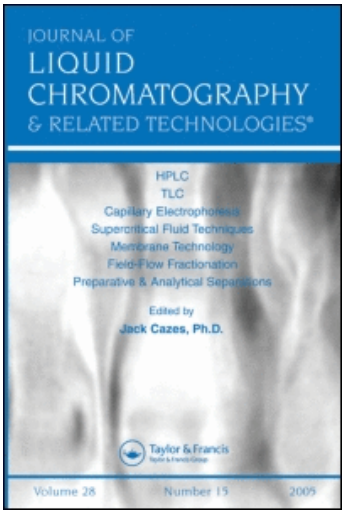

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Anthracene Isocyanate as a New Fluorescent Label for Compounds with an Alcoholic Group

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ANTHRACENE ISOCYANATE AS A NEW FLUORESCENT LABEL FOR COMPOUNDS WITH AN ALCOHOLIC GROUP.

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ABSTRACT

Anthracene isocyanate is used as a new fluoresceing reagent for the derivatization of compounds with an alcoholic hydroxyl. The reactivity of anthracene isocyanate and the fluorescence intensity of the resulting derivatives are compared with naphthyl isocyanate and the naphthylurethanes, respectively. Although the reactivity of anthracene isocyanate is only insignificantly lower, the formation of the anthracenecarbamicacidesters with primary and secondary alcohols may also be performed in the same way as the reaction with naphthyl isocyanate in 30 minutes at 95°C in xylene or toluene. The derivatization of substances possessing a tertiary alcoholic group is achieved at 140°C in xylene within 2 hours. The method shows good reproducibility and high sensitivity. The detection limits obtained with the anthraceneurethanes are in the low picomole range and are, in some cases, better by a factor of 10 than those of the naphthylurethanes.

INTRODUCTION

Compounds featuring an alcoholic component as sole functional group in the molecule are widely applied in chemistry and chemistry related sciences. For analyzing such compounds fluorimetric methods are applied, which

can be used in most cases only for single substances or groups of substances [1-11]. Moreover they often present poor reproducibility or too little sensitivity. By employing naphthyl isocyanate (NI) as a reagent for derivatization a wide range of structural as well as strongly differing alcohols were estimated [12]. Thereby the fluorescence of NI is transmitted to the total molecule and this is evaluated fluorodensitometrically.

It is known that the fluorescence intensity arises certainly with an increasing number of aromatics but at the same time the reactivity of the corresponding isocyanates decreases. Therefore, it seems worthwhile to find out whether the reactivity of the higher condensed aromatics guarantees a sufficient derivative-formation and how far the higher fluorescence quantum yield of these compounds is related to an enhancement of the fluorescence intensity of the resulting urethanes. This present paper describes the application of anthracene isocyanate (AI) for the quantitative determination of substances with an alcoholic group.

EXPERIMENTAL

Instrumentation:

A Perkin Elmer MPF 44 Spectrofluorimeter with TLC accessory was used for fluorescence measurements. The

peak areas were recorded with a Perkin Elmer recorder 56 and integrated with the Perkin Elmer Minigrator M2. Measurement mode: ratio. The solutions were applied to the plates with 2 µl capillaries (Drummond Scientific).

Reagents and syntheses:

Anthracene-2-isocyanate was synthesized essentially by the method of Fieser and Creech [18]. Chrysene-6-isocyanate was synthesized by the reaction of chrysene-6-amine with phosgene: 1.5 g of the chryseneamine - purified by recrystallization with benzene - were dissolved at a temperature of 70°C in 70 ml benzene dried with a molecular sieve. To the warm solution 37 g phosgene, 20% in toluene (Merck, Darmstadt, F.R.G.) was gradually added while stirring in a nitrogen atmosphere. After refluxing for 90 minutes the brown precipitate of the amine hydrochloride was filtered and the solution was evaporated under reduced pressure in a nitrogen atmosphere. Several recrystalizations from benzene and carbon tetrachloride gave 0,85 g chrysene-6-isocyanate with a m.p. of 155°C-156°C.

