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Acenaphthene fluorescence derivatisation reagents for use in high-performance liquid chromatography

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Abstract

Acenaphthene-5-sulphonyl chloride (AcNSCl), acenaphthene-5-sulphonyl hydrazine (AcNH) and 5-bromoacetyl acenaphthene (AcBr) were synthesised and applied to the analysis of primary and secondary amines, carbonyl compounds and carboxylic acids, respectively. AcNSCl was used in the HPLC assay of 20 amino acids and 5 peptides. The AcNSCl derivatives were found to be 10–25 times more fluorescent than their corresponding dansyl derivatives; between-run analyses gave coefficients of variation of 2.8–7.3%. AcNSCl has a pseudo-first-order rate constant 1.5 times greater than dansyl chloride for the reaction with amino acids. AcNH derivatives of the ketosteroids were 10 times more fluorescent than the corresponding dansyl derivatives. Fluocinolone cream was analysed with this reagent. AcBr derivatives of carboxylic acids gave a 15–24-fold increase in sensitivity in comparison with the corresponding naphthacyl bromide derivatives. This reagent was used in pharmacokinetic studies of ibuprofen.

1. Introduction

Dansyl chloride (DNSCl) was introduced by Weber [1] as a highly fluorescent label for the analysis of proteins and has subsequently become one of the most sensitive agents for pre-column derivatisation of amino acids prior to analysis by high-performance liquid chromatography (HPLC) [2]. It is reactive towards a variety of bases including primary and secondary amino groups. Chayen et al. [3] employed dansyl hydrazine for the determination of trace levels of carbonyl compounds and Distler [4] used naphthacyl bromide for the determination of carboxylic acids. The fluorescent characteristics

of each of these reagents is attributable to the substituted naphthalene nucleus. The structurally similar acenaphthene moiety is reported as having a fluorescence yield 2.6 times greater than that of naphthalene [5]. This observation prompted this investigation into the synthesis and application of acenaphthene-based derivatisation agents in the HPLC analysis of trace amounts of biologically important molecules.

The structural identification of naphthalene sulphonates was subject to confusion in the scientific literature and in some reports the 3-substituted product was thought to have been produced whilst in others the 5-substituted one was reported as being the product [6]. Consequently, a spectroscopic study was performed on the acenaphthene derivatives synthesised here in order to provide the correct structural assign-

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ments. The aim of the present work is to compare the properties and sensitivity of the acenaphthene derivatising agents with the corresponding dansyl and naphthacyl reagents. Analytical applications of the derivatives are also discussed.

2. Experimental

2.1. Chemicals

Acenaphthene, aluminium chloride, 1,2-dichloroethane, bromoacetylchloride, triethylamine, sodium carbonate and 2-naphthacyl bromide were obtained from Sigma Chemicals. Ibuprofen and flurbiprofen were gift samples obtained from Boots Chemical. Hydrochloric acid and acetonitrile (Hypersolv) were obtained from BDH Chemicals. Polygram precoated silica gel plastic sheets containing a fluorescent indicator were obtained from Camlab. Silica gel 0.125–0.250 mm, 60–120 mesh size was obtained from Fisons Chemicals, 1.5-ml amber-coloured polypropylene vials were obtained from Eppendorf.

2.2. Instrumentation

For isocratic separations a Kontron Analytical LC 414 pump was used in conjunction with a Rheodyne 7125 syringe loading sample injector.

Gradient elution chromatography was performed using a Hewlett-Packard 1090 M liquid chromatograph. A Perkin-Elmer LS 5 luminescence spectrometer with flow cell was used in detecting the fluorescent derivatives. For data analysis peak integration was performed using a Trilab 2000 from Trivector Scientific.

Mass spectroscopy was carried out on a DH88 Mass Spectroscopy Data System, NMR analysis was carried out on a Joel JNM EX 270 spectrophotometer and IR data was obtained on a Pye Unicam SP 100 spectrophotometer.

Class A glassware from Gallenkamp and Beaton Clark was used.

2.3. Synthesis of reagents

The synthetic route for the preparation of the derivatives is shown in Fig. 1. Acenaphthene (1) was bromoacetylated via a Friedel–Crafts reaction to yield 5-bromoacetyl acenaphthene (5) (AcBr). Acenaphthene-5-sulphonic acid (2) sodium salt was synthesised and then reacted with phosphorus pentachloride to yield acenaphthene-5-sulphonyl chloride (3) (AcNSCl). Further reaction of this product with hydrazine hydrate yielded the acenaphthene-5-hydrazide (4).

2.4. Acenaphthene-5-sulphonic acid sodium salt

An amount of 20 g acenaphthene was dissolved in 100 g of nitrobenzene and the solution

