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Note

High-performance liquid chromatography of fluorescamine-labelled amines in acid solvents

DAVID L. INGLES* and DAVID GALLIMORE
CSIRO Division of Food Research, P.O. Box 52, North Ryde, N.S.W. 2113 (Australia)
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Separation of fluorescamine-labelled amines by high-performance liquid chromatography has been applied to aliphatic diamines and polyamines[1,2] and to some catecholamines[3] using gradient elution with buffers, pH 8.0. Under such conditions the fluorescamine derivatives are chromatographed as the sodium salt forms of II (Fig. 1).

We obtained improved results by chromatography in acid solvents when the species of derivative chromatographed is the free carboxylic acid II (Fig. 1). Acid solvents have been shown[4] to have advantages also for thin-layer chromatography of acid-induced fluorophores from fluorescamine derivatives of 2-(4-imidazoyl) ethylamines.

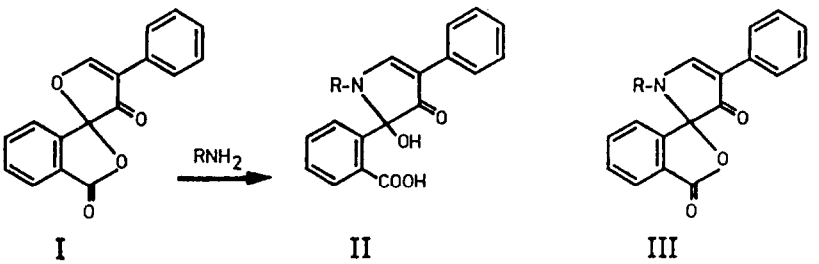
EXPERIMENTAL

Materials

Amines were purchased from Sigma (St. Louis, MO, U.S.A.). Fluorescamine was purchased from Hoffman-la Roche (Nutley, NJ, U.S.A.). Spherisorb 5 μm S5W and Spherisorb 5 μm ODS were purchased from Phase Separations (Clwyd, U.K.). All other organic solvents and chemicals were commercially available AR grade reagents.

Fluorescent derivative formation[5,6]

One ml of a standard amine solution (*ca.* 1 mg amine/10 ml) was mixed with



I II III

Fig. 1. Chemical structures of fluorescamine (I) and the acid (II) and lactone (III) forms of its amine derivatives.

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1 ml of 0.1 *M* sodium phosphate buffer, pH 8.0, or with 1 ml of 0.1 *M* triethylamine phosphate buffer, pH 8.0 and 1 ml of a solution of fluorescamine (20 mg/10 ml acetone) was then added rapidly while the mixture was agitated in a Vortex mixer. Aliquots (20 μl) of the fluorescamine derivatives were applied to the chromatograph columns as follows. (a) Solutions of derivative in sodium phosphate buffer were applied directly. (b) Solutions of derivative in triethylamine phosphate buffer were diluted first with acetone (10:1). (c) Solutions of derivative in sodium phosphate buffer (3 ml) were acidified to pH 4 with glacial acetic acid (10 μl) and extracted twice with chloroform (3 ml). The chloroform extracts were dried over anhydrous sodium sulphate and applied to the column.

Crystalline derivatives of ethylamine

Ethylamine was treated with fluorescamine as described above and the acidified mixture was extracted with chloroform as in (c). Concentration of the chloroform extract gave the crystalline free acid form II (Fig. 1) in quantitative yield. When the acidified sodium phosphate solution of the derivative was allowed to stand for 4 h before extraction with chloroform, concentration of the chloroform extract gave the crystalline lactone III (Fig. 1). The crystalline forms, II and III of the ethylamine derivative had the properties previously reported[5]. The crystalline derivatives were used in acetone or sodium phosphate buffer, pH 8.0, solutions (*ca.* 1 mg/10 ml) for the experiments reported in Figs. 6 and 7.

Equipment

The liquid chromatograph consisted of a high-pressure pump (Altex Scientific, Model 110) which delivered mobile phase at 1 ml/min to a stainless-steel column fitted with a sample injection valve (Altex No. 905-41) with a 20-μl loop. The eluted fluorescent derivatives were measured with an Aminco fluoro-monitor (American Instrument, U.S.A.) fitted with a high-intensity mercury vapour lamp, a Corning No. 7-51 primary filter, a Wratten No. 8 secondary filter and a quartz flow through cell (2 mm I.D.).