For quantitative experiments the urethanes formed by the reaction of 2-propanol with phenyl isocyanate, naphthyl isocyanate and anthracene isocyanate were synthesized [13,14]. The urethanes formed by the reactions of anthracene isocyanate with n-butanol,

cetanol, cholesterol and 1-phenylethanol were synthesized in the same way as the napthylurethanes [15-17].

Synthesis of chrysene-6-carbamidoacidisopropyl-ester: 0,25 g chrysene-6-isocyanate were disolved in 5 ml molecular-sieved benzene and mixed with 0,043 ml of an absolute isopropanol. After heating for an hour at 70°C, the precipitate formed in the cold was recrystallized in carbon tetrachloride. 0,09 g of the urethane was obtained. M.p.: 237°C.

The catalyst 1,4-Diazabicyclo[2,2,2]octane and n-butanol were purchased from Merck (Darmstadt, F.R.G.) and cetanol, cholesterol, codeine from Herba AG (Graz, Austria). The steroids were obtained from Chemie Linz AG (Linz, Austria), Ciba-Geigy (Basel, Switzerland), Schering Wien GmbH, Hoechst Austria, Pfizer Corp. Austria as well as Werfft-Chemie (all Vienna, Austria).

Chromatography:

The fluorodensitometric analyses were performed with thin-layer plates MN SilG with fluorescence indicator, 20x20 cm, from Macherey-Nagel (Düren, F.R.G.). The chromatograms were developed in benzene-ether (95+5) or in chloroform-benzene-ethanol (45+15+5). The used solvents were of an analytical grade.

For comparing fluorescence intensities 192 mg of the phenylurethane, 230 mg of the naphthylurethane,

292 mg of the anthraceneurethane and 330 mg of the chryseneurethane - in each case formed with isopropanol - were dissolved in dichloromethane and diluted 1:1000.

General procedure

For determining an unknown concentration of a compound with a primary, secondary or tertiary hydroxyl group 10 µl of the sample solution in xylene or toluene containing not more than 25 µg of the substance are pipetted in a conical vial. After adding an equimolar amount of triethylenediamine in 5 µl xylene or toluene and a 30-fold excess of a hot solution of anthracene isocyanate in 80 µl xylene or toluene the reaction mixture is heated in a drying oven for 30 minutes at 95°C in the case of primary and secondary alcohols and for 2 hours at 140°C in the case of tertiary alcohols. Then a 2-fold molar amount of diethylamine in 5 µl xylene is put to the still hot solution. The reaction mixture is shaken shortly and when cold, is centrifugated at 3000 rpm. 2 µl from the supernatent clear solution are spotted on the thin-layer plate and the chromatogram is developed in the appropriate solvent-system. After dipping in a saturated solution of polyethylene glycol 4000 in methanol the fluoresceing derivatives are measured at their maxima.

RESULTS AND DISCUSSION

The fluorescence quantum efficiency represents a characteristic magnitude, which is of great importance, above all with regard to the desired sensitivity of identification. If the ΦF-value reaches 0,29 for naphthalene and 0,46 for anthracene then it is twice as high for tetracene as for naphthalene. From this fact it can be seen that an increase of the fluorescence intensity and therewith an improvement of the detection limits is possible by using isocyanates with a greater aromatic adduct. In fig. 1 the fluorescence signals of solutions of equimolar concentrations of the phenyl-, naphthyl-, anthracene- and chrysenecarbamic-acidester of the n-propanol are compared. The measurement was performed in dichloromethane. As expected, an obvious rise of the fluorescence behaviour from phenylurethane to naphthylurethane then to anthraceneurethane can be seen, while the corresponding chryseneurethane, in spite of having a greater number of aromatics, shows even slighter fluorescence. On the other hand, the more highly condensed aromatic system makes one expect a reduction of the reactivity compared to the NI. Phenylisocyanate (PI) already reacts with primary and secondary alcohols at roomtemperature, whereas by employing NI as a reagent, in most cases, heating is