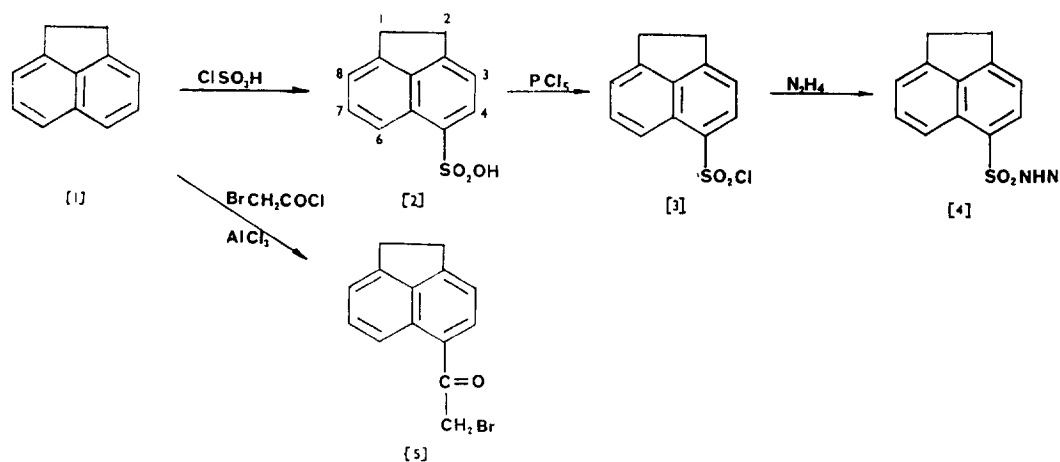


Fig. 1. Synthetic route for the preparation of AcNSO₃H [2], AcNSCl [3], AcNH [4] and AcBr [5].

cooled to 0°C. Then 9 ml of chlorosulphonic acid was added dropwise with constant stirring and with the temperature maintained below 5°C during the addition. The temperature of the mixture was then allowed to rise to 20°C over half an hour. After diluting with 500 ml of water, the aqueous layer was separated from the nitrobenzene layer. Solid sodium carbonate was added to neutralise the aqueous solution. The neutralised solution was treated hot with sodium chloride until precipitation was observed. The mixture was then cooled in an ice bath for 1 h and then filtered. Traces of nitrobenzene and water were removed by heating at 140°C for 36–48 h. Obtained was 25 g, yield 76%, of a pale yellow solid, m.p. >300°C, m.p. anilide derivative 178°C.

NMR: δ /ppm 2.95 (d, 4H, $2 \times \text{CH}_2$, H-1, H-2), 7.15 (d, 1H, Ar, H-3), 7.83 (d, 1H, Ar, H-4), 8.00 (d, 1H, Ar-6), 7.48 (t, 1H, Ar-7), 7.02 (d, 1H, Ar-8).

2.5. Acenaphthene-5-sulphonyl chloride

An amount of 10 g of AcNSO₃H sodium salt was ground in a mortar with 3.5 g of phosphorus pentachloride for 3 min. Following the evolution of hydrogen chloride, cracked ice and water were added and the mixture vigorously stirred to wash out phosphoric acid, the presence of which would lead to decomposition of the product. The product was then extracted into 100 ml of ethyl acetate, washed with 5% sodium bicarbonate solution and then with distilled water until neutral. After drying over anhydrous sodium sulphate and evaporation of the solvent by a gentle stream of nitrogen, crude AcNSCl crystals were obtained. An amount of 200 mg of the crude product, dissolved in 2–3 ml of toluene, was applied to a 2×30 cm column of silica gel H, prepared by filling a suitable glass column with a slurry of silica gel in toluene (25 g in 100 ml). Elution with toluene separated AcNSCl well ahead of its impurities as the first yellow band to be eluted. On crystallisation 150 mg of yellow crystals were obtained, yield 11.5%, m.p. 98–101°C. Elemental analysis: Calculated for C₁₂H₉O₂ClS: C 57.14%, H 3.57%, S 12.69%, Cl 13.88%; found: C 57.30%, H 3.80%, S 12.65%,

Cl 13.83%. NMR: δ /ppm 3.46 (s, 4H, $2 \times \text{CH}_2$, H-1, H-2), 7.35 (d, 1H, Ar, H-3), 7.48 (d, 1H, Ar, H-8), 7.73 (t, 1H, Ar, H-7), 8.25 (d, 1H, AR, H-4), 8.35 (d, 1H, AR, H-6). Mass spectrum: m/z 252 [M⁺], 153 (base peak).

2.6. Acenaphthene-5-sulphonyl hydrazine (AcNH)

An amount of 1 g of AcNSCl was dissolved in 3 ml of tetrahydrofuran (THF) in a three-necked pear-shaped flask fitted with a Pasteur pipette, thermometer and dropping funnel. The mixture was cooled in an ice bath to 10°C while stirring and bubbling a gentle stream of nitrogen through it. Then 0.4 ml of an aqueous solution of 85% hydrazine hydrate was added dropwise through the dropping funnel while maintaining the temperature between 10–15°C. Stirring was continued for a further 15 min. The lower aqueous layer was drawn off and the upper THF layer was filtered through a bed of celite filter aid. The celite was washed with 1 ml THF to remove any adsorbed hydrazide. Two 10-ml portions of water were added to the THF layer while stirring vigorously. A buff flocculent precipitate resulted and the reaction mixture was cooled in a refrigerator for 1 h to complete the precipitation. The product was filtered through a Buchner funnel and washed several times with water and dried. It was then recrystallised from ethanol. Obtained was 0.95 g, 95% yield, m.p. 132–134°C. Elemental analysis: Calculated for C₁₂H₁₂N₂O₂S: C 58.06%, H 4.83%, N 11.29%, S 12.90%; found: C 58.6%, H 5.00%, N 11.31%, S 12.80%. NMR: δ /ppm 3.23 (s, 2H, NH₂), 3.45 (s, 4H, $2 \times \text{CH}_2$, H-1, H-2), 5.72 (s, 1H, NH), 7.35 (d, 1H, Ar, H-3), 7.43 (d, 1H, Ar, H-8), 7.62 (t, 1H, Ar, H-7), 8.18 (s, 1H, Ar, H-4), 8.22 (d, 1H, Ar, H-6). Mass spectrum: m/z 248 [M⁺], 153 (base peak). IR: 1140, 1323, 1610, 3330 cm⁻¹.

