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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NAPHTHYL-URETHANES WITH FLUORESCENCE DETECTION

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SUMMARY

The method used previously for the fluorodensitometric determination of compounds with an alcoholic hydroxyl group was examined for its applicability in high-performance liquid chromatography. The conversion of alcoholic substances into urethanes was performed with naphthyl isocyanate. Chloroform–benzene–ethanol and *n*-heptane–diethyl ether, were used as eluents for the separation of urethanes of various polarity with silica gel columns. Reversed-phase material is also suitable. The detection limits ascertained by means of fluorescence detection are in the picomole range.

INTRODUCTION

Several workers have reported on the conversion of alcohols into urethanes with phenyl isocyanate and their high-performance liquid chromatographic (HPLC) separation[1–7]. Fluorescence detection of such compounds is not possible, however. In previous papers[8,9] we described the derivatization of substances with an alcoholic hydroxyl group using fluorescent isocyanates, followed by fluorodensitometric measurement. Both naphthyl isocyanate (NI) and anthracene isocyanate (AI) can be used for the derivatization of many alcoholic compounds with different structures. Owing to the greater number of rings in the AI molecule, its reactivity is lower, especially with tertiary alcohols. This may be balanced by increasing the excess of the reagent. However, it was found that this may lead to a complicated separation of the urethanes from the by-products. This paper describes the use of NI, enabling smaller amounts of reagent to be used in the determination of compounds with alcoholic hydroxyl groups by means of HPLC with fluorescence detection.

EXPERIMENTAL

Apparatus

An Altex Model 110A liquid chromatograph equipped with a Perkin-Elmer

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650-10S fluorescence detector and a Perkin-Elmer W+W 1100 recorder were used, together with a Rheodyne variable-volume injector. For the determination of the excitation and emission maxima 10^{-4} *M* solutions of the substances in benzene were prepared and measured with a Perkin-Elmer MPF 44 fluorimeter and recorded with a Perkin-Elmer 023 recorder.

Reagents and materials

A pre-packed 10-μm LiChrosorb Si 100 and a 5-μm LiChrosorb RP-18 column (25 cm × 0.46 mm I.D.) were purchased from Merck (Darmstadt, G.F.R.). Throughout this work doubly distilled water was used. The solvents and chemicals were of analytical-reagent grade from Merck, except for acetonitrile, which was obtained from Rathburn (Walkerburn, Great Britain). They were used without any further pre-treatment.

Procedure

A 1-ml volume of a toluene solution containing not more than 400 ng of the alcoholic compound is pipetted to 0.5 ml of a 1.5-fold molar excess of NI in toluene and to 0.5 ml of a 1 *M* solution of triethylenediamine (TED) in toluene. After heating (primary and secondary alcohols for 60 min at 95°C and tertiary alcohols for 120 min at 140°C, all solutions in xylene), 1 ml of a 3-fold molar excess of diethylamine in toluene is added while still hot. The reaction mixture is shaken briefly and centrifuged at 1000 *g* for 5 min. From the clear, supernatant solution a volume of 200 μl is used for injection on to the chromatographic column.

Determination of the substance 32-468 {4'-[2-hydroxy-3-(1,1-dimethylamino)propoxy]-spiro(cyclohexa-2',1-indan)-1'-one} in plasma

To 1 ml plasma, spiked with 100 ng of 32-468, are added 5 ml of 1 *M* sodium hydroxide solution and 10 ml of chloroform and the mixture is shaken for 20 min and centrifuged at 1000 *g* for 5 min. After taking an aliquot of 9 ml of the organic phase and evaporating it under reduced pressure at 35°C, the dry residue is further treated as described above.

RESULTS AND DISCUSSION

Separation on a LiChrosorb Si 100 column

Using silica gel as the stationary phase we used thin-layer chromatography (TLC) as a pilot technique for the selection of suitable mobile phases. We first used benzene–diethyl ether (95:5), which we had previously used in fluorodensitometric analyses. In order to obtain approximately results of the same quality as with TLC separation, less polar eluents have to be applied in HPLC. Instead of benzene, we therefore used toluene, xylene and *n*-heptane. With *n*-heptane–diethyl ether (95:5) a satisfactory separation of the urethanes listed in Table I from their by-products could be achieved.

