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## Note

# Characterization of selected fluorescamine–amino acid reaction products by high-performance liquid chromatography

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Fluorescamine was introduced several years ago as a reagent useful in the formation of fluorescent products with primary amines[1]. Use of the reagent has since been advocated in thin-layer chromatography (TLC)[2], in high-performance liquid chromatography (HPLC)[3], and in direct fluorimetry[4]. Fluorescamine has enjoyed wide acceptance, which is not surprising in the light of the speed of reaction at room temperature in aqueous solution and the intense fluorescence of derivatives formed[5].

These appealing features make the reagent especially well suited to HPLC studies. Initially[6] such studies were confined to systems in which the analytes, separated on an ion-exchange column, were passed through a post-column reaction coil, and the fluorescence generated by the reaction was subsequently monitored. Such systems have been shown to have considerable utility in, for example, protein analysis[7].

However, separation of pre-formed fluorescamine derivatives would considerably simplify the system and speed the process by removing the necessity for a post-column reaction coil. The previously discussed[8] quantitative conversion of primary amines to a single derivative lends support to such an approach, and recent studies[9,10] have confirmed its utility with other primary amines. Therefore, a study was initiated to investigate the determination of amino acids by separation of their fluorescamine reaction products.

Contrary to expectations, however, initial studies demonstrated that each of the several amino acids examined gave rise to two fluorescent derivatives, the relative proportions of which were dependent on the amino acid. Formation of multiple fluorescent derivatives would make separation of the numerous amino acids difficult. Therefore, this study was conducted to characterize the reaction products with the hope that characterization would allow procedural alterations giving rise to only one product. As previously noted[11], instability of the reaction product(s) under conditions necessary for isolation makes an indirect study necessary.

## EXPERIMENTAL

### *Materials*

A 30 cm × 4 mm I.D. μBondapak $C_{18}$ (Waters Assoc., Milford, Mass., U.S.A.) column was used throughout the study. μBondapak $C_{18}$ monomolecular layer of octadecyltrichlorosilane chemically bonded to Porasil beads having an average particle size of 10 μ.

Fluorescamine was obtained from Hoffman-La Roche (Nutley, N.J., U.S.A.), with the exception of the 2-, 3- and 4-aminobutyric acids, which were obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

Camphorsulfonic acid was obtained from Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.) and used as a 1% solution in water. Quaternary amines were purchesed from Eastman-Kodak (Rochester, N.Y., U.S.A.).

### *Equipment*

A Model ALC202 liquid chromatograph equipped with a Type M6000 pump and a Type U6K injector (Waters Assoc.) was used. Column effluents were monitored with an Aminco fluoro-microphotometer (American Instrument) equipped with a Corning 7-60 primary filter, a 65A secondary filter and a quartz flow-through cell (2 mm I.D.).

### *Preparation of mobile phase*

Camphorsulfonic acid ($3.0 \times 10^{-3}$ moles) was added to water (300 ml) and the pH was adjusted with a 5% solution of tetrapropylammonium hydroxide in water. This solution was diluted with methanol, deaerated and used as the mobile phase. Methanol was obtained from Matheson, Coleman and Bell (Spectroquality —MX475) and used as received.

### *Fluorescent derivative formation*

To a mixture of one volume of sample solution (0.5 nmole of amino acid/ml) and one volume of 0.1 *M* phosphate buffer, pH 8.4, was added one volume of fluorescamine solution (20 mg/10 ml in acetone or dioxane). The mixture was shaken for 1 min, and an aliquot (5 ml) injected into the chromatographic system.

## RESULTS AND DISCUSSION

A reversed-phase system was selected for characterization of the products because of the predictable effect of polarity on retention volumes of sample constituents[12]. Because of the amphoteric nature of the previously characterized reaction product, ion-pairing was used to effect the separation. The mobile phase was therefore selected in accordance with the principles of reversed-phase ion pairing previously presented[13,14].

Injection of the reaction solution into this system gave two peaks incompletely separated (Fig. 1). Different reaction conditions (variation of pH, of fluorescamine solvent, etc.) produced variations in the absolute peak areas, but did not change the ratios. It was thus apparent that the multiple products could not be explained in terms of an unhappy choice of reaction conditions.

