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N-CHLOROMETHYLPHTHALIMIDES AS DERIVATIZATION REAGENTS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new class of substances is described, the N-chloromethylphthalimides, which can be used as UV-sensitive reagents for the formation of derivatives suitable for high-performance liquid chromatography (HPLC).

The highly reactive chlorine in the N-methyl group reacts quantitatively with alkali metal or ammonium salts of CH-, OH- and NH-acid compounds. The salts of the free acids can be formed by direct addition of triethylamine to the reaction mixture. Hence the reagent is suitable for fast pre-column derivatization of free acids. Aprotic solvents are suitable reaction media. Acetonitrile is used with preference, as the reaction solution does not absorb at 254 nm and can be analysed directly by using HPLC.

If a crown ether is used as a phase transfer catalyst, alkali metal salts of carboxylic acids can be quantitatively converted within 15 min at 60°. The applications of this group of reagents in derivatization are illustrated by examples of mono- and dicarboxylic acids and barbiturates.

INTRODUCTION

The need to produce derivatives for high-performance liquid chromatography (HPLC) which are readily detectable by photometric methods has prompted numerous studies in recent years. Lawrence and Frei[1] and Blau and King[2] reviewed the UV-sensitive and fluorescence-sensitive reagents which were then in use, together with their fields of application. It is evident that the assay of compounds in trace amounts is often possible only if specific, selective derivatization reactions are used, as the detectability can be appreciably improved and the derivatization step usually serves also as a clean-up procedure in the analysis of a complex matrix.

This paper examines the suitability of N-chloromethylphthalimides for use as derivatization agents in HPLC, with special reference to their selectivity and reactivity.

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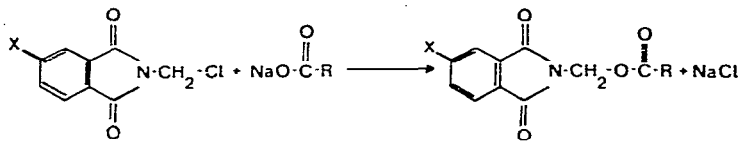
In Table I spectrophotometric data and references are listed for the substances studied, namely N-chloromethylphthalimide (ClMPI) and N-chloromethyl-4-nitrophthalimide (ClMNPI). The isomer of N-chloromethylphthalimide, N-chloromethylisatin (ClMIS), was also studied, as its chemical properties are very similar to those of the imide. The halogen atom of the N-methyl group in all of these compounds is very mobile and as a result it reacts with various proton-active groups.

TABLE I

UV-SPECTROSCOPIC DATA FOR N-CHLOROMETHYLPHTHALIMIDES



ClMPI : X = H
ClMNPI: X = NO_2

Compound	*Abbreviation*	λ_{max} *(nm) in acetonitrile*	$\varepsilon_{\lambda max}$	$\varepsilon_{254\ nm}$	*References*	*M.p. (°C)*
N-Chloromethyl-phthalimide	ClMPI	224	18,700	1390	3–6	135
N-Chloromethyl--4-nitrophthalimide	ClMNPI	235	23,900	9500	4	142
1-Chloromethylisatin	ClMIS	240			6–10	100

Table II gives a brief survey of the reactions in which this type of compound can take part. Details of reaction velocities and yields are not given, as the data in the literature are not readily comparable.

TABLE II

FUNCTIONAL GROUPS REACTING WITH N-CHLOROMETHYLPHTHALIMIDES AND N-CHLOROMETHYLISATIN

Functional group	*Type of reagent*	*References*
Carboxyl	ClMIS	7–9
	ClMPI	3, 5, 6
CH-acid	ClMIS	7
	ClMPI	3
NH-acid	ClMIS	7–9
Amine	ClMIS	7, 11
Hydroxyl	ClMIS	7, 12

As for the reaction mechanism, it can be stated that the N-chloromethylphthalimides, as well as N-chloromethylisatin in general react only with the salts of CH-, NH- and OH-acid compounds. For preference the sodium salts are used[7–9], although ammonium salts react satisfactorily in certain instances[5]. Any aprotic solvent will serve as the reaction medium. The numerous reactions open to this class of compounds and their wide field of application in analysis can be seen in Table II.

