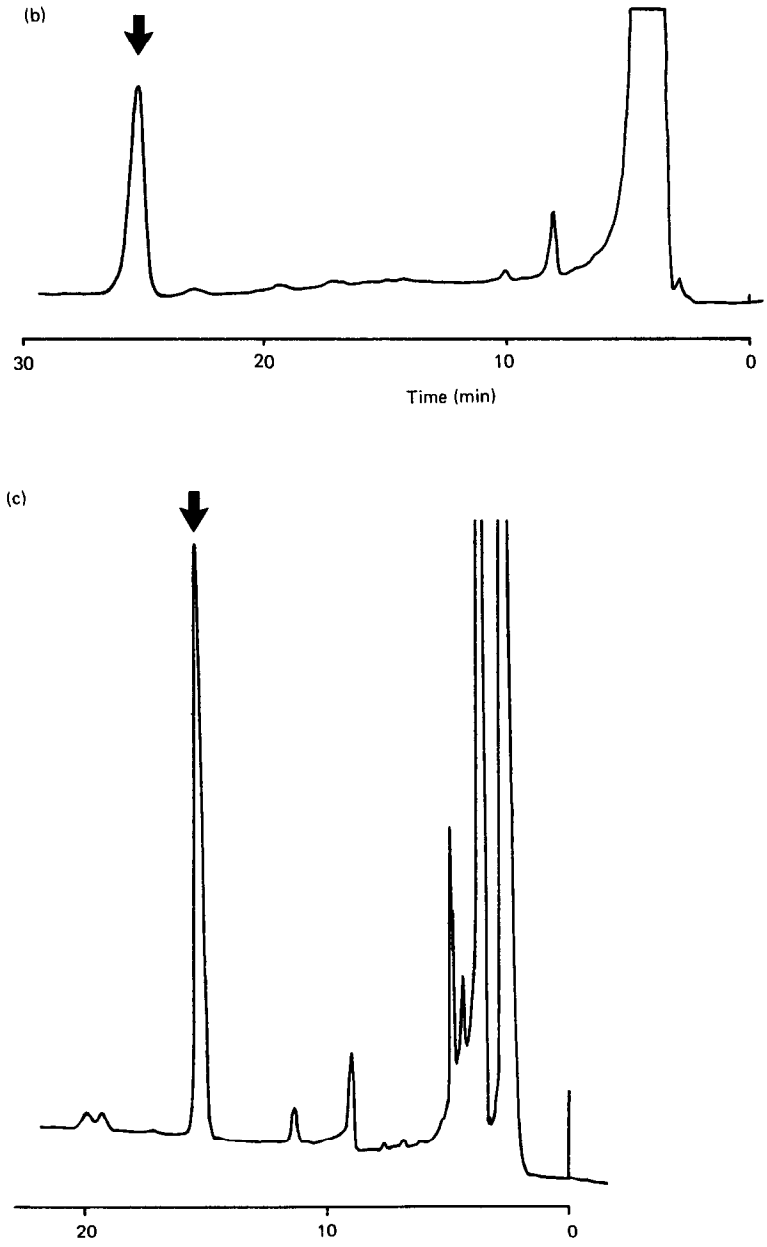

Fig. 3. Chromatography of standard esters and derivatisation reaction mixtures. (a) Standard esters of cholesterol (1), methanol (2) and testosterone (3). Normal-phase chromatography on Spherisorb S5W, 250 × 4.9 mm I.D. Mobile phase, chloroform–hexane (90:10) at 1.0 ml/min. Detection, UV at 360 nm. (b) T-2 toxin derivatisation reaction mixture. Early-eluting peaks are due to reagent. Reversed-phase chromatography on Hypersil 5 ODS, 250 × 4.9 mm. Mobile phase, acetonitrile–water (70:30) at 1.0 ml/min. Detection, UV at 380 nm. (c) Deoxynivalenol derivatisation reaction mixture. Peaks eluting earlier than 12 min appear in reagent blank. Conditions as for b except mobile phase was acetonitrile–water (90:10)

TABLE II

CHARACTERISTICS OF ACC DERIVATIVES OF ALCOHOLS (NORMAL PHASE)

Solvents: C = chloroform; H = hexane. Column: Spherisorb 5W.