Preparative Altex stainless-steel columns (250 × 10 mm I.D.) were used for chromatography with Spherisorb 5 μm S5W and analytical Altex columns (250 × 3.2 mm I.D.) were used for chromatography with Spherisorb 5 μm ODS.

The electrochemical detector consisted of a control module (Bioanalytical Systems, U.S.A., Model LC-4A) and transducer (Model LC-17) fitted with a glassy carbon electrode. The applied operating potential was 1.0 V.

Measurements were recorded on an Electronic Polyrecorder (TOA Electronics, Japan, Model EPR-23A).

RESULTS AND DISCUSSION

Fluorescamine (I) reacts with primary amines as shown in Fig. 1. Reaction occurs in sodium phosphate or other buffer at pH 8–9[5,6] and yields the fluorescent derivative II as the corresponding salt form.

Our first attempt at chromatography of such a salt form of II employed normal phase silica columns with ammoniated hexane–isopropyl alcohol as eluant (Fig. 2). Results were disappointing. The peaks were broad and resolution was poor. In order

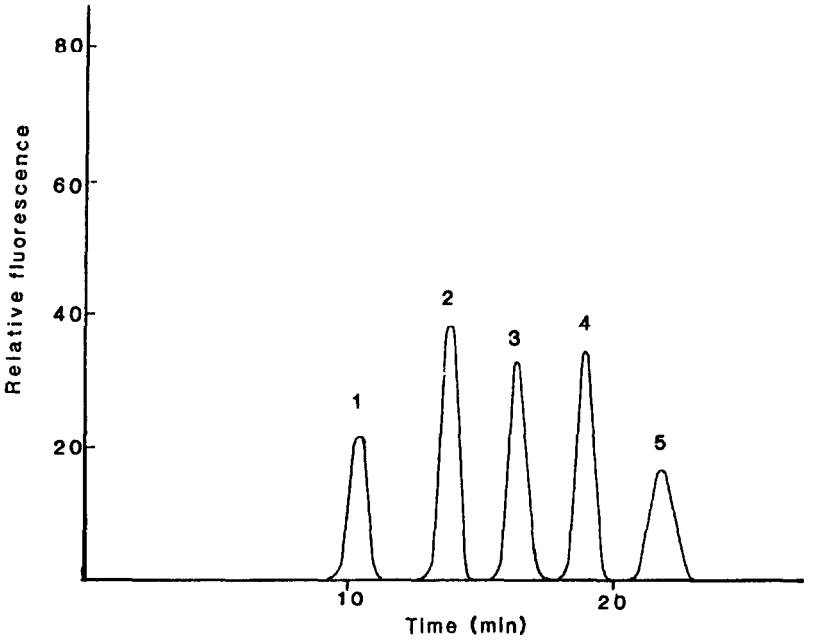

Fig. 2. Chromatogram of fluorescamine derivatives of amines on Spherisorb S5W. Isocratic elution with isopropanol–hexane–ammonia (55:45:2, v/v/v). Peaks: 1 = phenylethylamine; 2 = tryptamine; 3 = tyramine, ethylamine; 4 = methylamine; 5 = 5-hydroxytryptamine.