Increasing the proportion of water in the mobile phase increased retention

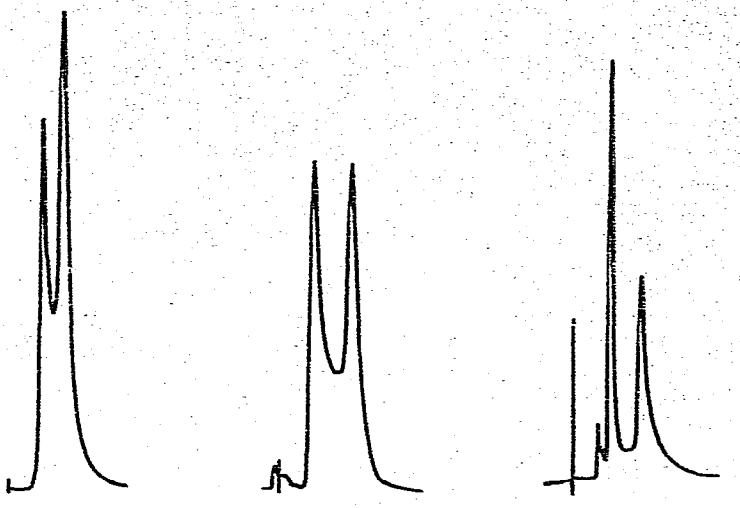


Fig. 1. Chromatogram of fluorescamine–alanine reaction products in a solvent of water ($3.0 \times 10^{-3}$ moles of camphorsulfonic acid) adjusted to pH 3.5 with tetrapropylammonium hydroxide and methanol (200 ml/300 ml). Flow-rate, 1.5 ml/min; pressure, 3100 p.s.i.; temperature, ambient.

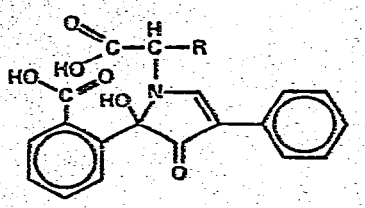
Fig. 2. Chromatogram of fluorescamine–alanine reaction products in a solvent of water ($3.0 \times 10^{-3}$ moles of camphorsulfonic acid) adjusted to pH 3.5 with tetrapropylammonium hydroxide and methanol (250 ml/300 ml). Conditions as in Fig. 1.

Fig. 3. Chromatogram of fluorescamine–alanine reaction products in a solvent of water ($3.0 \times 10^{-3}$ moles of camphorsulfonic acid) adjusted to pH 7.5 with tetrapropylammonium hydroxide and methanol (250 ml/300 ml). Conditions as in Fig. 1.

volumes of each component and the distance between peak maxima but, because of the extensive "tail" of the first peak, did not effect separation (Fig. 2). These chromatographic characteristics indicated that a rapid equilibrium between components of the two peaks might exist within the chromatographic system. The pH dependency of the proposed equilibrium was determined by altering the pH of the mobile phase. At pH 7.5, the two peaks were separated (Fig. 3). However, collection and reinjection of either fraction gave a chromatogram indistinguishable from the initial chromatogram in the area ratio of the two peaks.

On the basis of the foregoing studies, it appears that the two reaction products are reversibly related, and the reversible reaction proceeds more slowly under mildly alkaline conditions than in acid solution. Each component fluoresces at the same excitation and emission wavelengths, and the two differ considerably in polarity.

One component must be the previously indirectly characterized product I, and a logical candidate for the second component is the lactone II derived from I. The rate of lactone formation in aqueous solution has the general pH dependency exhibited in the present case, and such formation should not greatly affect fluorescence characteristics of the products. The decreased polarity of the lactone over the parent acid alcohol would also account for the retention characteristics of the second, more



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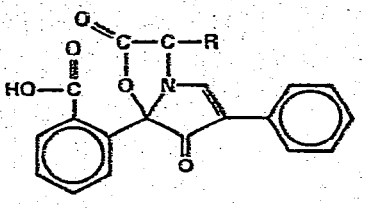

II

strongly retained product. Finally, the change in the ratio of products formed with a change in amino acid could be explained by noting the steric effect of the amino acid on lactone formation.