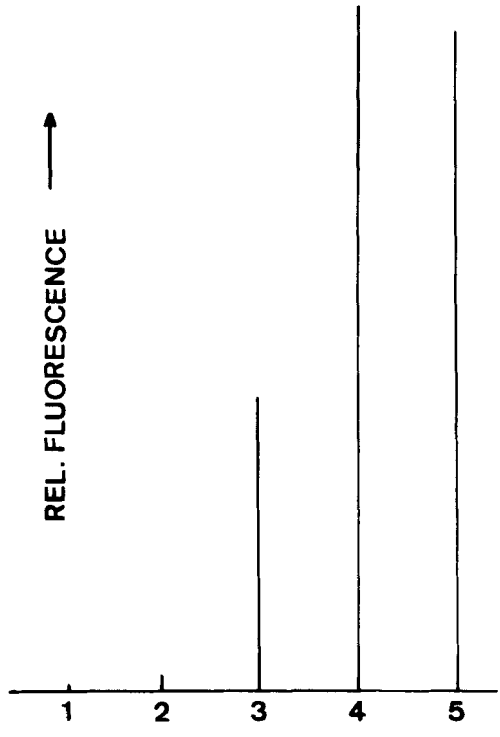

FIGURE 1

Values of measurement of 10^{-5} molar solutions of urethanes with isopropanol. 1 Blank, 2 phenylurethane, 3 naphthylurethane, 4 anthraceneurethane, 5 chryseneurethane.

necessary. This fact required long reaction times by derivating with AI. As fig. 2 shows, by means of the reaction of cetanol as well as with NI and with A-Iso differences of the reactivity can be observed. When catalyzing with triethylenediamine a quantitative formation of the naphthylurethane is attained after

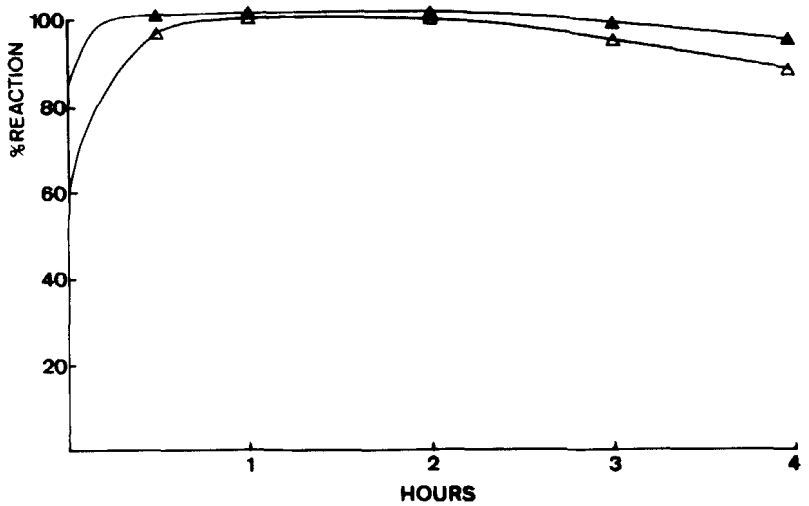

FIGURE 2

Kinetics of cetanol with isocyanate reagents at 95°C in toluene. ▲ with AI, △ with NI.

10 minutes, whereas the reaction of cetanol with AI is completed at 100%, after 30 minutes.

The derivatization of the secondary alcohol cholesterol happens with smaller reaction velocities than with the primary hydroxy compounds and it shows a different characterization of the kinetics too (fig. 3). The N-naphthylcarbamicacidester is already constituted quantitatively after 20 minutes and the N-anthracenecarbamicacidester after 50 minutes. The reaction of trihexyphenidyl, a tertiary alcohol, is considerably slower at 140°C with AI than with NI.