2.7. 5-Bromoacetyl acenaphthene

A 1-l three-necked flask was fitted with a mechanical stirrer and a dropping funnel. An

amount of 43 g acenaphthene was dissolved in 200 ml dichloroethane and 43 g bromoacetyl chloride was added to the homogenous solution. The mixture was cooled to -5°C in a freezing mixture of ice and salt. Then 38 g of aluminium chloride was added in small portions during 90 min with vigorous stirring, the temperature not being allowed to rise above 3°C . The stirrer was removed and the central neck stoppered. The side necks of the flask were respectively fitted with a capillary bleed tube and a vacuum line. The pressure was reduced for 30 min and an excess of crushed ice was added to separate the dichloroethane layer. The latter was washed successively with two 100-ml portions of dilute hydrochloric acid and 100 ml of 5% sodium carbonate solution. The dichloroethane layer was dried over magnesium sulphate, filtered and then distilled off under reduced pressure. The oily liquid obtained was transferred to a porcelain basin. After it had solidified, the top liquid layer was decanted and the remaining liquid blotted off with filter paper. The brownish coloured residue (yield 75%) contained about 50% unreacted acenaphthene as determined from the NMR spectrum. The residue was then purified by passing it through a 2×30 cm column of silica gel, 60–120 mesh, and eluting with toluene. Fractions of the eluate were examined by performing TLC on silica gel sheets impregnated with fluorescent indicator using toluene as the partitioning solvent. Unreacted acenaphthene was thus found to elute from the column first, followed by the yellow AcBr fraction. On crystallisation the AcBr gave a yield of 40%, m.p. $87\text{--}90^{\circ}\text{C}$. Elemental analysis: Calculated for $\text{C}_{14}\text{H}_{11}\text{OBr}$: C 61.09%, H 4.00%, Br 29.09%; found: C 61.50%, H 4.05%, Br 29.3%. NMR ^1H : CDCl_3/TMS , δ/ppm : 3.42 (s, 4H, $2 \times \text{CH}_2$, H-1, H-2), 4.57 (s, 2H, CH_2 , H-2), 7.32 (d, 1H, Ar, H-3), 7.39 (d, 1H, Ar, H-8), 7.63 (t, 1H, Ar, H-7), 8.08 (d, 1H, Ar, H-4), 8.68 (d, 1H, Ar, H-6); ^{13}C : CDCl_3/TMS , δ/ppm : 30.30, 30.64 (C-1 and C-2), 32.83 (C-2), 118.02 (C-3), 120.74 (C-8), 122.30 (C-6), 126.67, 129.76 (C-5 and C-5a), 130.93 (C-7), 133.01 (C-4), 139.78, 146.29, 154.47 (C-2a, C-2b and C-8a), 192.69 (C-1). Mass spectrum: m/z 277

$[\text{M}^+]$, 197 (base peak). IR (KBr) C = O: 1685 cm^{-1} .

2.8. Derivatisation of amino acids with AcNSCl and DNSCl

Solutions of 1 ml containing $20\ \mu\text{mol}$ of a single individual amino acid were dispensed into separate 10-ml volumetric flasks, and a similar amount of alanine was added to each, to act as an internal standard. The flasks were made up to volume with $0.2\ \text{M}$ sodium bicarbonate solution, pH 8.5. The resulting solution (A) was diluted 1:10 times to give solution (B). The (A) solutions were derivatised with DNSCl and the (B) solutions were derivatised with AcNSCl in the following manner. A 1-ml portion of the amino acid–alanine solution under study was pipetted into a 3-ml amber Eppendorf vial together with 1 ml of a 0.015% solution of the derivatising agent in dry acetone. After mixing the vial was capped tightly and maintained at 47°C for 30 min. The reaction was quenched by the addition of $200\ \mu\text{l}$ of 2% ethylamine and then allowed to reach room temperature. Separation of $20\ \mu\text{l}$ of the amino acid derivative solution was achieved using a Hypersil $5\ \mu\text{m}$, C_{18} , 250×4.6 mm I.D. column. A mobile-phase gradient was employed with 5% THF in 10 mM pH 4.2 acetate buffer (solvent A) and 10% THF in acetonitrile (solvent B). The gradient profile employed was 10% solvent B increasing to 40% solvent B over a 30 min period. This was maintained for a further 15 min and increased to 100% solvent B for 3 min. A flow-rate of 1 ml/min was maintained throughout. Reproducibility of these reaction conditions was determined by analysing a solution of valine containing $20\ \mu\text{mol}/\text{ml}$ to which had been added $20\ \mu\text{mol}$ alanine as internal standard. Within-run precision was determined by performing six analyses of a single sample in parallel with each other. Between-run precision was determined by performing the analysis of the same sample on six separate occasions. The fluorescent enhancement achieved with the AcNSCl reagent compared to the DNSCl reagent was determined from the ratio of the

chromatographic peak areas of the derivative peaks measured at their optimum fluorescence wavelengths and calculated for equivalent concentrations.

2.9. Peptide analysis with AcNSCl and DNSCl

Three peptides were investigated, Asp-Glu-Gly, Met-Leu-Phe and Val-Ala-Ala-Phe, at concentrations of 60 $\mu\text{g/ml}$ in water. The solutions were diluted by a factor of ten prior to analysis with AcNSCl.