The usefulness of these derivatization reagents for carboxyl groups will now be

examined in detail, and the reaction kinetics, with and without catalysts, will also be discussed. In a further section, the formation of barbiturate and phenol derivatives will be described.

Determination of carboxylic acids as phthalimidomethyl or 4-nitrophthalimidomethyl esters using HPLC

With optical detectors, the HPLC assay of carboxylic acids with poor chromophores is of very low sensitivity. By derivatization of the carboxyl groups to esters with UV-sensitive reagents, the detectability of the acids can be greatly improved and, moreover, the high polarity of the acids is decreased, so that, in addition, the derivatives generally possess better chromatographic properties.

Acid derivatives which are sensitive to UV include the corresponding benzyl[13], *p*-nitrobenzyl[14,15], phenacyl[15,16], *p*-nitrophenylacyl[17,18], *p*-bromophenacyl[18–20] and 2-naphthacyl[21] esters. All of these derivatives have been chromatographically investigated and good HPLC separations of the acid–ester mixtures were achieved in every instance. By this method it also proved possible to separate *cis–trans* isomers such as C_{18}' and C_{18}'' as well as isomers with the double bond in a different position[19].

It can be seen from Table I that, as expected, the nitration of ClMPI caused a shift of λ_{max} towards the visible region of the spectrum. The molar extinction coefficient, ε, is higher for ClMNPI than for ClMPI at λ_{max}, but at 254 nm, the wavelength which is most commonly used for fixed-wavelength detectors in HPLC, ε, for the nitro compound is higher by a factor of 7 and therefore the detectability of the analogous 4-nitrophthalimidomethyl derivatives is correspondingly greater. In this study both reagents were used, although it was realised that in the future preference would be given to ClMNPI because of its greater sensitivity enhancement.

EXPERIMENTAL

HPLC system

All analyses were carried out on a Hewlett-Packard 1010B apparatus with a Hewlett-Packard 1036A fixed-wavelength detector. The columns were HIBAR columns filled with LiChrosorb RP8 of average particle size 7 μm (Merck, Darmstadt, G.F.R.).

Reagents

The solvents were of analytical-reagent grade and were supplied by Merck. They were used without further purification. The crown ether 18-Crown-6-(1,4,7,10, 13,16-hexaoxacyclooctadecane) of purum quality was supplied by Fluka (Buchs, Switzerland).

N-Chloromethylphthalimide was synthesized using the method of Böhme *et al.*[3]. The reagent was recrystallised from dry benzene–cyclohexane and could be kept in dry storage at room temperature for several months without decomposition. N-Chloromethyl-4-nitrophthalimide was synthesized by a procedure analogous to that described by Böhme and co-workers[3,4], and 4-nitrophthalimide was produced by the method of Huntress and Shriner[22]. The yields obtained corresponded with the data in the literature.

Some of the fatty acids and dicarboxylic acids were of analytical-reagent grade

and others of purum quality. All of the barbiturates were of the degree of purity required for pharmaceutical purposes.

Derivatization reaction

Method A (used for acid concentrations of 0.5–10.0 mM). The sample of a non-volatile, organic acid is dissolved in methanol or diethyl ether and carefully evaporated to dryness in a 50-ml reaction vessel. The residue is taken up in an approximately 3 molar excess of triethylamine (TEA) and 20 ml of acetonitrile (or dimethylformamide), and a 3 molar excess of ClMPI or ClMNPI is then added.

The vessel is closed and kept at 60° (in a drying oven) for 1 h and the contents are then added with stirring to about 100 ml of water at room temperature. A copious precipitate settles out, which is a mixture of excess of reagent and the ester in question. This residue can be freed from the reagent by several crystallizations from benzene–cyclohexane and can be obtained in a form suitable for analysis in this way.

Method B (used for acid concentrations of 0.001–0.5 mM). The samples of non-volatile organic acid, which are dissolved in water, methanol or diethyl ether, are carefully evaporated to dryness in reaction vessels that can be firmly closed (ampoule flasks) of 1 or 5 ml volume. The residue, together with an excess of the alkylating reagent (ClMPI or ClMNPI) and triethylamine (as in method A), is taken up in 0.5–3 ml of acetonitrile. The vessel is firmly closed and kept at 60° for 1 h.