From Table I it can also be seen how the urethanes are eluted with increasing polarity of their alcoholic component. It takes about 28 min for a run when working isocratically, because the by-products are eluted relatively late. Naphthylamine, which appears as the last peak in the chromatogram, may be eliminated by extraction

TABLE I

CAPACITY FACTORS (k') OF URETHANES AND BY-PRODUCTS USING *n*-HEPTANE–DIETHYL ETHER (95:5) AS THE ELUENT

Flow-rate: 4 ml/min.

Compound	k'	*Compound*	k'
Naphthylamine	31.84	O-Octanol-N-naphthylurethane	4.19
N,N-Diethyl-N′-naphthylcarbamide	18.74	O-Cetyl alcohol-N-naphthylurethane	3.93
N,N′-Dinaphthylcarbamide	10.84	O-Cholecalciferol-N-naphthylurethane	2.67
O-3-Methyl-1-butanol-N-naphthylurethane	4.96	O-Cyclohexanol-N-naphthylurethane	2.62
O-1-Butanol-N-naphthylurethane	4.83	O-Cholesterol-N-naphthylurethane	2.56

with 1 *M* hydrochloric acid and the time for one run is then shortened by about 40% (*cf.*, Fig. 1) and may be further shortened by changing to a more polar eluent after the appearance of the urethane peak.

As the first tests demonstrated, the application of a 30-fold molar excess of the reagent, as chosen for the fluorimetric quantitations on TLC, is unsuitable. The separation of the by-products from the urethanes in this instance involves great difficulties. To overcome this problem, the reagent concentration had to be decreased in order to decrease the peaks of the by-products. This is particularly effective with primary and secondary alcoholic compounds, which react quantitatively within a few

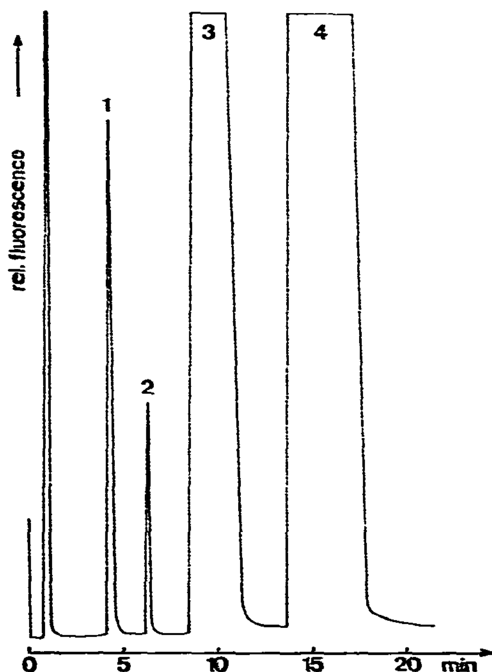


Fig. 1. Chromatogram of the reaction mixture of 200 ng of cetyl alcohol with NI after HCl extraction. Mobile phase, *n*-heptane–diethyl ether (95:5); flow-rate, 4 ml/min. Peaks: 1 = urethane; 2, 3, 4 = by-products.

TABLE II

PEAK AREAS (ARBITRARY UNITS) OF THE REACTION PRODUCTS OF 1-BUTANOL WITH NI

Reaction temperature, 95°C; reaction time, 60 min; injection volume, 10 µl.

Molar excess of reagent	*Amount of alcohol (ng)*		
	200	*75*	*10*
30-fold	896	404	105
3-fold	891	401	102
1.5-fold	891	400	102

minutes with a 30-fold molar excess of reagent. When the reaction time is prolonged to 60 min sufficient derivatization is also achieved after the reagent concentration is decreased to 95% of the initial amount. Table II, which shows the results for the formation of urethane by reaction of 1-butanol with NI, indicates that with a 1.5-fold molar excess of the reagent no reduction in the reaction rate occurs. Steroids with a primary or secondary hydroxyl group, which have low reactivity, need a reaction time of 60 min at 95°C. By increasing the reaction temperature to 140°C compounds with a tertiary hydroxyl group may also be derivatized with a 1.5-fold molar excess of NI within 120 min.

The excess of NI, which is difficult to elute owing to its high polarity, is destroyed by adding diethylamine.

The smallest detectable amounts of urethanes and their excitation and emission maxima are given in Table III. All of the investigated compounds with an alcohol group show a linear relationship between the amount of N-naphthylcarbamic acid ester applied and the peak area. The detection limit is between 1 and 20 ng; the highest tested concentration was 100 ng in an injection volume of 200 µl.