Alcohol	*Solvent system*	*Retention (k')*	*Detection limit* (fmol)*
Methanol	C	0.3	7.4
DEG (diester)	C	1.0	44.4
DEG (monoester)	C	>25	–
Testosterone	C	3.1	18.6
17α-methyl testosterone	C	3.1	18.6
Cholesterol	C	0.1	7.4
Methanol	C:H, 20:80	5.1	–
Hexanol	C:H, 20:80	2.6	–
Octanol	C:H. 20:80	2.4	–
1,5-Dihydroxypentane (diester)	C:H, 50:50	3.7	–
DEG (diester)	C:H, 50:50	23.4	–

* Values obtained from samples of purified standard esters using a Perkin-Elmer LS-4 fluorimeter with excitation and emission wavelengths of 30 nm and 460 nm respectively. Signal-to-noise ratio, 5:1.

When employing 10-nm slits, both excitation and emission spectra exhibit broad maxima, and minor errors in calibration are unimportant.

Sterols, trichothecene mycotoxins and DEG were selected as typical solutes lacking UV characteristics adequate for HPLC detection. Testosterone and 17α-

TABLE III

CHARACTERISTICS OF ACC DERIVATIVES OF ALCOHOLS (REVERSED PHASE)

Columns: H = Hypersil ODS; S = Spherisorb ODS. Solvents: A = acetonitrile; T = THF; W = Water.

Alcohol	*Solvent system*	*Retention k'*	*Detection limit* (fmol)*	*Column*
Methanol	A	0.4	6**	H
Methanol	A:W, 70:30	3.0	45	H
Cholesterol	T:A, 50:50	0.5	5	H
Testosterone	A	3.4	18	S
Testosterone	T:A. 30:70	0.2	5	H
T-2 Toxin	A:W, 80:20	4.2	110	H
Deoxynivalenol	A:W, 90:10	6.6	105	H
DEG (diester)	A:W, 80:20	4.8	215	H
DEG (monoester)	A:W, 80:20	0.7	35	S

* Values obtained from samples of purified standard esters (methanol, cholesterol, testosterone and DEG esters) or derivatisation reaction mixtures (T-2 toxin and deoxynivalenol) using a Perkin-Elmer LS-4 fluorimeter with excitation and emission wavelengths of 360 nm and 460 nm respectively. Signal-to-noise ratio, 5:1.

** Detection limit of 1.7 fmol for methyl ester under identical conditions but with excitation at 250 nm.

methyltestosterone, although detectable at 240 nm, were chosen as representative sterically hindered analytes. Representative chromatograms of sterol ester standards and derivatised trichothecenes are shown in Fig. 3. Both normal-phase and reversed-phase HPLC were acceptable; under normal-phase conditions it was desirable to react the excess reagent with methanol, rather than water, to avoid the formation of strongly-retained anthracene-9-carboxylic acid. Injection of free ACC in normal phase gives rise to baseline problems; under reversed-phase conditions hydrolysis on-column was acceptable as the acid elutes rapidly. Chromatographic detection limits (for a signal-to-noise ratio of 5:1), solvent systems and retention times are listed in Tables II and III.

CONCLUSIONS

Anthracene-9-carbonyl chloride has favourable properties as a fluorescence and UV derivatising reagent for hydroxylated compounds. ACC may be prepared by a simple one-step reaction from the commercially available parent acid. It reacts readily with hydroxyls in a range of environments, without nucleophilic catalysis, which should not be employed. It is particularly useful for the determination of diesters because of a structure-dependent quenching of fluorescence, which in combination with UV detection, allows confirmation of identity of the derivative.

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