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Note

Separation and determination of aliphatic amines by high-performance liquid chromatography with ultraviolet detection*

EVELINE SOLON BARREIRA, JOSÉ PAZ PARENTE and JOSÉ WILSON DE ALENCAR*
Laboratório de Produtos Naturais, Dept.° de Química Orgânica e Inorgânica, Cx. Postal 3010, Universidade Federal do Ceará, Fortaleza-Ce 60.000 (Brazil)
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The separation and determination of primary and secondary amines by high-performance liquid chromatography (HPLC), using different derivatives with fluorescence and chemiluminescence detection, have been the subject of numerous investigations in recent years[1–8]. It has been demonstrated that electrochemical detection (ED) is extremely favourable with respect to its sensitivity and selectivity for the analysis of electroactive compounds in biological fluids[9,10]. Derivatization reagents of potential utility for HPLC–ED have been reviewed[11]. However, there is only one report on the liquid chromatography of tertiary amines using an acetic anhydride solution of citric acid as colour reagent[12]. Tertiary amines do not yield stable derivatives when subjected to reactions with acid chlorides, and covalent derivatization procedures are of little use for such compounds[13].

Aliphatic and aromatic amines have also been separated by HPLC using phenyl isocyanate as a derivatizing reagent on a reversed-phase system with UV detection[14].

A possible reagent for amines is that recommended for hydroxyl compounds, *viz.*, 3,5-dinitrobenzoyl chloride (DNBC)[15]. The use of a benzoylation reaction using benzoyl chloride and pyridine to form a UV-absorbing derivative has been described with respect to its utility in improving the detection limit for non-absorbing hydroxyl-containing steroids by liquid chromatography[16]. The separation of some perbenzoylated carbohydrates by HPLC has been described[17]. Hippuric acid has been prepared using the Schotten–Baumann reaction[18].

Reversed-phase separations of the derivatives of aliphatic amines, diamines and polyamines with benzoyl chloride have been reported[19,20–22] and the investigation of N-alkylbenzamides by reversed-phase liquid chromatography has been studied[23,24]. The conversion of an amine to a sulphonamide has been used to enhance detectability in the UV region[13] and the HPLC separation and quantitation of polyfunctional amines as their *m*-toluoyl derivatives has been reported[25]. However, none of the procedures described is selective for amino groups if alcoholic groups are present in the molecule.

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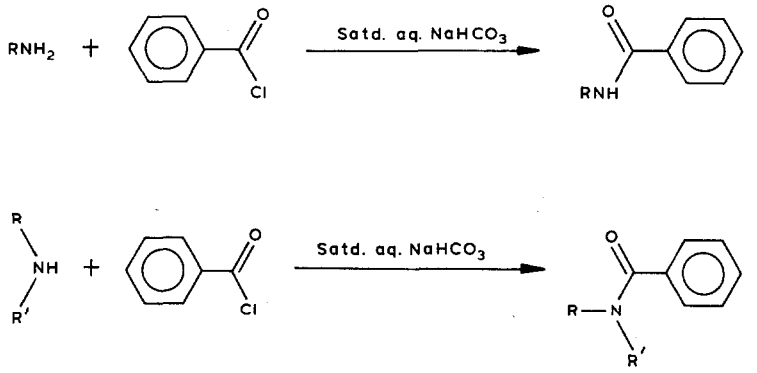

Fig. 1. General reactions of primary and secondary amines with benzoyl chloride.

In this work we used a modified Schotten–Baumann reaction in which sodium hydroxide was replaced with sodium hydrogencarbonate according to Fig. 1.

The major advantage of this reaction is that alcoholic groups present in the molecule do not interfere in the reaction and the benzoylation of the amino groups occurs selectively.

EXPERIMENTAL

Apparatus

A Varian Model 5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a 254-μm UV detector, a linear recorder and a MicroPak MCH-5 reversed-phase column (300 × 4.6 mm I.D.) was used for the chromatographic separation and detection of the products. Injections were made by a pressure-tight 10-μl syringe.

A Varian 604 scanning UV–visible recording spectrophotometer (Varian Techtron, Springvale, Australia), a Perkin-Elmer IR 1320 (Perkin-Elmer, Norwalk, CT, U.S.A.), a Varian EM 360 (60 MHz) NMR spectrometer, a Hewlett-Packard MS 5995A (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a Mettler FP 52 melting-point apparatus (Mettler, Greifensee-Zürich, Switzerland) together with the HPLC apparatus, were used to check the purity and structures of the amine derivatives.

Reagents

Benzoyl chloride and the standard amines were of analytical-reagent grade. The eluent was a mixture of methanol (HPLC grade) (Merck Schuchardt, Darmstadt, F.R.G.) and doubly distilled water.