This system, with *n*-heptane–diethyl ether as the mobile phase, can be applied particularly to the separation of reaction mixtures of urethanes with less polar alcohol groups. For urethanes with a polar alcoholic component it is less suitable because

TABLE III

DETECTION LIMITS, MEASURED AT A SIGNAL-TO-NOISE RATIO OF 3:1, AND EXCITATION AND EMISSION MAXIMA

Injection volume, 200 µl; mobile phase, *n*-heptane–diethyl ether (95:5).

Derivatized alcoholic compound	*Limit of detection (ng)*	*Excitation maximum (nm)*	*Emission maximum (nm)*
Isopentyl alcohol	4	310	350
1-Butanol	4	310	355
Cetyl alcohol	1	308	350
Cholecalciferol	20	318	352
Cholesterol	6	319	356
Cyclohexanol	4	318	350
1-Octanol	1	310	350

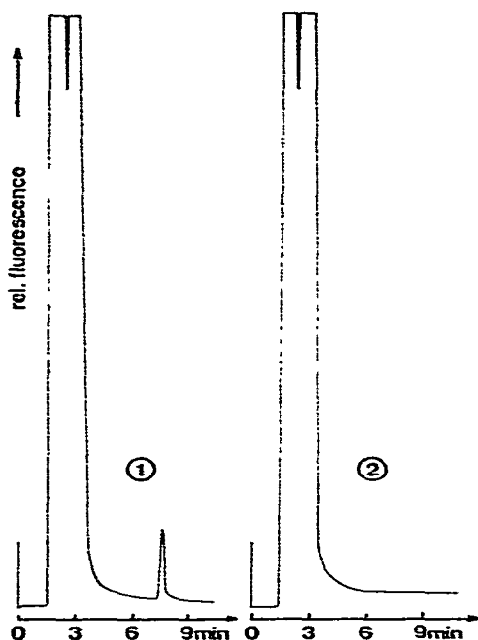


Fig. 2. Chromatogram of the reaction mixture of 200 ng of quinine with NI (1) compared with the blank (2). Mobile phase, chloroform–benzene–ethanol (45:30:0.2); flow-rate, 2 ml/min.

under these conditions separation from the polar decomposition products is difficult. For this purpose, chloroform–benzene–ethanol (45:30:0.2) was found to be a suitable eluent. Whereas the relatively polar by-products of derivatization eluted very late with *n*-heptane–diethyl ether as the eluent, they now appeared within 4 min, followed by the urethane. In contrast to the *n*-heptane–diethyl ether eluent, the excess of reagent could now be increased again. The resulting stronger peaks of the by-prod-

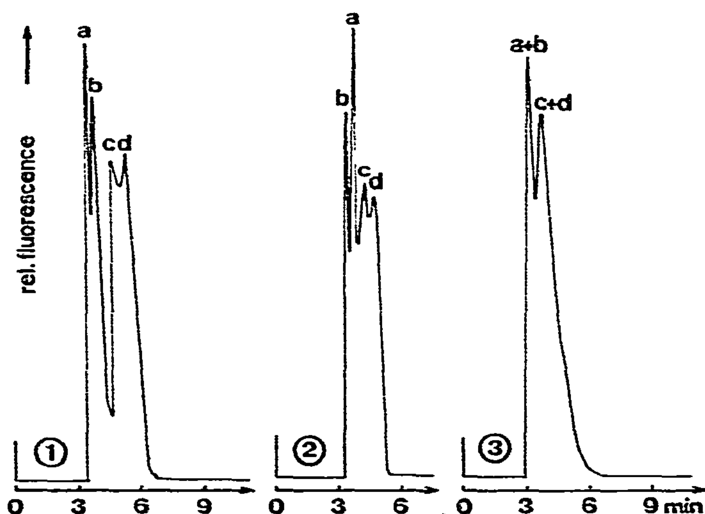


Fig. 3. Chromatogram of the urethane formed from 200 ng of cetyl alcohol and NI. Mobile phase, chloroform–benzene–ethanol: (1) 45:30:0.7; (2) 45:30:5; (3) 15:15:20. Peaks: (a) urethane; (b), (c), (d), by-products.

ucts are eluted very rapidly owing to the higher polarity of the chloroform–benzene–ethanol mixture.

Fig. 2 shows as an example the reaction of quinine with a 1.5-fold molar excess of NI. The run is finished within a few minutes when working isocratically. However, by derivatizing with a 30-fold molar excess of NI separation from the by-products can also be observed.