After 3 hours reaction time about 53% of the fluorescence of the naphthylcarbamicacidester are attained with the anthracenecarbamicacidester.

For the checking of the quantity of the conversion the urethanes with isopropanol, n-butanol, 2-butanol, cetanol, cholesterol and 1-phenylethanol were synthesized as described [15-17]. In all the other cases the percent values should be refered to the highest fluorescence intensities which can be obtained and which are ascertained according to the kinetics. The synthesis of the chrysene-6-isocyanate and of the urethane of chrysene-6-isocyanate with n-propanol is not described

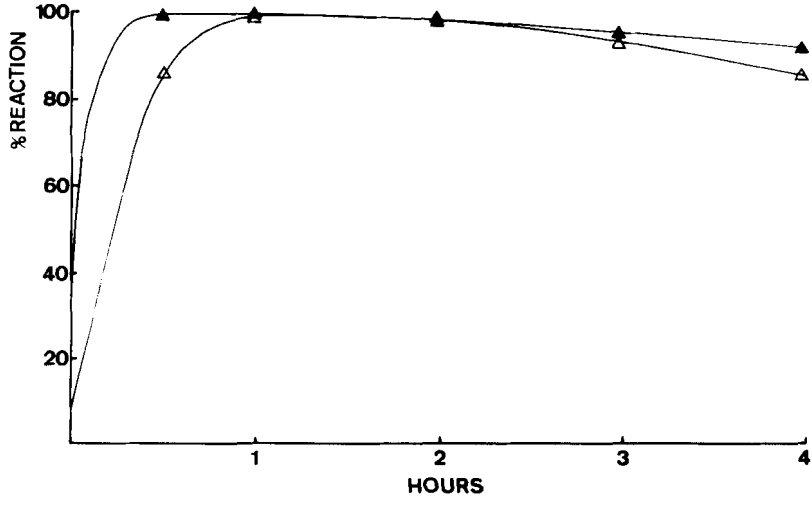

FIGURE 3

Kinetics of cholesterol with isocyanate reagents at 95°C in toluene. ▲ with AI, △ with NI.

in the literature. Chrysene-6-isocyanate was synthesized by minor modifications of the method from Fieser and Creech described for AI [18].

Another aspect of the investigations is related to the transferability of the excess of the reagent chosen for NI to the derivatization with AI. For this purpose cetanol was converted with a 50-, 30-, 15- and 5-fold molar excess of AI at 95°C by catalyzing with triethylenediamine (fig. 4). The best reaction rate is

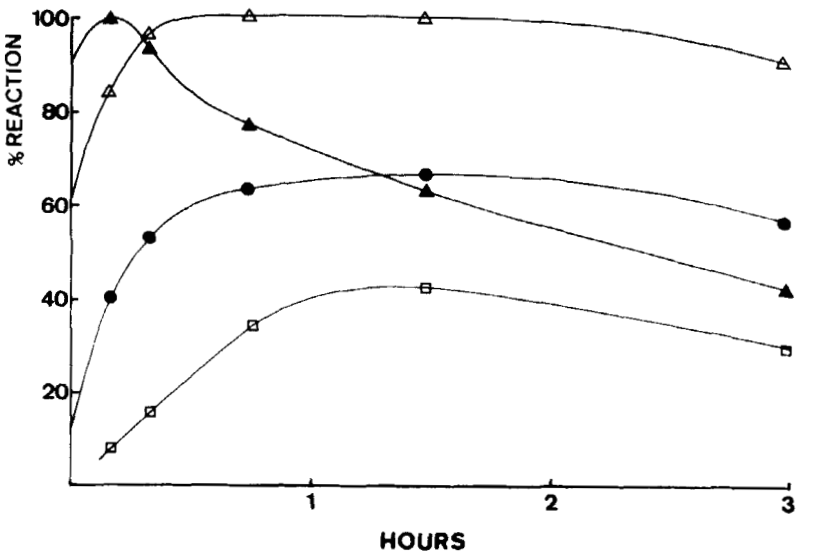

FIGURE 4

Influence of the molarity of the reagent to the reaction rate of cetanol with AI at 95°C in toluene. ▲ 50-fold, △ 30-fold, ● 15-fold and □ 5-fold excess of the reagent.