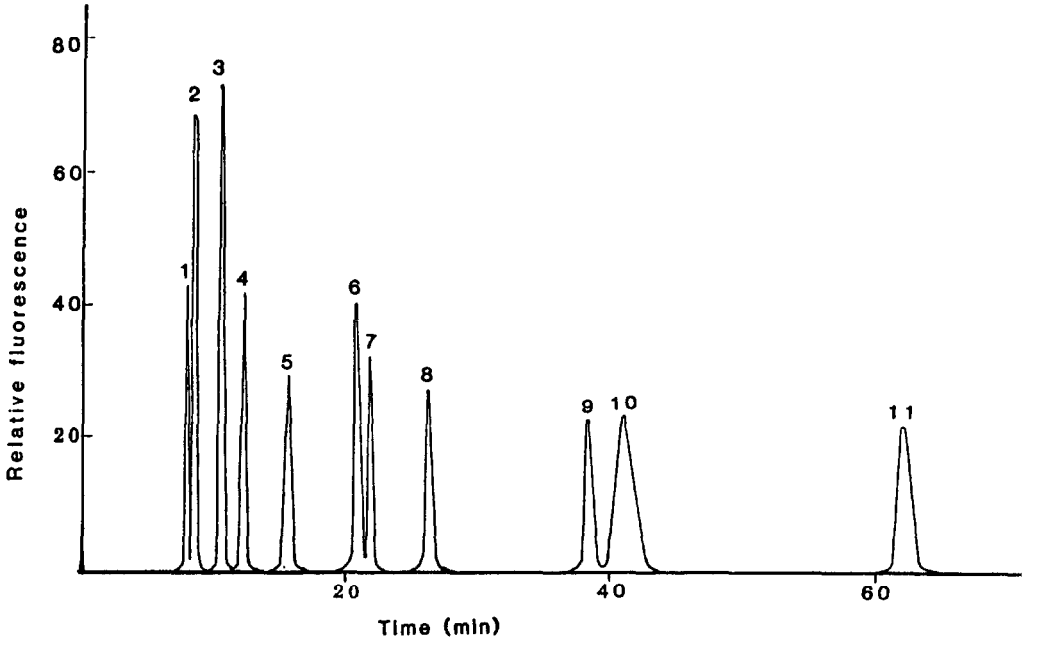

Fig. 3. Chromatogram of fluorescamine derivatives of amines on Spherisorb S5W. Isocratic elution with acetone–hexane (25:75, v/v) containing 1% chloroacetic acid. Peaks: 1 = octylamine; 2 = heptylamine; 3 = butylamine; 4 = phenylethylamine, 5 = ethylamine; 6 = tryptamine; 7 = methylamine; 8 = tyramine; 9 = 5-hydroxytryptamine; 10 = dopamine; 11 = 1-propanolamine.

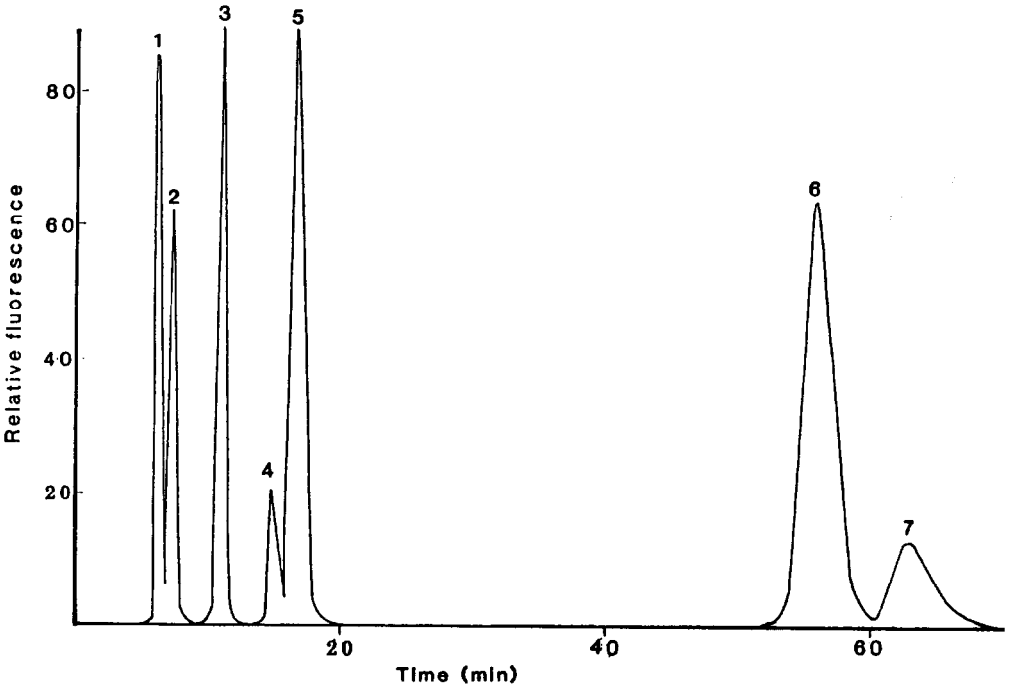

Fig. 4. Chromatogram of fluorescamine derivatives of amines on Spherisorb ODS, 5 μm. Isocratic elution with acetone–water (35:65, v/v) containing 1% chloroacetic acid. Peaks: 1 = ethanolamine; 2 = methylamine; 3 = dopamine; 4 = 5-hydroxytryptamine; 5 = tyramine; 6 = phenylethylamine; 7 = tryptamine.

to achieve compatability of the injected sample with the mobile phase the derivative was prepared in triethylamine–phosphate buffer pH 8.0, and the final solution was diluted with acetone. This dilution seriously reduced the sensitivity of the analysis.