An attempt was made to use chloroform–benzene–ethanol as the eluent for the analysis of urethanes with a non-polar hydroxyl group, *e.g.*, of butanol or cetyl alcohol. As can be seen in Fig. 3, this solvent system is not appropriate for the separation of urethanes with a lipophilic component. Starting from the mixture chloroform–benzene–diethyl ether (45:30:0.2) the amount of ethanol was gradually increased in an attempt to achieve the separation of by-products. However this was not successful, just as adding of more polar solvents, *e.g.*, acetic acid was also not successful.

The possibility of urethane formation and subsequent HPLC separation with chloroform–benzene–ethanol was also investigated for the determination of 32-468 in plasma.

This substance is a β-blocking agent and possesses a secondary alcoholic hydroxyl group and a secondary amino group in the side-chain, both of which are suitable for derivatization with NI. Three different structures of the derivatives would therefore be conceivable, namely the N-substituted, the O-substituted and the di-substituted products. A closer investigation of the resulting derivative was not performed as this is of no importance for the determination. In comparison with ephedrine[8], the reaction takes place more slowly. As can be seen from Fig. 4, employing a 1.5-fold molar reagent excess, in this instance also within 120 min, a low tendency to react may be observed. When employing a 15-fold molar excess of NI the derivatization is virtually completed within 60 min.

For the determination of 32-468 in plasma the optimal extraction conditions with different organic solvents at different pH values were sought. By shaking once with chloroform at pH 11 an average recovery of 99.8% could be attained. Fig. 5 shows the chromatogram of the reaction product of 300 ng of 32-468 with NI after chloroform extraction from plasma. The detection limit of the urethane was 60 ng for an injection volume of 200 μl.

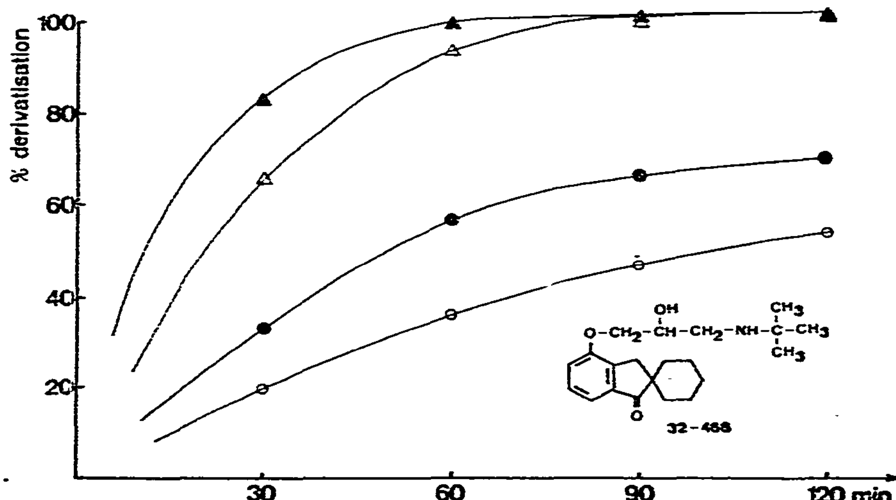


Fig. 4. Kinetics of reaction of 32-468 *versus* the concentration of the reagent. Molar excess: ▲, 150-fold; △, 15-fold; ●, 3-fold; ○, 1.5-fold.

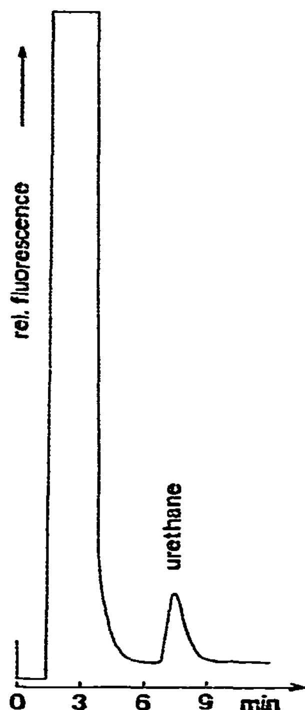


Fig. 5. Chromatogram of the urethane formed from 32-468 and NI. Mobile phase, chloroform–benzene–ethanol (45:30:0.2); flow-rate, 2.5 ml/min.

Separation on a reversed-phase column

As a further possibility, analysis with a reversed-phase support was considered. With such a system, the urethanes must be soluble in acetonitrile–water and methanol–water mixtures. As the solubility tests showed, the N-naphthylcarbamic acid esters with 1-butanol, 2-propanol, 2-phenylethanol or similar alcohols are soluble, whereas the N-naphthylcarbamic acid esters with cetyl alcohol and cholesterol are insufficiently polar and therefore insoluble. In this instance the system with *n*-heptane–diethyl ether in combination with a LiChrosorb Si 100 column is suitable.