After cooling, the solution can be assayed directly by HPLC; any colourless needles that may precipitate are triethylammonium chloride and do not affect the determination.

Method C (used for acid concentrations of 0.001–0.5 mM). Unless it is already in the form of an aqueous solution of carboxylic acid salts, the sample of organic acids (including volatile acids), dissolved in water or methanol, is neutralized to phenolphthalein with methanolic potassium hydroxide solution. The salt solutions are immediately placed in the reaction vessels and carefully evaporated to dryness.

An excess of alkylating medium and crown ether in a molar ratio of 10:1 in acetonitrile is added to the residue and the reaction vessel is then placed in a drying oven for 1 h at 60° with shaking at intervals. The solution can be assayed directly.

For the derivatization of barbiturates, methods A, B and C are used. Quantitative derivatization of the phenolic hydroxy group may be effected by method C via the alkali metal phenolates which are readily formed using methanolic potassium or sodium hydroxide solution. Method C is to be used for the alkali metal salts.

RESULTS AND DISCUSSION

Derivatization studies

In order to evaluate a derivatization reaction for use in analysis, it is necessary to investigate the rate of reaction and the yield. The reagents tested (ClMPI and ClMNPI) react almost quantitatively (>97%) with both the lower and higher fatty acids and with the dicarboxylic acids (C_2–C_6).

The NH-acid compounds, as represented by the barbiturates, are also quantitatively derivatized. Moreover, the phenolic hydroxyl group can be converted quantitatively into the corresponding ether.

ClMPI and ClMNPI react satisfactorily only with salts of CH-, OH- and NH-

acid compounds; these salts may be the alkali metal or, as in the case of TEA, the ammonium salts. The reaction with alcoholic OH groups does not go to completion under the given conditions (methods A–C) owing to inadequate alcoholate formation, and similarly, conversion is incomplete and variable with primary and secondary amines. Tertiary amines tend to form quaternary ammonium compounds with the chloromethylimides, and in methods A and B this leads to side-reactions with the excess of TEA. The reaction is appreciably slower than that with the acid; nevertheless, if the excess of TEA is too small, it is possible that the derivatization will not reach 100% yield as no further TEA remains for the formation of the required salt. ClMPI and ClMNPI react very slowly with water, which is an advantage as it is not critical if small amounts of water are still present in the samples to be derivatized.

The rate of the esterification or etherification reaction does not depend very much on the reaction medium, provided that it is an aprotic water-miscible solvent. We prefer to use acetonitrile, as this serves also as a component of the mobile phase in HPLC and, moreover, shows no UV-absorption at 254 nm. TEA salts of the acids generally react quantitatively within 30–40 min in acetonitrile at 60°.

As can be seen from Fig. 1, the alkali metal salts react very slowly when the reaction, is not catalysed, so that complete conversion is unlikely to occur. The use of TEA or 4-dimethylaminopyridine (DMAP) greatly accelerates the reaction, probably by promoting solubility.