reached at a 50-fold excess, whereby the reaction proceeded quantitatively within 5 minutes at room-temperature. After 10 minutes the conversion is 100% at 95°C. This seemingly favourable amount of reagent cannot be used in practice, because the solubility of AI in the reaction solvent is too small and the resulting precipitate complicates the spotting on the thin-layer plates considerably. At a 30-fold molar excess of the reagent quantitative formation of the derivative exists after 25 minutes. This is the most favourable amount of reagent because lower concentrations do not react completely. As these attempts show the reactivity of the AI is somewhat smaller than that of the NI. This for the most part may be adjusted for compounds with a secondary or tertiary hydroxyl group which are not so reactive, either by increasing the reaction temperature or by increasing the reaction time.

With these optimized conditions of reaction the substances listed in table 1 were transformed into the corresponding N-anthracenecarbamicacidesters. For derivating primary alcohols a reaction time of 30 minutes at 95°C in xylene was chosen. For the formation of the urethanes with secondary alcohols 30 minutes are also sufficient. Only for codeine and cholesterol are 50 minutes necessary in order to derivate them

TABLE 1

Solvent systems and hRf-values of the investigated and fluoresceing anthracene urethanes.

-: These urethanes showed no fluorescence

Compound	hRf-values	solvent system
Atropin	6	2
1-Butanol	45	1
Cetanol	50	1
Cholesterol	60	1
Codein	9	2
Dexamethasone	26	2
Dianabol	-	-
Fluorometholone	-	-
Hydrocortisone	36	2
Methyltestosterone	-	-
Prednisone	21	2
Testosterone	-	-
Trihexyphenidyl	11	2

completely, whereby also here the degree of conversion after 30 minutes already runs to 85-90%. The derivatisation of compounds with a tertiary alcoholic group with AI can only be performed at 140°C in xylene for 2 or more hours.

A nonuniform reaction pattern is investigated with steroid hormones. Steroids possessing a primary, secondary and/or tertiary alcoholic hydroxyl were tested and their reaction behaviour with AI compared with those of NI.

Dexamethasone, hydrocortisone and prednisone have a primary OH-group in the 21-position and a tertiary in the 17-position. Dexamethasone and hydrocortisone, in addition, have a secondary hydroxyl in the 11-position. At the conversion with NI nearly a quantitative derivative formation my be obtained with dexamethasone after 30 minutes at 95°C in xylene and with prednisone after 60 minutes at 50°C in acetone. But the products obtained show no fluorescence. Hydrocortisone reacts with NI within 1 hour in acetone at 50°C to about 95% and has a very weak fluorescence. The derivatization with AI ensues with all the three steroids within 1 hour in acetone at 50°C to about 90% whereby the resulting urethanes fluoresce strongly blue. As can be shown by comparing analysis the urethane formation of these three compounds occurs exclusively

on the primary hydroxyl in the side chain. The secondary and the tertiary group do not react with the isocyanate.

Testosterone has as a recative group the secondary hydroxyl in the 17-position. The reaction with NI in xylene at 95°C is completed quantitatively after 30 minutes, but also here no fluorescence may be observed. With AI no conversion to the urethane is noticeable within 1 hour at 50°C in acetone.

Fluorometholone having the reactive secondary hydroxyl group in the 11-position and the tertiary in the 17-position forms in contrast to testosterone carbamicacidesters neither with NI nor with AI. This fact may be explained by the small reactivity of the C 11-OH compared to the C 17-OH.

Dianabol and methyltestosterone featuring solely a tertiary hydroxyl group in the 17-position also do not react at prolonged reaction times, neither with NI nor with AI.

