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RAPID, SENSITIVE AND SPECIFIC DERIVATIZATION METHODS WITH 9-(HYDROXYMETHYL)ANTHRACENE FOR THE FLUORIMETRIC DETECTION OF CARBOXYLIC ACIDS PRIOR TO REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION

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SUMMARY

Three derivatization procedures are described for the pre-column fluorescence labelling of carboxylic acids. The methods are based on esterification with 9-(hydroxymethyl)anthracene. The carboxylic acid function is activated with 2-bromo-1-methylpyridinium iodide, N,N'-carbonyldiimidazole or N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride, respectively. Benzoic acid was completely converted into the corresponding ester with all three methods. About 100 fmol of the acid could be detected after high-performance liquid chromatographic analysis of the derivative. The methods are well suited for the analysis of carboxylic acids in plasma.

INTRODUCTION

A number of derivatization methods have recently been described for the fluorescence labelling of compounds with a carboxylic acid function, in order to enhance the fluorescence sensitivity of these compounds in high-performance liquid chromatography (HPLC)[1–10]. The fluorogenic reagents are usually derived from polyaromatic hydrocarbons, *e.g.,* anthryldiazomethane[2] and bromoacetylpyrene[4], or from 7-methoxycoumarin[3,7,9,10], as, *e.g.,* 4-bromo-7-methoxycoumarin[3]. The derivatization reactions are frequently non-specific; other functional groups, such as phenols, thiols and imides, are also converted if the reactions are carried out with alkyl halides[5,8] or diazomethyl derivatives[2,9].

In the present study the application of three derivatization reactions for the sensitive detection of compounds with a carboxylic acid function in HPLC analysis is investigated. All the methods are based on the formation of an ester by coupling the acid with 9-(hydroxymethyl)anthracene (HMA).

EXPERIMENTAL

Materials

9-(Hydroxymethyl)anthracene (HMA), N,N'-carbonyldiimidazole (CDI) and

N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (DAC) were obtained from Fluka (Buchs, Switzerland), 1-hydroxybenzotriazole (HOBT), anthracene (gold label grade), 2-bromopyridine and methyl iodide from Janssen Chimica (Beerse, Belgium), and triethylamine and benzoic acid from E. Merck (Darmstadt, F.R.G.). Ibuprofen was kindly supplied by The Boots Company (Vianen, The Netherlands). Acetone, chloroform, dichloromethane, diethyl ether, hexane and methanol were purchased from J. T. Baker Chemicals (Deventer, The Netherlands). All these reagents and solvents were of analytical reagent grade. The solvents were further purified by distillation from glass. Anthracene and 9-(hydroxymethyl)anthracene (HMA) were recrystallized twice from absolute ethanol and stored over phosphorus pentoxide. The other compounds used came from various sources and were used as such.

2-Bromo-1-methylpyridinium iodide (BMP) was synthesized following the procedure described by Saigo *et al.*[11] for the synthesis of 2-chloro-1-methylpyridinium iodide. The ester (BAMA) of HMA and benzoic acid was synthesized on a preparative scale by the carbodiimide method described below. Purification of the reaction product was performed by preparative liquid chromatography and by twice recrystallizing the resulting product from ethanol. The product was stored over phosphorus pentoxide. Its identity was confirmed by IR spectroscopy and by mass spectrometry.

Instrumentation

HPLC. The apparatus consisted of a Model 6000 A solvent delivery system and a U6K injector (Waters Assoc., Milford, MA, U.S.A.), equipped with a Perkin-Elmer Model 650 fluorescence detector (excitation wavelength 365 nm, emission wavelength 415 nm, slit width 10 nm). A 30 cm × 1.9 mm I.D. steel column, slurry packed with LiChrosorb RP-18 (10-μm particles; E. Merck) was used under ambient conditions. A methanol–water mixture (90:10, w/w) was deaerated ultrasonically and employed as the mobile phase at a flow-rate of 1 ml/min.