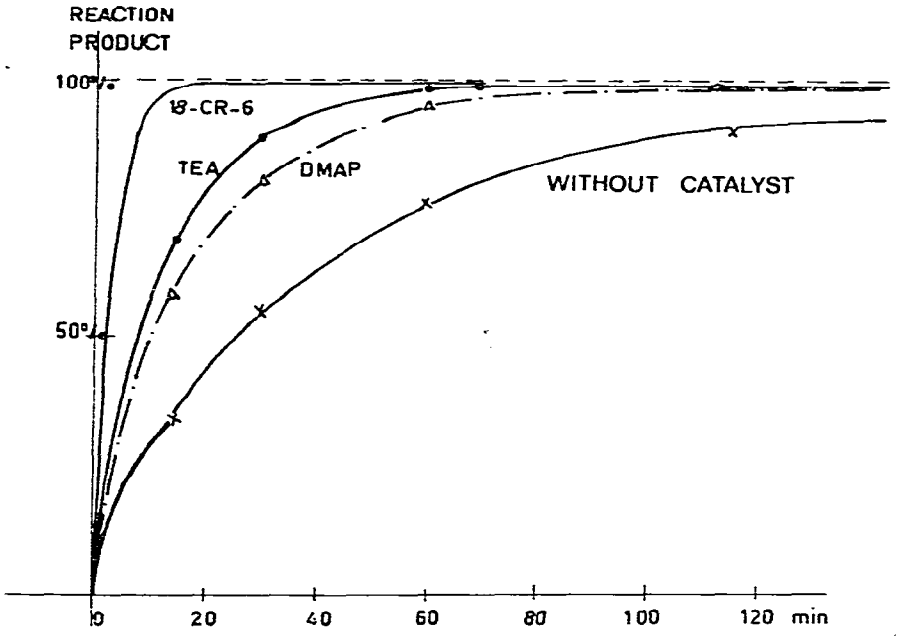


Fig. 1. Reaction rate of sodium acetate with N-chloromethylphthalimide in acetonitrile at 60°.

Phase-transfer catalysis with a crown ether causes the greatest increase in reactivity in these alkylation reactions, and according to Durst and co-workers[20,23] it has likewise proved very effective in the formation of phenacyl esters. After a reaction time of 15 min, the conversion of sodium acetate is virtually complete.

C_2–C_{18} fatty acids, including unsaturated fatty acids, and also the C_2–C_6 dicarboxylic acids, show slight decreases in reactivity as the chain length increases; the long-chain acids react like TEA salts (method B) within 60 min.

At present no data can be given on the reaction kinetics of the derivatization of the barbiturates, although it has been established by NMR spectroscopy that both NH groups react.

Chromatographic separations

Separations were carried out at room temperature with a fixed-wavelength detector at 254 nm. Fig. 2 shows the isocratic separation of the phthalimidomethyl fatty acid esters of a sunflower oil. The latter had been saponified with methanolic potassium hydroxide solution and extracted with acid.

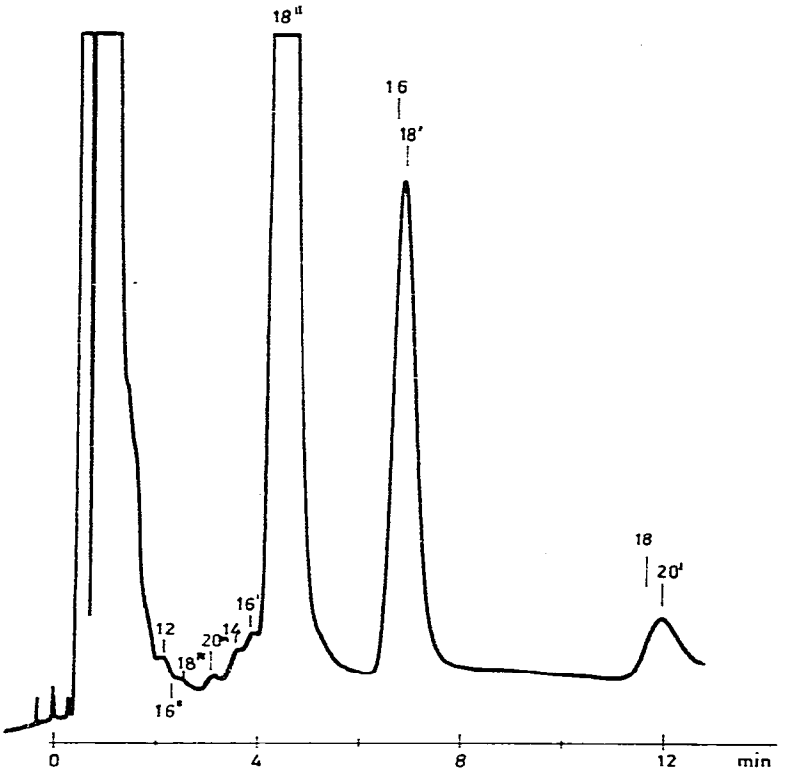


Fig. 2. Separation of the phthalimidomethyl esters of the fatty acids from sunflower oil. Column: HIBAR (Merck), C_8 LiChrosorb (7 μm), temperature 20°. Mobile phase, acetonitrile–water (9:1); flow-rate, 1.5 ml/min; detection, UV at 254 nm; range, 0.4. Peak designation: the numbers indicate the chain lengths of the given carboxylic acid and the superscripts indicate the number of double bonds.