The anthraceneurethanes obtained are separated from the by-products in the same solvent systems used for the naphthylurethanes (hRf-values see table 1). It appeared that the hRf-values of the anthraceneurethanes are at about 5-25% higher in the more unpolar solvent system benzene-ether, as those of the naphthylurethanes. With the more polar solvent system chloro-

form-benzene-ether the hRf-values of the anthracene-urethanes are about 5-30% lower than those of the naphthylisocyanates.

The reaction course may be investigated very easily visually by thin-layer chromatography, because the emission maxima of the N-anthracenecarbamicacid-esters are already in the visible range (see table 2).

TABLE 2

Detection limits, excitation - and emission maxima of the investigated anthraceneurethanes.

Compound	Detection limits	λ Ex (nm)	λ Em.(nm)
Atropin	0,4 ng	395	430
n-Butanol	0,4 ng	390	428
Cetanol	3 ng	395	430
Cholesterol	0,7 ng	392	425
Codeine	0,4 ng	380	420
Dexamethasone	1 ng	391	420
Hydrocortisone	1 ng	378/396	422
Prednisone	1 ng	380/394	418
Trihexyphenidyl	1 ng	380	421

As it is obvious by viewing a developed chromatogram under the UV-lamp at 366 nm, by-products still arise which are composed of anthraceneamine and N-anthryl-N',N'-diethylcarbamide besides the alcohol explored. Both these products present like the urethane blue fluorescence while three other decomposition products existing in insignificant amounts fluoresce yellow-green and orange, respectively.

Fig. 5 shows the chromatogram of the conversion of hydrocortisone with AI. The good stability of the urethanes hence may be shown, that within 24 hours no decrease of the fluorescence intensity may be confirmed when the thin-layer plate is stored in the dark and covered with another plate. By dipping a chromatogram in a methanolic solution of polyethylene glycol 4000, not only a considerable increase of the fluorescence intensity and therewith better detection limits are caused, but also higher stability of the derivatives is ensured. As can be seen from fig. 6 a chromatogram treated in such a manner also can be measured after several days without lost of the fluorescence properties.

The measurement of the highly fluoresceing spots is performed at the maxima stated in table 2. This means a bathocromic shift at about 70 nm compared with the naphthylurethanes. In the case of hydrocortisone