Preparation of standard samples

Standard samples were prepared by mixing free amine (1 mol) with a saturated sodium hydrogencarbonate solution (*ca.* 5 mol) followed by the dropwise addition of benzoyl chloride (1.1 mol) at room temperature with magnetic stirring for 4 h. The corresponding amide was extracted with chloroform, dried over sodium sulphate and evaporated.

Hippuric acid was prepared from glycine by the same procedure, except that after completion of the reaction the reaction mixture was poured on to hydrochloric acid and the precipitate was crystallized from water.

Derivatization procedure

The general procedure for derivatization was as follows. Aliquots of amines (5–10 μl) were mixed with saturated sodium hydrogencarbonate solution (50 μl) followed by the addition of benzoyl chloride (5–10 μl), and the whole reaction mixture was stirred magnetically for 4 h. The benzoylation was stopped by addition of water (0.4 ml) and the amide was extracted with chloroform (3 × 0.4 ml). After washing of the chloroform phase with water (2 × 0.5 ml), the extract was dried over sodium sulphate, filtered and concentrated and the sample was subjected to analysis

Liquid chromatographic separation and quantitation

Derivatized samples in a methanol solution free of particulate matter were injected (10 μl) into the chromatographic system with a pressure-tight syringe. The Micro-Pak MCH-5 column was at ambient temperature. The eluent of water–methanol (50:50), was pumped isocratically at 0.6 ml/min. The peaks were monitored at 254 nm.

The peak heights of the unknown samples were compared quantitatively with those of standard samples having the same retention times. Moreover, each peak was collected and subjected to gas–liquid chromatography–mass spectrometry (GLC–MS).

RESULTS AND DISCUSSION

Selectivity and reaction conditions

The reaction of aliphatic alcohols with benzoyl chloride in the presence of sodium hydrogencarbonate is slower than that of the corresponding amines. To illustrate this, we compared as an example the IR spectra of the products of the benzoylation of (a) isobutylamine, (b) isobutyl alcohol and (c) isobutyl alcohol (1 mol) + isobutylamine (1 mol). The IR spectra of (a) and (c) showed a single carbonyl absorption at 1625 cm^{-1} (amide) whereas (b) showed three carbonyl absorptions at 1690 cm^{-1} (free acid), 1710 cm^{-1} (ester) and 1775 cm^{-1} (acid chloride). When the amino and the hydroxyl groups coexist in the same molecule the benzoylation of the amino group becomes even more selective.

An example of this is illustrated by the reaction of ethanolamine with benzoyl chloride in a saturated sodium hydrogencarbonate solution. The product of the reaction showed a single HPLC peak (see Fig. 2). GLC–MS analysis of the substance corresponding to this HPLC peak (see Fig. 3) showed it to be $C_6H_5CONHCH_2CH_2OH$. Although the reaction is generally complete in 4 h at room temperature, in order to ensure complete derivatization a reaction time of 4–16 h was used.

Chemical stability

The derivatized compounds in the dry state or in methanol solution are stable for several days at ambient temperature. This enables samples to be prepared long before the chromatographic run.

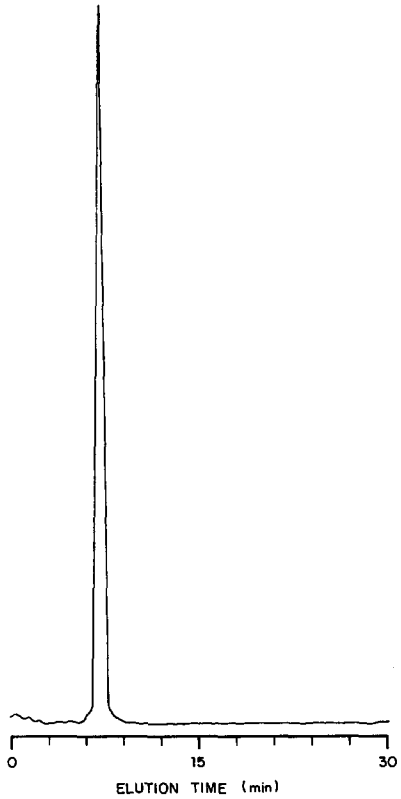

Fig. 2. Elution profile of the N-benzoylethanolamine. Chromatographic conditions as in Fig. 4.

UV detection

N-Alkyl- and N,N-dialkylbenzamides show a strong UV maximum at *ca.* 230 nm. The molar absorptivity at 230 nm is *ca.* 10000 l mol^{-1} cm^{-1}, which allows detection down to the 5-ng level. UV monitoring at 230–260 nm allows the detection of the derivatives down to the 5–10-ng level.