Free fatty acid samples were derivatized by method B. The chromatographic identification of the individual saturated acids was facilitated by the fact that using the isocratic procedure the logarithms of the retention times yield a linear relationship when plotted against the number of carbon atoms of the acids. The same applies

to unsaturated homologous acids, although here the linear relationship has a different slope. The situation is closely analogous to the gas chromatographic identification of fatty acid methyl esters under isothermal conditions.

It can also be seen that the retention time decreases as the number of double bonds in a carbon chain increases, as for example in the C_{18} acids.

Highly unsaturated long-chain fatty acids, which can be determined only with great difficulty by gas chromatography owing to the very long retention times of the methyl esters, can be quickly and efficiently separated and assayed by HPLC using the UV-sensitive fatty acid esters and a mobile phase gradient programme.

As expected, the molar extinction coefficient (ε) is the same for all phthalimidomethyl monocarboxylic acid esters; dicarboxylic acid esters extinction coefficients that are twice as high. This phenomenon can be used conveniently for standardization in quantitative work[24].

Using the 4-nitrophthalimidomethyl esters and a wavelength of 254 nm, a lower limit of detection of 1–2 ng for acetic acid and of 10 ng for stearic acid are readily attainable.

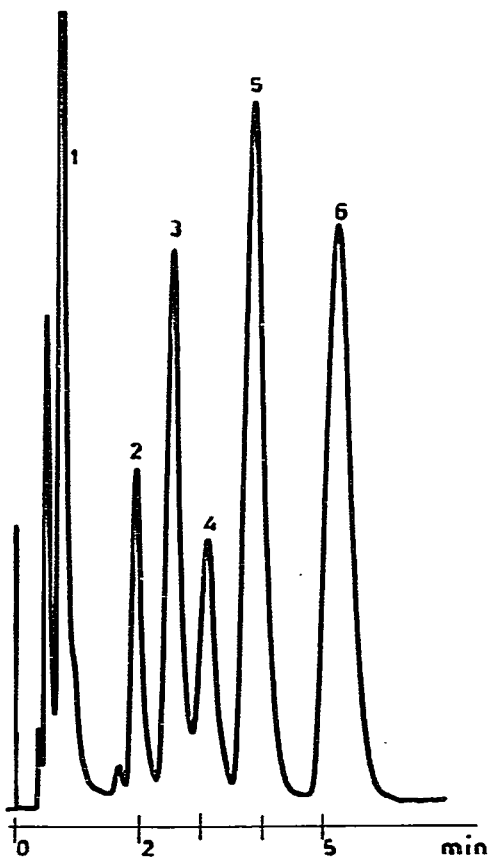


Fig. 3. Separation of the phthalimidomethyl diesters of the C_2–C_6 dicarboxylic acids. Column as in Fig. 2. Mobile phase, acetonitrile–water (3:2); flow-rate, 1.5 ml/min; detection, UV at 254 nm. Peak designation: 1 = 1-hydroxymethylphthalimide; 2 = ClMPI; 3 = malonic acid diester; 4 = succinic acid diester; 5 = glutaric acid diester; 6 = adipic acid diester.

As can be seen in Fig. 3, the dicarboxylic acid esters can also be separated successfully. Under the isocratic conditions described here the oxalic acid diester peak largely coincides with the reagent peak.

The reaction rate and degree of esterification of dicarboxylic acids with ClMPI or ClMNPI were not studied in detail, although they seemed to be comparable to those for the fatty acids.

The highly unstable α-ketocarboxylic acids can be derivatized successfully without decomposition. Fig. 4 illustrates the separation of three acids occurring in the Krebs citric acid cycle (α-ketoglutaric, succinic and fumaric acids) in the presence of excess of reagent, which had been derivatized by using method B or C. The reaction can also be carried out at room temperature.