N-terminal analysis

An amount of 100 μl of the peptide solution under study was lyophilised by freeze drying and 50 μl 0.2 M sodium bicarbonate solution added. Then 50 μl of a 0.5% solution of the derivatising agent in dry acetone was added and the mixture incubated at 47°C for 30 min. The reaction was quenched with 100 μl of 2% ethylamine. The mixture was then lyophilised and hydrolysed with 100 μl 6 M HCl at 105°C for 16 h in a sealed glass vial. After further lyophilisation the residue was dissolved in 100 μl of mobile phase prior to HPLC analysis as described for the analysis of amino acids.

Amino acid composition

An amount of 100 μl of the peptide solution was lyophilised, 100 μl 6 M HCl was then added and the solution hydrolysed at 105°C for 16 h in a sealed glass vial. A 50- μl portion of the hydrolysate was then lyophilised and dissolved in 15 μl 0.2 M sodium bicarbonate solution. Then 15 μl of labelling agent was added and the solution incubated at 47°C for 30 min. The reaction was quenched with 100 μl 2% ethylamine and 20 μl was subjected to HPLC analysis of constituent amino acids.

2.10. Derivatisation of 17-ketosteroids with AcNH and DNH

Solutions of progesterone (0.2 mg/ml) and TFA (2.5%) were prepared in toluene. AcNH or DNH (1 mg/ml) were prepared as solutions in ethanol–toluene (1:9, v/v). Of each of the ster-

oids 100 μl , TFA and the labelling agent were placed in an amber-coloured vial and the solvent removed under vacuum at 60°C. The residue was reconstituted in 100 μl acetonitrile and 20 μl of the resulting solution analysed by gradient elution chromatography using a Hypersil C₁₈, 5 μm , 250 \times 4.6 mm I.D. column. The flow-rate was maintained at 2 ml/min using a binary solvent mixture. Solvent A was 0.5 g/l Tris buffer, pH 7, and solvent B was 90% acetonitrile in water. A linear gradient of 50% solvent A to 70% solvent B over 20 min was used for DNH derivatives whilst the same gradient over a 10-min period was used for AcNH derivatives. To determine the effect of AcNH concentration on derivative formation, solutions of progesterone were derivatised under identical conditions except that the volume of AcNH reagent utilised was varied from 10 to 100 μl .

Fluocinolone acetonide cream was analysed by normal-phase HPLC following extraction and derivatisation. An amount of 11.25 g of Synalar Cream was transferred to a separating funnel with the aid of 100 ml of cyclohexane. Then 50 ml of methanol was added and the mixture shaken vigorously for 3 min. After standing for 15 min the lower layer was transferred to a second separating funnel containing 140 ml of distilled water and 100 ml of chloroform. The separating funnel was shaken for 3 min, the phases allowed to separate and a 3-ml portion of the chloroform placed in a screw-capped test tube containing 0.3 ml of hydrocortisone internal standard solution, 0.028% w/v in ethanol. Standard solutions of fluocinolone acetonide were prepared to cover the concentration range 0.02–1.00 mg/ml and 1 ml of each was dispensed into a screw-capped test tube containing 0.3 ml of internal standard solution. The solutions were then reacted with 1 ml of AcNH, 3 mg/ml, and derivatised following the procedure described by Chayen et al. [3] for the derivatisation of carbonyl compounds with DNH. The dried extracts were reconstituted in 200 μl of mobile phase (dioxane–toluene 10:90) prior to separation on a 500 \times 1 mm I.D., 10 μm silica microbore column. Excitation and emission wavelengths were as given in Table 1.

Table 1
Fluorescence characteristics of acenaphthene derivatives

	(DNS) Dansyl derivative		AcNSCl derivative		Fluorescence enhancement (AcN/DNS)
	λ_{ex} (nm)	λ_t (nm)	λ_{ex} (nm)	λ_t (nm)	
Alanine					27
Arginine					25
Asparagine					24
Aspartic acid					35
Cysteine					25
Glutamic acid					30
Glutamine					30
Glycine					30
Histidine					25
Isoleucine	250	470	230 (300)	420	24
Leucine					26
Lysine					20
Methionine					25
Phenylalanine					22
Proline					20
Serine					27
Threonine					37
Tryptophan					25
Tyrosine					25
Valine					27
	DNH derivative		AcNH derivative		
Progesterone	340	520	230 (300)	350	10
Fluocinolone acetonide					10
	Naphthacyl derivative		AcBr derivative		
Propionic acid					25
Hexanoic acid	22				
Ibuprofen	250 (290)	410	250 (290)	450	16
Flurbiprofen					15

2.11. Derivatisation of carboxylic acids with AcBr

A modification of the method for the analysis of short-chain fatty acids using naphthacyl bromide (NBr) was followed [4]. All solutions were prepared in acetonitrile. An amount of 100 μ l of 0.04 M AcBr (or NBr) was added to 100 μ l 0.01 M carboxylic acid sample in a 1.5-ml amber-coloured vial. Then 100 μ l 3% triethylamine and 200 μ l acetonitrile were added and the contents of the vial thoroughly mixed and heated in a water bath at 75°C for 5 min. On cooling, 500 μ l

acetonitrile was added to give a total volume of 1 ml. When using AcBr as derivatising agent it was necessary to carry out a further dilution of 1 in 15 of the AcBr derivatives in order to be able to evaluate off-range peaks. Of the resulting solution 20 μ l was subjected to HPLC analysis using a Hypersil 5 μ m, C₁₈, 250 \times 4.6 mm I.D. column and a mobile phase of 90% acetonitrile in water at a flow-rate of 1 ml/min. The effect of the molar concentration of the labelling agent on fluorescence intensity was determined over a four-fold concentration range. Excitation and emission wavelengths of the carboxylic acid

derivatives are shown in Table 1. Reproducibility of the derivatisation reaction was determined by analysing 100 μl of 0.01 M ibuprofen containing an equivalent amount of flurbiprofen as internal standard. Within-run precision was determined by performing six analyses of a single sample in parallel with each other. Between-run precision was determined by performing the analysis of the same sample on six separate occasions.