Fluorimetry and UV absorbance. Excitation and emission spectra were obtained with a Perkin-Elmer Model 204 fluorescence spectrophotometer. Fluorescence quantum yields were determined with reference to anthracene in ethanol at ambient temperature[12,13] by comparison of the areas under the curve of the emission spectra of anthracene with those of the derivatives, after correction for the absorbances of the compounds under investigation (all other parameters kept constant). To avoid collisional quenching and inner-filter effects, the absorbance of the solutions of which the fluorescence was measured was kept below 0.01 absorbance units.

UV absorption spectra were recorded on a Shimadzu UV-200 spectrophotometer. Molar absorptivities were determined from UV absorbance measurements with a Perkin-Elmer Model 139 UV-Vis spectrophotometer.

Thin-layer chromatography (TLC). Thin-layer plates (E. Merck) of 5 × 10 cm and 20 × 20 cm, precoated with silica gel 60F and having a layer thickness of 0.25 mm, were used. Reaction mixtures originally containing 0.1 μg/μl acid were analyzed by spotting an aliquot of the mixture on a plate together with solutions of the acid under investigation and the reagents. The plates were developed in the ascending mode with eluents consisting of chloroform–methanol or chloroform–hexane mixtures. After evaporation of the eluent the spots were visualized under UV irradiation of 254 nm and 365 nm.

Derivatization procedures

The reactions were performed in stoppered 1.5-ml polypropylene tubes. The residues obtained after evaporation were dissolved in 1 ml HPLC eluent; 10-μl portions of the resulting solutions were injected into the chromatograph.

BMP method. To a solution of 0.01–1.0 μg benzoic acid in 100 μl dichloromethane, 1 mg BMP, 1 mg HMA and 10 μl triethylamine were added. After vortex mixing for 15 sec the mixture was allowed to stand at room temperature for 30 min and evaporated to dryness under a stream of nitrogen.

CDI method. A mixture of 0.01–1.0 μg benzoic acid in 10 μl chloroform and 3 mg CDI was kept at 25°C for 5 min. Then 1 μl of 4 M hydrochloric acid was added and the mixture was heated at 60°C for 30 sec. After addition of 0.5 mg HMA in 50 μl chloroform, vortex mixing for 15 sec and heating at 60°C for 20 min, the mixture was evaporated to dryness under a stream of nitrogen.

DAC method. To 0.01–1.0 μg benzoic acid in 10 μl chloroform a solution of 5 μg HOBT in 100 μl chloroform was added. After evaporation to dryness under nitrogen, 30 μg DAC in 30 μl chloroform and 5 μl triethylamine were added. The resulting solution was kept at 0°C for 30 min. Then a solution of 20 μg HMA in 100 μl chloroform was added; the mixture was allowed to stand at room temperature for 10 min and was then evaporated to dryness under a stream of nitrogen.

RESULTS AND DISCUSSION

Derivatization procedure

Benzoic acid was used as test compound in the derivatization studies with HMA. The three derivatization procedures are based upon activation of the carboxylic acid function with BMP, CDI or DAC. Without activating either the carboxylic acid or the alcohol (reagent), only minute amounts of the esters are formed under these mild reaction conditions. In the case of the BMP and the DAC methods the reaction mixture is made alkaline by the addition of triethylamine; with the CDI method no base is needed.

The BMP method as described above was developed by us, based on the work of Saigo *et al.*[11]. Contrary to the CDI and DAC methods, the BMP reaction could be carried out in one step. At reaction temperatures higher than 25°C the reaction yield decreases. The BMP reaction could also be performed at 5°C with satisfactory results.

The use of CDI and DAC as activators for the esterification of carboxylic acids with alcohols is well documented. Both types of compounds have been applied in the fluorescence or UV labelling of carboxylic acids prior to HPLC analysis[1,14]. HOBT has been added as a catalyst in the DAC method[1]. The derivatizations with CDI and DAC are, in our experience, best conducted in two separate steps. First the carboxylic acid is treated with the activator at relatively low temperatures. Then HMA is added and the esterification is accomplished at higher temperatures. Lower yields were obtained when the reactions were run in one step at temperatures higher than those used during the first step of the final procedure (0°C for the DAC method; 25°C for the CDI method). Possibly, the compounds formed from benzoic acid and the activator are unstable at higher temperatures. In the CDI method the excess of CDI had to be destroyed, with hydrochloric acid, prior to the second reaction step, to prevent side reactions.