It can therefore be applied successfully to the derivatization of thermolabile substances such as penicillin G, and the water-soluble sodium salts of the penicillins mostly employed in pharmaceutical dosage forms which can be used directly for the

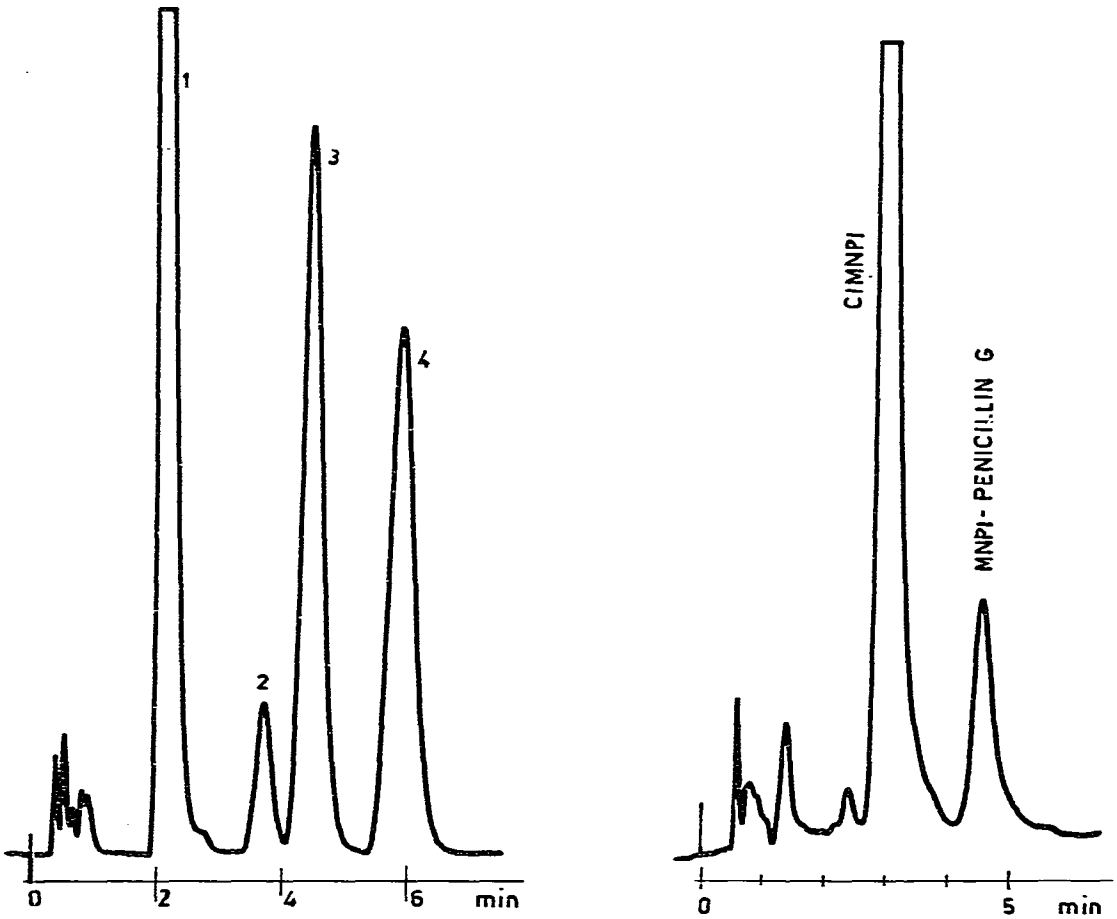


Fig. 4. Separation of Krebs cycle acids as 4-nitrophthalimidomethyl diesters. Separation system as in Fig. 2. Peak designation: 1 = ClMNPI; 2 = α-ketoglutaric acid diester; 3 = succinic acid diester; 4 = fumaric acid diester.

Fig. 5. Chromatogram of penicillin G 4-nitrophthalimidomethyl ester. Column and detection as in Fig. 2. Mobile phase, acetonitrile–water (1:1); flow-rate, 1.5 ml/min.

purpose. Fig. 5 shows an application of the principle. If necessary, the excess of the reagent can be destroyed by addition of triethylamine after derivatization. The product of this reaction precipitates.