2.12. Analysis of ibuprofen in rat plasma

Serial samples of plasma were obtained from rats previously administered with 50 mg/kg ibuprofen at a localised site of inflammation. An amount of 50 μl of flurbiprofen internal standard (2 $\mu\text{g}/\text{ml}$) was added to 100 μl of plasma sample. Then 25 μl of 2 M HCl was added and the mixture vortexed for 15 s. Then 2 ml of iso-octane–2-propanol (85:15) was added and the ibuprofen extracted by rotary mixing for 5 min. The mixture was centrifuged at 3000 rpm for 10 min, after which time the upper organic layer was removed by pipette and evaporated under a stream of nitrogen at 45°C. Amounts of 25 μl of AcBr reagent (5 mg/ml in acetonitrile) and 10 μl 3% triethylamine catalyst were added to the residue and then vortex mixed for 30 s. The mixture was transferred to an amber-coloured vial and incubated at 75°C for 5 min. The solvent was evaporated under vacuum and the residue reconstituted in 25 μl acetonitrile prior to HPLC analysis using the method for carboxylic acid analysis previously described. Plasma samples from three different rats were collected over a 7 h period and the ibuprofen concentration determined by comparison with a calibration curve covering the concentration range 0–80 $\mu\text{g}/\text{ml}$.

3. Results and discussion

3.1. Synthesis of 5-substituted acenaphthenes

Aromatic sulphonation of acenaphthene proceeds via an electrophilic substitution mechanism with the production of a mixture of 3-, 5-

and 3,5-substituted sulphonic acids [6]. Under the reaction conditions employed the 5-substituted sulphonic acid was found to be the major product formed. This was confirmed by ^1H NMR spectroscopy and by confirmatory evidence of the anilide melting point [7]. AcNSCl and AcNH were prepared using the AcNSO₃H and the syntheses were confirmed by ^1H NMR and MS. Friedel–Crafts acetylations of acenaphthene have been reported to give both 5-acetyladenaphthene and 3-acetyladenaphthene [8], the proportion of the 5-isomer being predominant (98%) when the reaction is carried out in dichloroethane. The identity of the product formed in this study was confirmed as being 5-bromoacetyl acenaphthene by ^1H and ^{13}C NMR.

3.2. Fluorescence characteristics of acenaphthene and dansyl derivatives

The fluorescence excitation and emission wavelengths for the AcNSCl and DNSCl derivatives, observed in the solvent systems employed for chromatographic analysis, together with the ratio of the fluorescence intensities (AcNS/DNS) are shown in Table 1. As predicted from the relative quantum yields of acenaphthene and naphthalene, the fluorescence intensities of the acenaphthene derivatives are greater than those of the corresponding dansyl derivatives. The fluorescence enhancement observed in the amino acids when derivatised with AcNSCl is 20–37 times greater than that observed when derivatised with DNSCl. The maximum excitation and emission wavelengths are shifted to shorter wavelengths in the acenaphthene derivatives. The fluorescence intensity is a function of both the quantum yield of the parent fluorophore and the solvent composition. The fluorescence characteristics of solutions of DNSCl derivatives are dependent on the solvent being used [9]. With increasing dielectric constant of the solvent the emission wavelength is shifted to longer wavelengths and generally the quantum yield decreases. A similar effect is observed for the AcNSCl derivatives. Since the separation of amino acids is best performed using a gradient

elution system in which the dielectric constant of the mobile phase decreases with increased elution time, the fluorescence intensity of the derivatives is expected to increase at longer retention times. This effect offsets the slight loss in sensitivity expected due to the band spreading of late eluting components.

Fluorescence intensity as a function of pH was found to be constant over the pH range 1–14 for $5 \cdot 10^{-5}$ M AcNSO₃H in aqueous buffer solutions. Fig. 2 demonstrates the stability of the fluorophore to changes in pH. Protonation of the dimethylamino function at low pH reduces the fluorescent intensity of the DNSCI derivatives to that comparable with naphthalene. Due to the absence of the dimethylamino group in acenaphthene this effect is not encountered in the

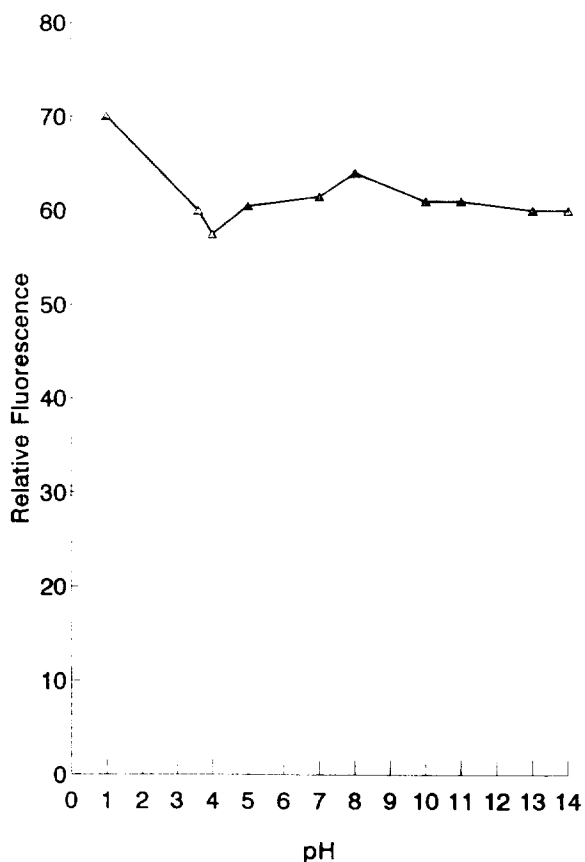


Fig. 2. Fluorescence emission of acenaphthene sulphonic acid versus pH.