To test this possibility, fluorescamine was reacted with 2-, 3- and 4-aminobutyric acid, and the ratio of the two products determined (Table I). The peak area ratios are explicable if the first peak is considered to be the acid alcohol and the second the lactone which is, as well, in accordance with polarity considerations. The ratio of lactone formed is then seen to increase from 2-aminobutyric acid to 3-aminobutyric acid because the six-membered lactone formed from the latter is more stable than the five-membered ring of the former. The seven-membered ring necessary for lactonization of 4-aminobutyric acid is too large for perceptable formation.

Likewise, examination of the ratio of products formed with alanine, valine, leucine, and isoleucine lends further support to the lactonization explanation. Substitution in the $\beta$ position decreases lactonization via a steric effect, and the proportion of II formed decreases with increasing substitution at this position. As expected, substitution in the $\alpha$ position, as with $\alpha$-aminoisobutyric acid, is a much more dramatic deterrent to lactonization.

Lactone formation should not occur with peptide derivatives since the free carboxylic acid is located some distance from the primary amine. As confirmation of this, reaction of fluorescamine with L-alanyl-L-alanine was shown to give only one peak (Table I).

TABLE I

RETENTION AND RESPONSE CHARACTERISTICS OF SELECTED FLUORESCAMINE–AMINO ACID REACTION PRODUCTS

| *Amino acid reactant* | *Retention volume (ml)* | | *Acid alcohol/lactone peak area ratio* |
|---|---|---|---|
| | *Acid alcohol derivative* | *Lactone derivative* | |
| 2-Aminobutyric acid | 6.0 | 10.2 | 2.5 |
| 3-Aminobutyric acid | 5.4 | 8.4 | 0.64 |
| 4-Aminobutyric acid | 4.5 | — | No lactone formed |
| Alanine | 5.0 | 8.6 | 2.84 |
| Valine | 6.8 | 16.2 | 5.4 |
| Leucine | 8.7 | 21.0 | 3.7 |
| Isoleucine | 8.9 | 22.0 | 4.8 |
| $\alpha$-Aminoisobutyric acid | 4.2 | 7.8 | 12.3 |
| L-Alanyl-L-alanine | 28.0 | — | No lactone formed |
| L-Alanine-*tert.*-butyl ester | 4.5 | — | No lactone formed |

Therefore, all evidence indicated that the second product of the fluorescamine–amino acid reaction is the lactone, and attention may then be directed toward the promotion or suppression of lactonization. One approach to suppression is to esterify the amino acid prior to reaction with fluorescamine. The alkoxide group of an ester "leaves" with considerably less facility than the hydroxide of an acid. The utility of this approach was investigated with the *tert.*-butyl ester of L-alanine, and no lactone was formed (Table I). However, the necessity for a cumbersome esterification step prior to fluorescamine reaction decreases the attractiveness of its use, and alternate approaches are therefore under study.

## REFERENCES


1 M. Weigele, S. L. Debernardo, J. P. Tengi and W. Leimgruber, *J. Amer. Chem. Soc.*, 94 (1972) 5927.
2 K. Imai, P. Bohlen, S. Stein and S. Udenfriend, *Arch. Biochem. Biophys.*, 161 (1974) 161.
3 S. Stein, P. Bohlen, J. Stone, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 203.
4 J. Sterling and W. G. Haney, *J. Pharm. Sci.*, 63 (1974) 1448.
5 S. Udenfriend, *J. Res. Nat. Bur. Std.*, 76 (1972) 637.
6 A. M. Felix and G. Terkelsen, *Arch. Biochem. Biophys.*, 157 (1973) 177.
7 P. Bohlen, S. Stein, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 213.
8 S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele, *Science*, 178 (1972) 871.
9 K. Samejima, *J. Chromatogr.*, 96 (1974) 250.
10 K. Imai, *J. Chromatogr.*, 105 (1975) 135.
11 S. DeBernardo, M. Weigele, V. Toome, K. Manhart and W. Leimgruber, *Arch. Biochem. Biophys.*, 163 (1974) 390.
12 P. J. Twitchett and A. C. Moffat, *J. Chromatogr.*, 111 (1975) 149.
13 D. P. Wittmer, N. O. Nuessle and W. G. Haney, *Anal. Chem.*, 47 (1975) 1422.
14 J. A. Korpi, D. P. Wittmer and W. G. Haney, *Fed. Anal. Chem. Spectrosc. Soc., Indianapolis, Ind., October 6th, 1975.*