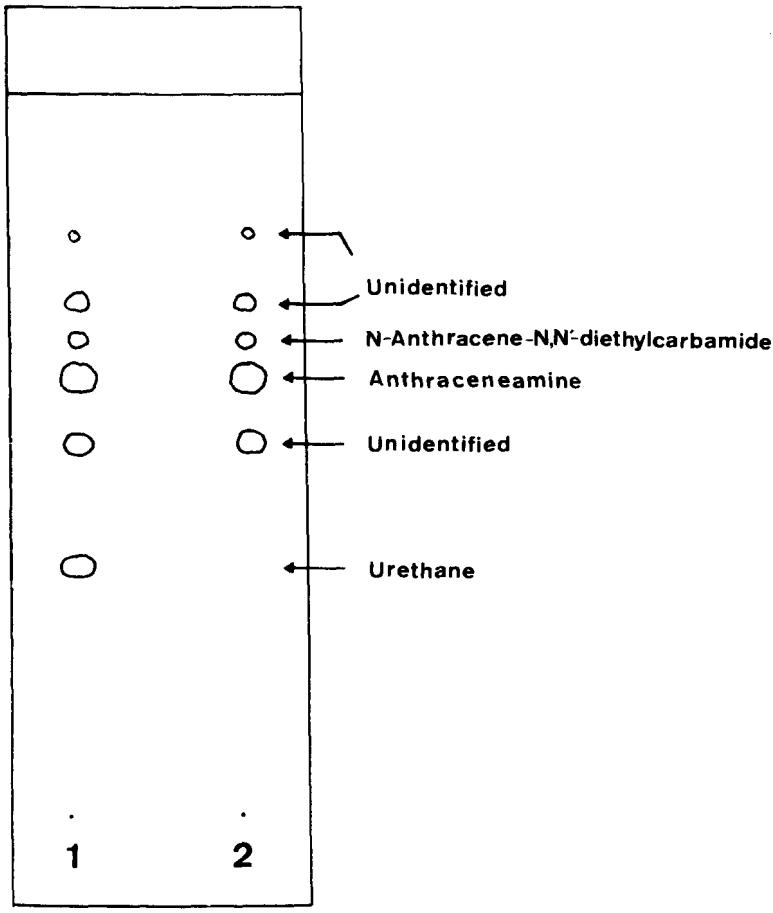

FIGURE 5

Chromatogram of the reaction mixture of hydrocortisone with AI (1), blank (2) by viewing at 366 nm. Solvent system: Chloroform-benzene-ethanol (45+15+5).

and prednisone two excitation maxima may be observed. Although the maxima being in the longer wavelength range are distinguished by a somewhat higher intensity, the determinations were carried out at their excitation

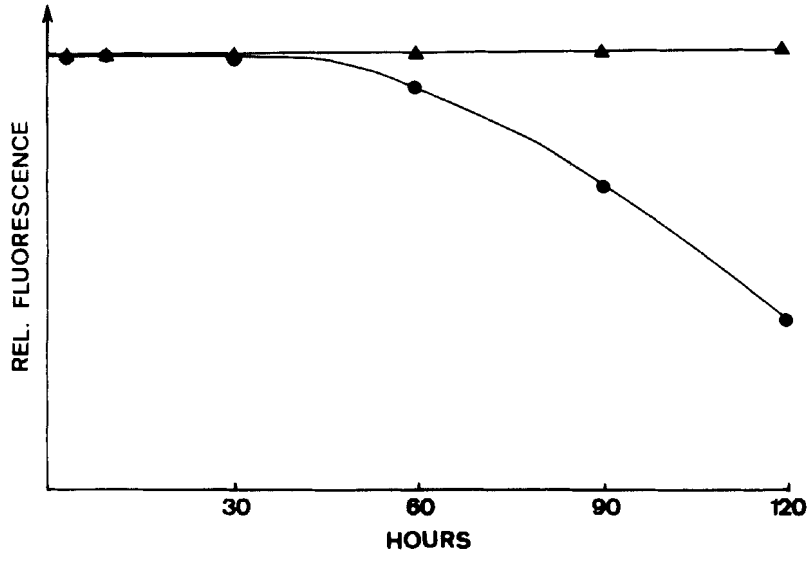

FIGURE 6

Stability of the urethane formed from cholesterol and AI with (▲) and without (●, 24-fold intensified) dipping in a solution of polyethylene glycol 4000 in methanol.

maxima in the lower wavelength range because otherwise the difference to the emission maxima is very small.

The range of concentration in which a quantitative estimation is possible depends on the examined alcohol. Generally, a linear relationship is obtained from the maximal sensitivity up to 3 orders of magnitudes. Because of the poor reactivity of the tertiary alcohols lower detection limits were obtained with this class of compounds than with primary and secondary alcohols, with the exception of benactyzine and trihexyphenidyl (see

table 2). Compared to the naphthylurethanes the detection limits of all the anthraceneurethanes are better, in some cases even by a factor 10. The correlation coefficients run between 0,997 and 0,999. The reproducibility of the method was tested by means of 10 determinations with 20 ng of n-butanol respectively. The value of the relative standard deviation amounts to 1,8%.

CONCLUSION

It can be confirmed that the reactivity of the AI in comparison with NI for the derivatization of substances with an alcoholic OH is not diminished to the expected extent. The higher fluorescence quantum yield of the AI also leads to a lowering of the detection limits, whereby the emission maxima of the anthracenecarbamicacidesters are in the visible range throughout. In contrast to the NI this reagent may also be applied for the derivatization of dexamethasone and prednisone. The use of still more intensive fluoresceing and more reactive isocyanate-reagents is in preparation.

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