AcNSCI derivatives. In strongly alkaline solution the amido group of primary-amine DNSCI derivatives ionises and causes a hypsochromic shift in the absorption maxima and of the emission maxima by about 10 nm [10]. This is also likely to occur for the AcNSCI derivatives; however, since high pHs are not normally employed for chromatographic separations, the AcNSCI derivatives are acceptable as being insensitive to pH change for practical purposes.

3.3. Rate of derivatisation reaction

The reaction between DNSCI or AcNSCI and an amino acid is bimolecular and the reaction appears second-order overall. However, if the concentration of the derivatising agent is in large excess, the reaction will become pseudo-first-order and the rate will depend upon the concentration of the amino acid A as described by the equation:

$$\ln [A] = -kt + \ln [A_0]$$

where $[A]$ is the concentration of amino acid at time t and $[A_0]$ the initial concentration of amino acid at time t_0 . Fig. 3 shows the rate of formation of AcNS-valine and DNS-valine. The first-order rate constants being 0.298 and 0.18 min⁻¹, respectively. Under these reaction conditions AcNSCI reacts 1.5 times faster than DNSCI.

3.4. Reproducibility and recovery

In a study of the dansylation reaction Tapuhi et al. [11] showed that the addition of a primary amine to the dansylation reaction mixture after 35 min quenches the reaction and eliminates the possibility of side reactions occurring and thus enhancing reproducibility. The results from reproducibility studies using acenaphthene and dansyl reagents with the amino acid valine and the arylpropionic acid ibuprofen are shown in Table 2. In both cases the acenaphthene derivatives demonstrated a slight improvement in the precision of the determinations compared with the dansyl derivatisation. For the analysis of

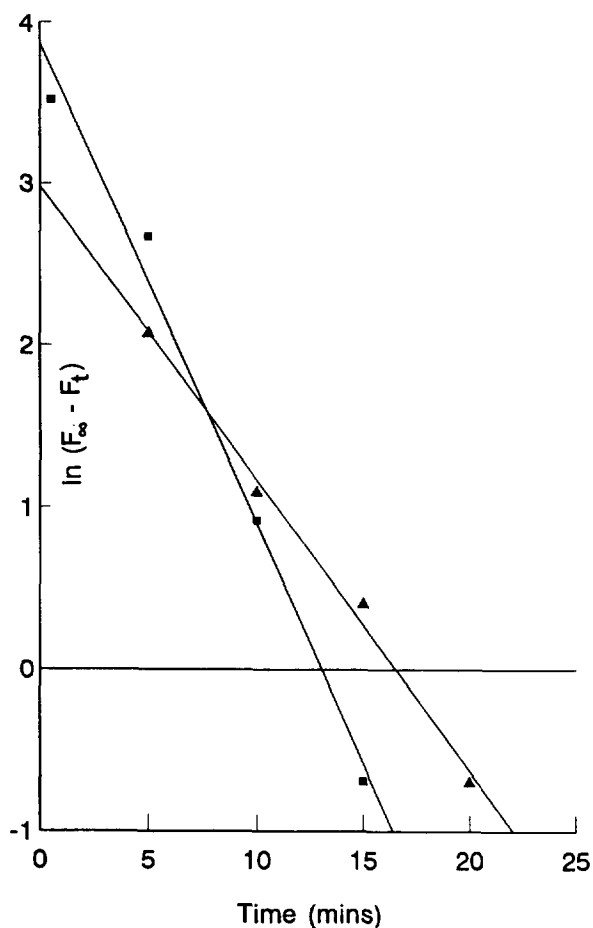


Fig. 3. Rates of formation of AcNS-valine (■) and DNS-valine (▲).

valine with AcNSCI linearity was observed over the concentration range 0–200 ng/ml.

Table 3 shows recoveries for assays performed in triplicate using each of the newly synthesised

acenaphthene derivatives. In each case the amount of analyte recovered was within 2% of the amount of analyte added. These results demonstrate an acceptable level of precision and accuracy for the derivatisation procedures using these derivatisation reagents.

3.5. Amino acid and peptide analysis

Fig. 4 shows the chromatographic separation of a mixture of nine amino acids together with the chromatogram for the blank. After the reagent hydrolysis product has eluted from the column at 5 min the baseline is clear of interference and is ideally suited to the analysis of amino acid mixtures. Relative to the corresponding DNSCI derivatives the AcNSCI derivatives have retention times which are approximately 1.2 times longer due to the greater hydrophobicity of the acenaphthene nucleus.