TABLE I

DERIVATIZATION OF DIFFERENT ACIDIC COMPOUNDS

+, Reaction is quantitative; +/−, reaction takes place, but is not quantitative; −, reaction does not take place.

Compound	Method		Compound	Method	
	DAC	CDI		DAC	CDI
Aliphatic carboxylic acids			*Aliphatic carboxylic acids*		
Acetic acid	+	+	Diclofenac	+	−
Lauric acid	+	+	Ibuprofen	+	+
Myristic acid	+	+	Indomethacin	+/−	+/−
Sorbic acid	+	+	Phenylacetic acid	+	+/−
Stearic acid	+	+			
Aromatic carboxylic acids			*Carboxylic with other acidic functions*		
Benzoic acid	+	+	Acetylsalicylic acid	+/−	−
Nalidixic acid	+/−	−	Aminocaproic acid	+/−	+
Nicotinic acid	+/−	−	Mandelic acid	+/−	+
Phthalic acid	−	+	Salicylic acid	−	+
Probenecid	+	+	p-Sulphamoylbenzoic acid	−	+/−
Other (than carboxylic acidic) functions					
Barbituric acid	−	−			
Mercaptopurine	−	−			
Phenol	−	−			
Purine	−	−			
Sulphanilic acid	−	−			

The derivatization yield was measured by comparison of the peak height of the ester (BAMA) after HPLC analysis of the reaction mixture and a solution of the purified ester, respectively. With all three procedures, 100% conversion of benzoic acid into BAMA was achieved.

A number of other compounds with carboxylic acid functions and some compounds with other acidic groups were tested with the CDI and the DAC methods. The reaction mixtures were investigated by TLC analysis. The appearance of one new, fluorescent spot in the chromatograms and the disappearance of the acid spot was taken as evidence for conversion of the acid under investigation into the corresponding ester. The complete disappearance of the acid spot from the chromatogram indicated that at least 80–90% of the acid had been converted. Not all of the carboxylic acids thus tested were completely esterified, but all were derivatized at least to some degree in one or both procedures. The results are summarized in Table I. The compounds with other acidic groups were not derivatized to any detectable degree with either of the two procedures (Table I). Similar results were obtained with a few compounds after derivatization with the BMP method.

HPLC analysis

Representative chromatograms from the HPLC analysis of the derivatization mixtures obtained with the DAC method are shown in Fig. 1. The lipophilicity of BAMA and the choice of the stationary phase —silica gel modified by the introduc-
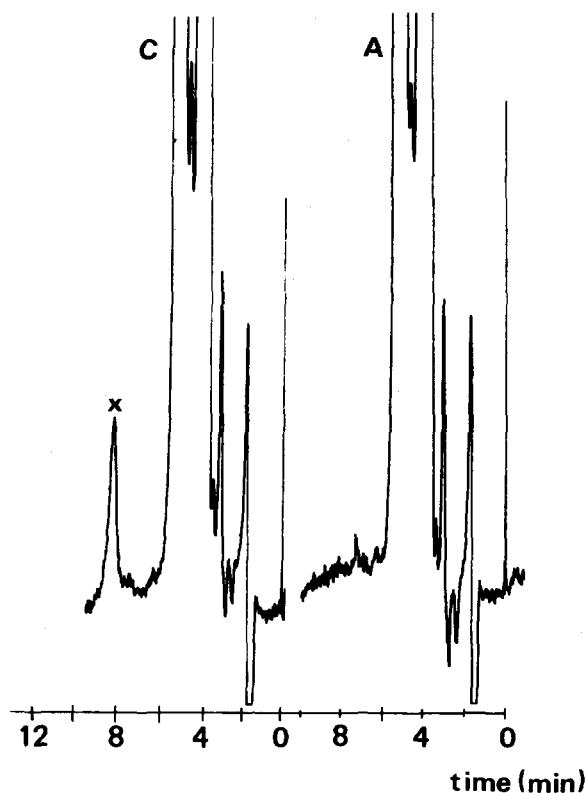
Fig. 1. Chromatograms obtained after derivatization of benzoic acid with HMA (DAC method): A, blank chromatogram; C, derivatization of 0.01 μg benzoic acid; × = MABA.