The broadness of the peaks was attributed to ionization of the carboxyl group of II and strong adsorption by the silica of such ionized forms.

When buffered solutions of fluorescamine-labelled amines are acidified to pH 4.0 the derivative exists in solution as the free carboxylic acid II (Fig. 1). The acids may be extracted in chloroform or ethyl acetate and such extracts have been employed for examination and analysis of amine identity by field desorption-mass spectrometry.[7]

Fluorescence yields are not decreased in acid solution[6] and chloroform extracts of the derivatives were stable over several hours. Chromatography of such chloroform extracts in acidic solvents is demonstrated in Fig. 3. The improved resolution shown is attributed to reduced ionization of the carboxyl group of II in acid. Optimum results were achieved with chloroacetic acid compared with acetic acid, dichloro- and trichloroacetic acids.

Satisfactory results were achieved also with reversed-phase silica columns run in acid solvents (Figs. 4 and 5). Injections of chloroform extracts of the fluorescamine-labelled amines are not compatible with the 1% chloroacetic acid–acetone–water solvents used for reversed-phase columns. However, aqueous solutions of the

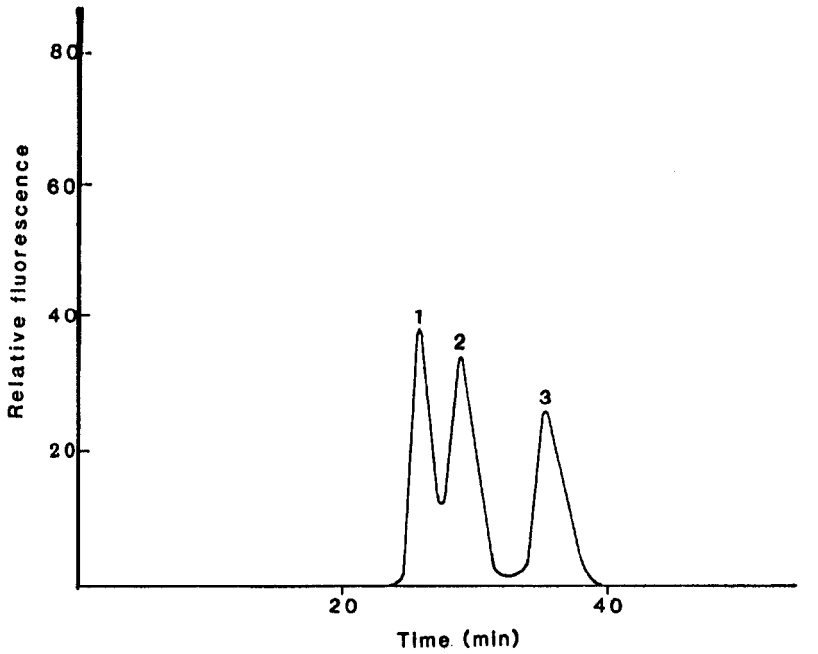

Fig. 5. Chromatogram of fluorescamine derivatives of amines on Spherisorb ODS, 5 μm. Isocratic elution with acetone–water (22.5:77.5, v/v) containing 1% chloroacetic acid. Peaks: 1 = histamine, 2 = putrescine; 3 = cadaverine.

fluorescamine derivatives in sodium phosphate buffer are compatible and such solutions are stable for extended periods[6].

The diamines, histamine, putrescine and cadaverine were resolved (Fig. 5) by reducing the acetone content of the mobile phase.

The solvents used for chromatography with the reversed-phase silica contain 1% chloroacetic acid and 65% or more water and permit detection by electrochemical means. When such a detector was included in the effluent line positive responses were obtained with both the free acid form of the fluorescamine derivative II and the lactone form III (Fig. 1). The resolution achieved with fluorescamine-labelled ethylamine II and its lactone form III is shown in Fig. 6. Both peaks 1 and 2 were obtained with electrochemical detection (oxidative mode) but only peak 1 was shown by the fluorescence detector.