Five peptides were hydrolysed according to the method described in the Experimental section and the resulting solutions derivatised with AcNSCI and DNSCI. The peptides studied were Asp-Glu-Gly, Met-Leu-Phe, Val-Ala-Ala-Phe, Val-Gly-Asp-Glu and Val-Gly-Ser-Glu. In all cases the constituent amino acids were identified from the chromatograms obtained using both derivatising reagents. N-terminal analysis was performed by derivatising the peptides prior to hydrolysis, again the terminal amino acids were identified in each case from the chromatograph. Fig. 5 demonstrates the analysis of Val-Gly-Ser-Glu by this method. Val-AcNS was found to give a 100% recovery when subjected to the acid hydrolysis procedure, indicating that the AcNSCI

Table 2
Reproducibility studies of acenaphthene derivatives

	Amount injected on-column (pg)	Within-run precision (C.V.%)	Between-run precision (C.V.%)
AcNS-valine	10.6	1.48	4.37
DNS-valine	106	2.33	5.13
AcBr-ibuprofen	9.7	0.62	2.90
NBr-ibuprofen	97	3.25	2.00

Using peak-height ratios, $n = 6$.

Table 3
Recoveries of analytes following derivatisation

Sample	Derivatising agent	Amount added	Amount found	Recovery ^a (%)
Valine in buffer (ng/ml)	AcNSCl	100	104	104.0 <i>n</i> = 6
Fluocinolone in cream (mg/100 g)	AcNH	25	25.3	101.5 <i>n</i> = 3
Ibuprofen in plasma (ng/ml)	AcBr	100	102	102.0 <i>n</i> = 6

^a *n* = number of samples analysed.

amino acid derivatives are stable to hydrolysis and could be used in qualitative amino acid analysis of protein hydrolysates. It should be noted that Gray [12] found that DNS-serine and

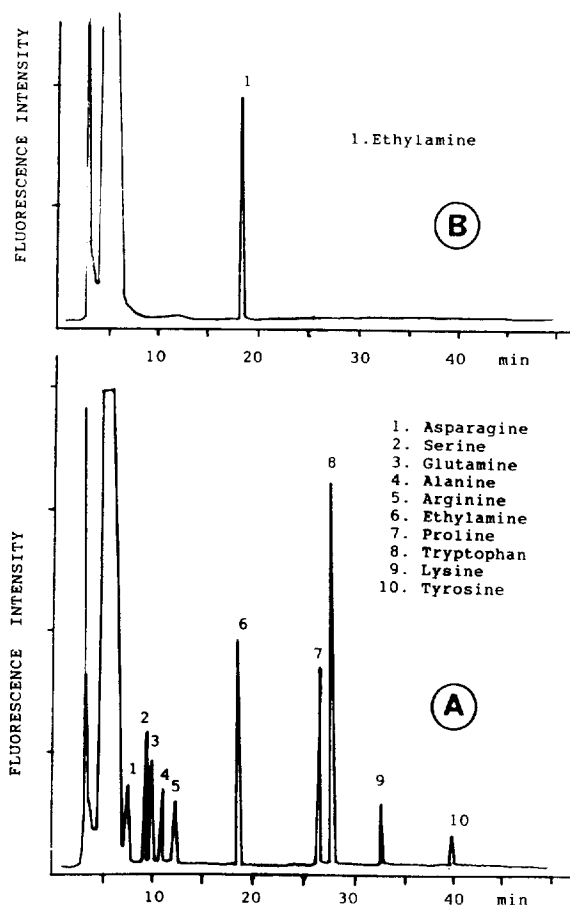


Fig. 4. (A) Chromatogram of a mixture of 1.25 nM of each of nine AcNS-amino acids. (B) Reagent blank.

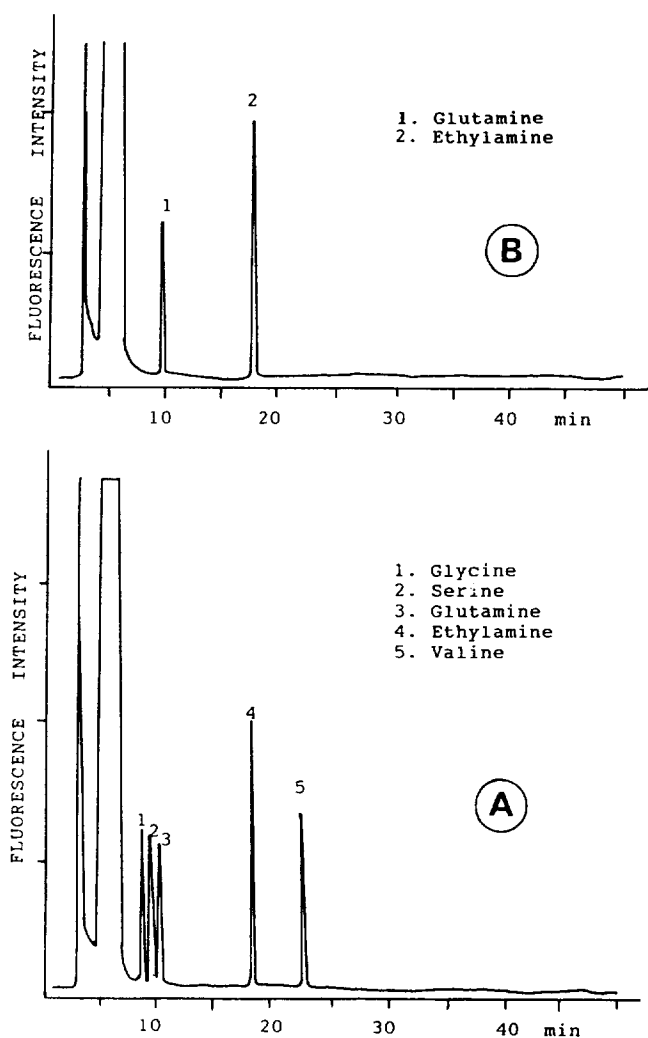


Fig. 5. (A) Chromatogram of constituent amino acids of 0.3 nM of the peptide Val-Gly-Ser-Glu following acid hydrolysis. (B) Chromatogram of terminal amino acid only.