tion of octadecyl groups— makes it necessary to use a methanol–water mixture with a high methanol content (90%) in order to achieve reasonable retention times. Better selectivity is usually obtained with eluents of higher water contents. However, the advantage of a high methanol concentration in the eluent is that HMA and many other fluorescence labels (and their derivatives) with $\pi-\pi^*$ transitions show better fluorescence sensitivity under these conditions (see below). In the case of very lipophilic derivatives the retention times may become too long even with pure methanol

TABLE II

UV ABSORPTION AND FLUORESCENCE DATA OF ANTHRACENE AND SOME DERIVATIVES

Compound	Solvent	A (365 nm)	Q_F	BW (cm^{-1})	IFS
Anthracene	Ethanol	1820	0.30*	1365	0.40
Anthracene	Methanol	1829	0.29	1375	0.39
9-Hydroxymethylanthracene	Methanol	7474	0.05	1860	0.20
9-Hydroxymethylanthracenebenzoate	Methanol	8213	0.10	1680	0.49

* Taken from ref. 12.

as the mobile phase, necessitating the use of a less apolar stationary phase. No improvement in detection sensitivity was observed when the mobile phase was deoxygenated by flushing with nitrogen gas, as compared with the ultrasonic treatment of the eluent.

Calibration curves obtained with all three methods showed good linearity, *e.g.*, $y = (0.28 \pm 0.30)x + 8.75 \pm 0.40$ ($r = 0.998$) was the equation for the calibration line after analyzing ten samples containing 0.1–1.0 μg benzoic acid according to the CDI method. The errors are the standard deviations. Under the prevailing conditions the detection limit for BAMA was found to be 100 fmol (signal-to-noise ratio 3).

Fluorescence sensitivity

The detection sensitivity of a fluorescing compound is determined not only by its retention behaviour (k' and peak shape) and by instrumental factors, but also by its molar absorptivity, A, at the excitation wavelength, its quantum yield of fluorescence, Q_F, and, inversely, the width of the emission band (BW) at half peak height.

The intrinsic fluorescence sensitivity (IFS) is a suitable parameter for the evaluation of the detection sensitivity of labelled compounds[12,15]

$$\mathrm{IFS} = Q_F A/\mathrm{BW}$$

in which BW is expressed in wavenumbers (cm^{-1}). Values of A, Q_F and IFS of HMA, BAMA and anthracene are presented in Table II. It is of interest that the Q_F value of the labelled ester (BAMA) is about one third of that of anthracene, and that the
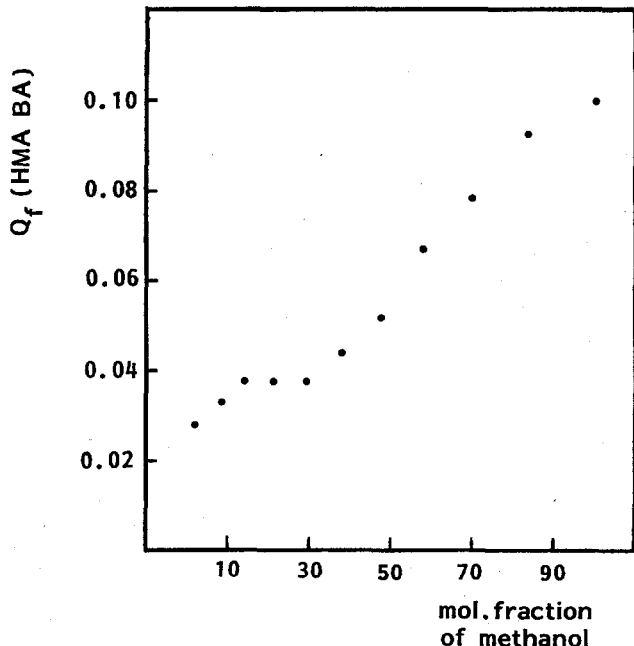

Fig. 2. Quantum yield of fluorescence of BAMA *versus* the molar fraction of methanol in water–methanol mixtures.