Advantages and disadvantages of the method

Advantages

(1) Alcohols are almost unreactive towards benzoyl chloride under the described conditions.

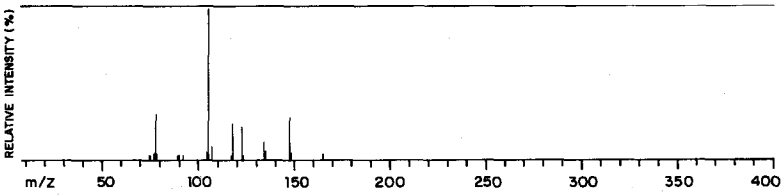

Fig. 3. Mass spectrum (70 eV) of the N-benzoylethanolamine.

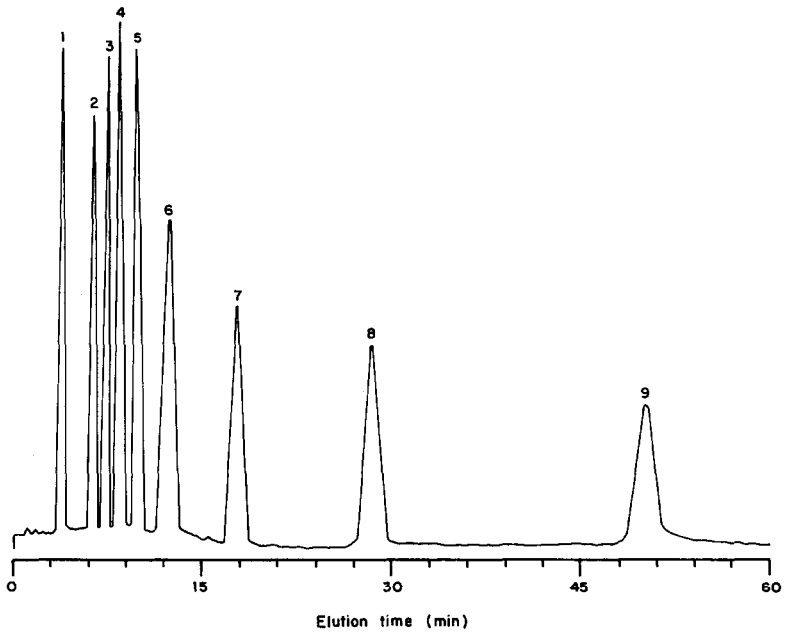

Fig. 4. Elution profile of eight amines and glycine derivatized with benzoyl chloride. Chromatographic conditions: column, MicroPak MCH-5, 300 × 4.6 mm I.D.; UV monitoring at 254 nm, 1.0 a.u.f.s.; solvent, water–methanol (50:50). The peaks correspond to the following compounds (*ca.* 1 mg/ml of each derivative in methanol): 1 = glycine; 2 = diethanolamine; 3 = ethanolamine; 4 = methylamine; 5 = ethylamine; 6 = isopropylamine; 7 = isobutylamine; 8 = piperidine; 9 = diisopropylamine.

(2) The derivatives are not sensitive to pH of eluents, so chromatography is not limited to solvent restrictions.

(3) The derivatives are stable for several days even in solution.

(4) Volatile amines can be analysed as less volatile derivatives.

(5) Standards are easy to prepare.

(6) The chromatographic method is sensitive to nanomol amounts of the sample. Using reversed-phase chromatography the reproducibility of the results is favoured.

(7) Benzoyl chloride is easily handled.

(8) No thermal treatment is needed and the reaction is complete at room temperature.

(9) The derivatives are sufficiently volatile to be analysed by GLC–MS, except when many hydroxyl groups are present in the molecule, *e.g.*, N-benzoyl-2-deoxy-aminoglucose.

Disadvantages

(1) Saturated sodium hydrogencarbonate solution is needed for the reaction to occur. This implies in solvent extraction of the derivatives from the reaction mixture prior to chromatographic run.

(2) Sodium benzoate is formed during the reaction. This is relevant when the sample to be derivatized contains acidic groups.

(3) The reaction time is longer than those cited in the literature, specially if hydroxyl groups are involved.

(4) The method for obvious reasons cannot be applied to tertiary amines.

Practical applications

Reversed-phase HPLC together with an aqueous methanol solvent and sensitive UV detection can be a useful technique for characterizing derivatized amines. Fig. 4, for example, shows the separation of a test mixture of N-alkyl and N,N-dialkylbenzamides with detection at 254 nm.

One of the applications of the method was found in determining piperidine in black pepper. The results however, are not presented in this note.

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