As a final application, the isocratic separation of a barbiturate mixture derivatized with chloromethylphthalimide by method B is shown (Fig. 6). The reaction kinetics have not been studied in detail; yields of more than 90% were consistently achieved in trial preparations. The attachment of two chromophores greatly increases the molar extinction coefficients of most barbiturates, and at the same time their detectability at 254 nm improved to about 4 ng.

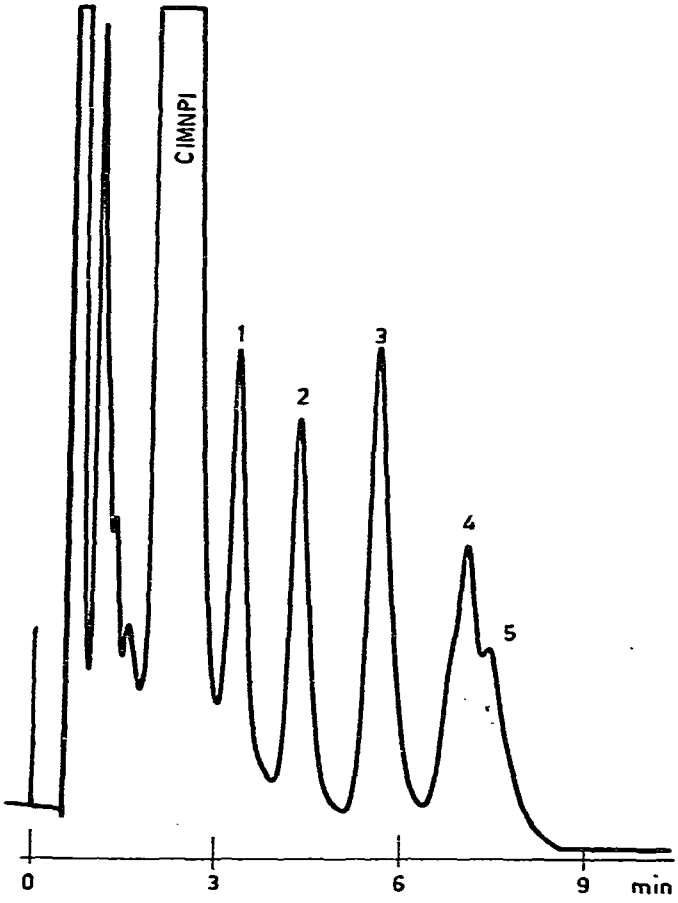


Fig. 6. Separation of a 4-nitrophthalimidomethyl-substituted barbiturate mixture. Separation system as in Fig. 3. Peak designation: 1 = methylphenobarbital derivative; 2 = phenobarbital derivative; 3 = cyclobarbital derivative; 4 = amobarbital derivative; 5 = secobarbital derivative.

The detection sensitivity of the barbital is enhanced by a factor of about 80 by the derivatization (at 254 nm). In this comparison it is assumed that the product peak height times retention time is comparable when isocratic elution is used.

The difference in the molar extinction coefficients at 254 nm between the derivatized barbital and barbital itself is about 10^3 (see Table I). With a signal-to-noise ratio of 5:1, 5 ng of derivatized barbital can be detected.

Recently, Clark and Chan[25] described the post-column ionization of barbiturates. The lower limit of detection of butalbital was 12.2 ng. In strongly alkaline solutions the barbiturates are ionized and a fully conjugated system of double bonds is formed. This results in a bathochromic shift of the UV absorption maximum and the absorbance is enhanced.

CONCLUSIONS

The advantage of the reagents described here over others such as 1-benzyl-3-*p*-talyltriazene[13,26] and 1-*p*-nitrobenzyl-3-*p*-talyltriazene[27] is that they are not limited to a particular functional group. From all of the examples described above it can be seen that the N-chloromethylphthalimides react relatively quickly and almost quantitatively with salts of NH and OH compounds and are thus very suitable UV-sensitive derivatization reagents. The derivatives possess good chromatographic properties and the limits of detection are in the nanogram range.

At present further studies are being undertaken to investigate the use of the reagents with important compounds with acidic functional groups, in particular compounds that occur in plants and other biological materials.

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