By applying acetonitrile-water (60:40) as the eluent the urethanes listed in Table IV may be separated easily from their decomposition products with an RP-8 column. The reactions were carried out with a 1.5-fold molar excess of NI. As Fig. 6 demonstrates, another peak appears after the urethane peak when working isocrati-

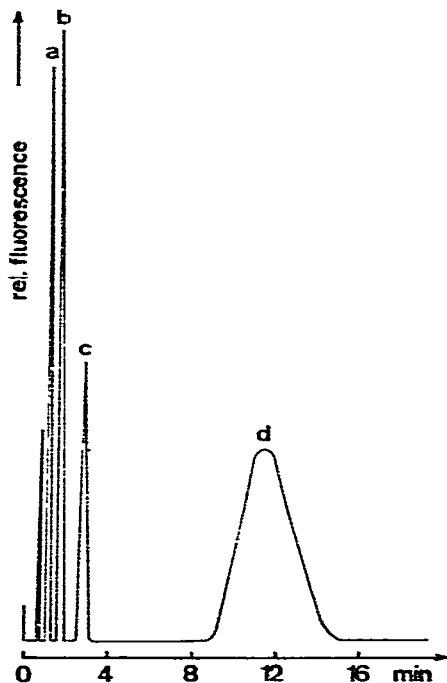
TABLE IV

CAPACITY FACTORS (k') OF NAPHTHYLURETHANES USING ACETONITRILE–WATER (60:40) AS THE ELUENT

Flow-rate: 4 ml/min.

Derivatized alcohol	k'	*Derivatized alcohol*	k'
2-Phenylethanol	2.95	2-Methyl-2-propanol	2.46
Benzyl alcohol	2.83	2-Butanol	2.42
Codeine	2.58	1-Butanol	2.40
2-Propanol	2.49		

cally. This may be eliminated within a short time by changing the eluent to pure acetonitrile after the appearance of the urethane peak.



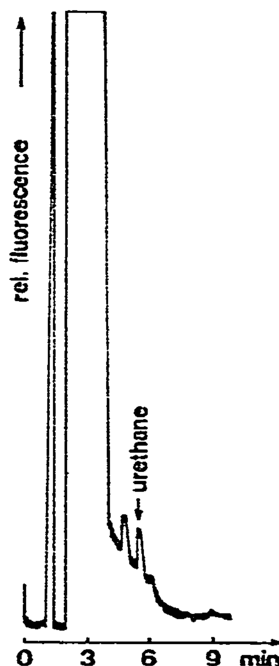


Fig. 6. Chromatogram of the reaction mixture of 200 ng of codeine with NI. Mobile phase, acetonitrile–water (60:40); flow-rate, 4 ml/min. Peaks: (a), (b), (d), by-products; (c), urethane.

Fig. 7 Detection limit of the urethane formed from n-butanol and NI; 10 pmol per 100-μl injection volume. Mobile phase, acetonitrile–water (60:40); flow-rate, 2 ml/min.

Fig. 7 gives an example of the urethane with 1-butanol, illustrating that the detection limits for all derivatizations were roughly the same. Linearity was observed from the detection limit to about 400 ng. The correlation coefficients for the function $y = ax + b$ were between 0.997 and 0.999.

REFERENCES

1 H. R. Schulten and I. Stoeba, *Z. Anal. Chem.*, 293 (1978) 370.
2 I. Stoeba and R. Reupert, *Wasser*, 51 (1978) 273.
3 M. Furukawa and T. Yokoyama, *Nippon Kagaku Kaishi*, 7 (1976) 1084.
4 Z. Stanke, *Z. Chem.*, 17 (1977) 230.
5 J. Cazes and J. Carter, *Soc. Plast. Eng. Tech. Pap.*, 22 (1976) 406.
6 R. J. Argauer and J. D. Warthen, *Anal. Chem.*, 47 (1975) 2472.
7 B. Björkquist and H. Toivonen, *J. Chromatogr.*, 153 (1978) 265.
8 R. Wintersteiger and G. Wenninger-Weinzierl, *Z. Anal. Chem.*, 309 (1981) 201.
9 R. Wintersteiger, *J. Liq. Chromatogr.*, in press.