DNS-proline did not give complete recovery when subjected to acid hydrolysis, and care should therefore be taken in quantitative determinations.

3.6. Steroid analysis

Steroids incorporating two carbonyl groups in the structure, such as progesterone, have been shown to form a mixture of mono- and bis-derivatives on reaction with DNH which can be individually identified by HPLC [13]. The relative amounts of each of the mono- and bis-derivatives is dependent on the concentration of DNH used. Fig. 6 shows the ratio of bis/mono formed over the concentration range 0.1–1.0 mg of AcNH used. This represents approximately 0.5–5.0 times molar excess of reagent. Under the reaction conditions used complete conversion to

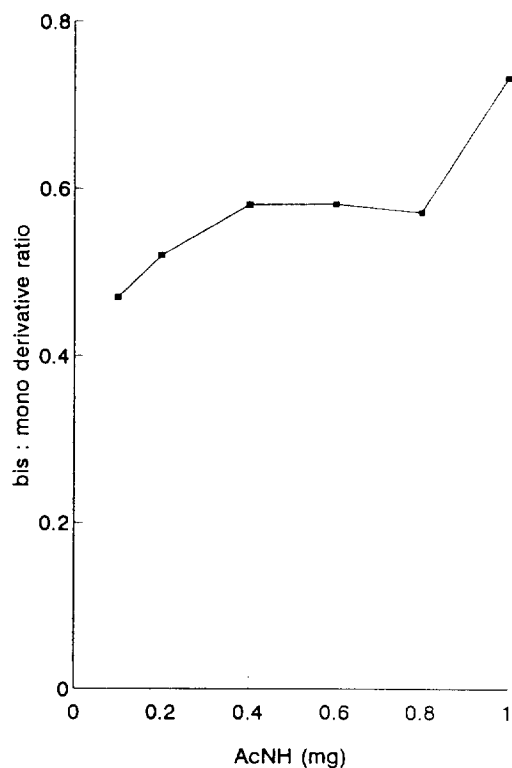


Fig. 6. Variation in the amounts of bis- and mono-derivatives of progesterone with increasing amounts of AcNH derivatising agent in the reaction mixture.

the bis-derivative was not achieved even in the presence of excess reagent.

Trace amounts of ketosteroids have been determined by reaction of the keto group with DNH to form highly fluorescent hydrazones [3]. The conjugate ketone Δ^4 -3-one reacts in the cold whereas the 17-ketosteroid requires an elevated temperature. The derivatisation of flucinolone acetonide with AcNH was performed in a boiling water bath for a period of 10 min. Chromatography of the resulting reaction mixture using a 15-fold molar excess of AcNH produced only a single peak for the bis component in contrast to the mixed mono and bis product at lower reagent concentrations. Linearity was observed over the concentration range 0–500 ng on column. The sample was analysed in triplicate and found to contain 0.0253% active ingredient, representing 101.5% of the label claim and validating this method.

3.7. Carboxylic acid analysis

2-Naphthacyl bromide is a fluorescent label used in the analysis of carboxylic acids [4]. The analogous compound 5-acenaphthacyl bromide was found to produce some 15–20 times more fluorescence when used to derivatise carboxylic acids than the naphthacyl derivative, see Table 1. On-column limits of detection for the short-chain carboxylic acids propanoic and hexanoic acid and the arylpropanoic acids ibuprofen and flurbiprofen were found to be 0.5 and 2.5 pmol, respectively. For the analysis of ibuprofen linearity was observed over the concentration range 0–200 ng/ml and the within- and between-run coefficients of variation were 0.62% and 2.9%, respectively.

The AcN-Br reagent was used to determine the concentrations of ibuprofen in rat plasma following regional administration of the drug to an inflamed site [14]. The levels of drug circulating in the plasma, following this method of delivery, were expected to be low and thus provide a suitable test for the sensitivity of the method. The precision of the method was found to be acceptable and was used to study the pharmacokinetic profile of ibuprofen. A typical

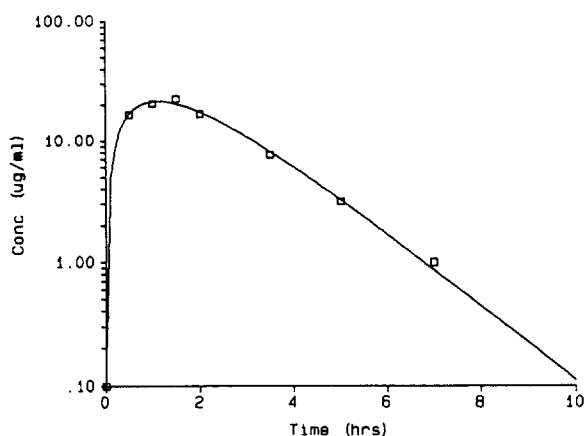


Fig. 7. Concentration–time profile of ibuprofen in rat plasma following administration of the drug at a regional site of delivery.

concentration–time profile is shown in Fig. 7. The mean half life from three subjects was determined as 1.57 h, compared with a value of 1.65 h determined at higher levels by HPLC–UV analysis [14].

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