These procedures enabled measurement of the rate of conversion of the free acid form of the fluorescamine derivative to its non-fluorescent lactone form under the conditions applying during chromatography (Fig. 7). Before injection the fluorescamine derivative is in buffer, pH 8.0, and therefore stable. After injection into the acid environment of the mobile phase in the column conversion to lactone occurs, the extent of which depends upon the elution time for a particular amine. Amines eluted in 20 min or less (Fig. 4) suffer $<5\%$ conversion to lactone. For an elution time of 1 h the conversion is only 15%. Since internal standards of amines are equally affected during analysis it is concluded that lactone formation with its concomitant loss of fluorescence is not a major source of error in the chromatography procedures.

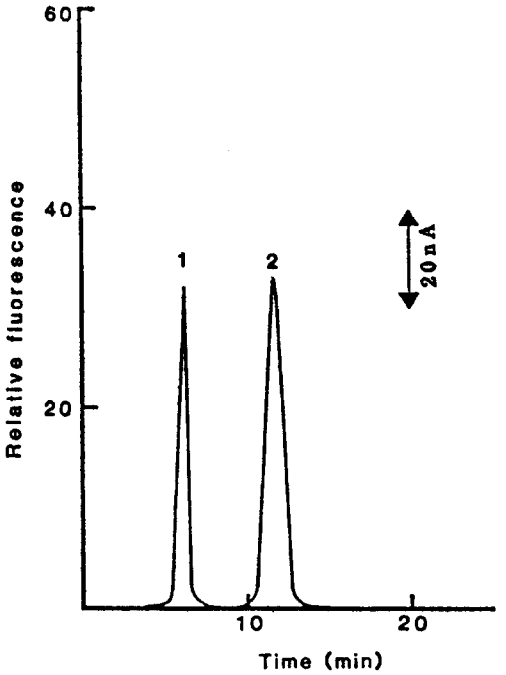

Fig. 6. Chromatogram of the fluorescamine derivative of ethylamine on Spherisorb ODS, 5 μm. Isocratic elution with acetone–water (35:65, v/v) containing 1% chloroacetic acid. Peaks: 1 = ethylamine derivative in free acid form (II, R = ethyl), 2 = ethylamine derivative in lactone form (III, R = ethyl).

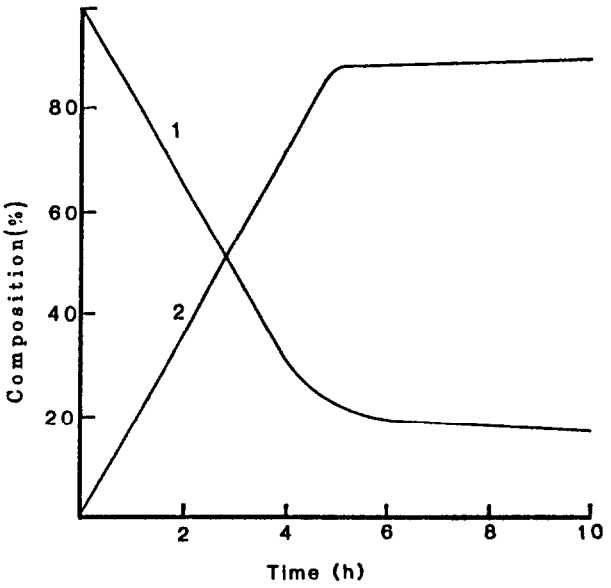

Fig. 7. Rate of conversion of the fluorescamine derivative of ethylamine (II, R = ethyl) to the non-fluorescent lactone derivative (III, R = ethyl) in acetone–water (35:65, v/v) containing 1% chloroacetic acid. Curve 1 shows the concentration of the free acid form (II, R = ethyl) and curve 2 the concentration of the derived lactone form (III, R = ethyl).

The use of reversed-phase silica columns run in acid solvents (*cf.* Figs. 4 and 5) has advantages. The fluorescamine derivatives can be applied directly without further treatment and the eluted derivatives can be monitored by fluorescence or electrochemical detection. Such a reversed-phase procedure has been applied to the analysis of amines in foods, particularly tyramine and phenylethylamine, and these results will be reported elsewhere[8].

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