IFS value of BAMA is nevertheless larger than that of anthracene due to the much higher molar absorptivity of BAMA. The IFS value of BAMA in methanol is 0.49, which is of the same order of magnitude as those of the fatty acid esters of 4-hydroxymethyl-7-methoxycoumarin in methanol–water mixtures[15].

The fluorescence quantum yield of many compounds is strongly dependent on the type of solvent in which they are dissolved. Q_F values of BAMA at different molar fractions of methanol in methanol–water mixtures are shown in Fig. 2. As expected, Q_F decreases with increasing water content of the mixture. As the values of A and BW are not seriously affected by the water content, the IFS values for BAMA also drop with increasing water content. This opposite to the situation with the 4-hydroxymethyl-7-methoxycoumarin esters, which are best detected in eluents containing a high concentration of water[15]. HMA-labelled compounds are lipophilic in nature and will therefore require comparatively high methanol concentrations in the eluent for HPLC analysis. The sensitivity of detection for HMA esters is therefore close to that attained under optimum conditions.

The derivatization procedures can also be used for the coupling of carboxylic acids with fluorescence labels carrying an amine group. This was tested with benzoic acid as the sample and 1-naphthylmethylamine and N-naphthylethylenediamine as fluorescence labels. Although complete derivatization was achieved, the resulting amides showed comparatively poor IFS values (results not shown). A search for amines which will provide better sensitivity is presently underway in our laboratory.
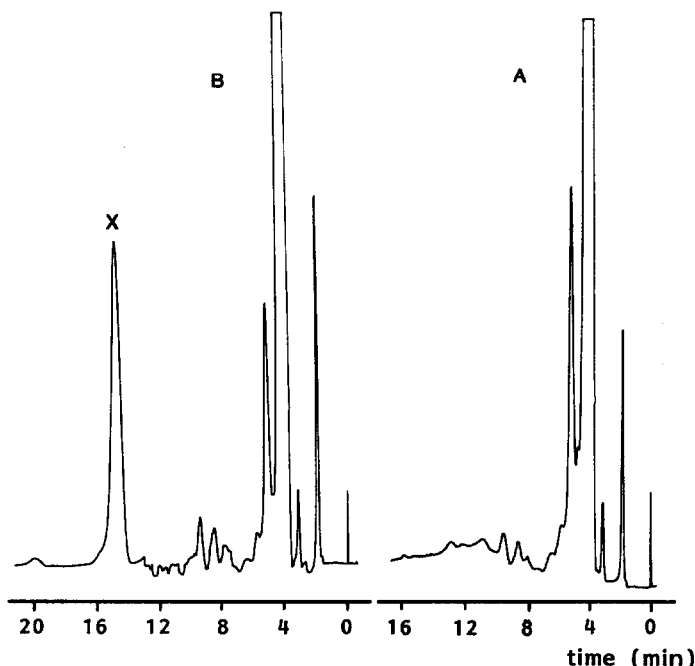

Fig. 3. Chromatograms obtained after derivatization of ibuprofen with HMA after extraction from plasma (DAC method): A, blank chromatogram; B, derivatization of 1 μg ibuprofen; × = Ester of HMA and ibuprofen.

Application to the analysis of ibuprofen in plasma

The potential of the derivatization with HMA for the analysis of carboxylic acids in blood plasma was investigated with ibuprofen, an analgesic anti-inflammatory drug.

A plasma sample of 150 μl, containing 1 μg of ibuprofen, was acidified by vortex mixing with 10 μl 4 *M* hydrochloric acid and then extracted with 1 ml of dichloromethane. After 5-min centrifugation (2500 *g*) the aqueous layer was discarded; the dichloromethane layer was transferred to a clean vial and evaporated under a stream of nitrogen. The residue was treated as described in Experimental following the DAC method. Ten microlitres of the final solution were subjected to HPLC analysis; a blank plasma sample was treated in the same way. The resulting chromatograms are shown in Fig. 3. The ibuprofen derivative peak is well separated from the reagent blank peaks and the peak due to plasma constituents.

ACKNOWLEDGEMENTS

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