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Note

Fluorescent labelling of amino acids with 9-anthryldiazomethane and its applications to high-performance liquid chromatography

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We have recently developed 9-anthryldiazomethane (ADAM)[1] as a reagent for the fluorescent labelling of carboxylic acids. ADAM has widely been used[2–5] in high-performance liquid chromatography (HPLC) for the precolumn derivatization of biologically significant carboxylic acids such as fatty acids[1–3] and prostaglandins[5]. It can react with carboxylic acids at room temperature without the presence of a catalyst, and even in the presence of water. Accordingly, it was expected to be useful also for the derivatization of carboxylic acids carrying polar residues such as hydroxyl or amino groups. However, preliminary experiments gave only poor yields of amino acid esters. This may be due to the formation of zwitterions between the carboxylic and amino groups. Prevention of the zwitterion formation seemed to be necessary for the improvement of reaction yield. The present study deals with the use of additives, such as sodium dodecyl sulphate (SDS), which intercalate between the amino and carboxylic groups. The resulting improved method for the derivatization was then applied to the separation and determination of amino acids by HPLC.

EXPERIMENTAL

Reagents and chemicals

ADAM was purchased from Funakoshi Yakuhin (Tokyo, Japan), alanine, phenylalanine and SDS from Wako Pure Chemical Industries (Osaka, Japan). Other amino acids, organic amines and arene- and alkenesulphonates were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Non-ionic surfactants were purchased from Nikko Chemicals (Tokyo, Japan), acetonitrile and methanol (for HPLC) from Wako. Other organic solvents were obtained from Kokusan Kagaku (Tokyo, Japan). Water was purified by use of a MILLI-Q water purification system (Millipore, Bedford, MA, U.S.A.) before use.

Reagent solutions

A solution of ADAM (1 mg/ml) was prepared by dissolving the reagent in

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acetone. It was kept in a refrigerator and used within 2 days. Amino acid solutions were prepared by dissolving the acids in water (100 μg/ml), and diluting 2 ml of the solutions in isopropanol to 20 ml. Solutions of additives (0.02 *M*) were prepared by dissolving organic amines, non-ionic surfactants, hydrochloric acid and arene- and alkanesulphonates in isopropanol–water (19:1).

HPLC

The HPLC system consisted of a Model 638 liquid chromatograph (Hitachi, Tokyo, Japan) equipped with an autosampler Model 710B (Waters, Milford, MA, U.S.A.), a RF-530 spectrofluoromonitor (Shimadzu Seisakusho, Kyoto, Japan) and an integrator Model 7000B (System Instrument, Tokyo, Japan). Besides the spectrofluoromonitor, a Model 635M multi-wavelength UV-monitor (Hitachi, Tokyo, Japan) was also used for the detection of samples at higher concentrations.

The separation was performed with a C_{18} reversed-phase column (150 × 4.0 mm I.D.) packed with TSK gel LS-410 (Particle size 5 μm; Toyo Soda, Tokyo, Japan). The column temperature was kept at 50°C. All the chromatographic separations were carried out isocratically using acetonitrile–1% SDS–phosphoric acid (450:550:1) as the mobile phase at a flow-rate of 1.0 ml/min.

Derivatization of amino acids

To 1 ml of a sample solution were added 1 ml of an additive solution and 1 ml of ADAM solution. The mixture was allowed to stand at 50°C for 3 h and then cooled to room temperature. Isopropanol was then added to make exactly 5 ml. An aliquot of 5 μl of the resultant mixture was directly injected for HPLC.

Fluorescence spectra of the ADAM derivative of alanine

To 1 ml of alanine solution (1 mg/ml) was added 1 ml of ADAM solution and 1 ml of 0.02 *M* SDS solution and the resultant mixture was allowed to stand for 3 h at 50°C. An aliquot of 20 μl was injected for HPLC. The fraction containing the ADAM derivative of alanine was collected and extracted with diethyl ether. The whole procedure was repeated several times and the ether layers were combined. The combined ether fraction was separated and evaporated to dryness, and the residue was redissolved in 10 ml of an appropriate solvent. The excitation and emission spectra of the solution were measured with a Shimadzu Model RF-500 fluorescence spectrophotometer equipped with a xenon discharge lamp.

RESULTS AND DISCUSSION

Fluorescent labelling of the amino groups of amino acids for HPLC has extensively been studied using dansyl chloride (Dns-Cl)[6] or *o*-phthalaldehyde (OPTA)[7,8]. On the other hand, fluorescent labelling of the carboxyl groups of amino acids has been hampered because of the lack of suitable reagents. Halogenoalkyl[9–13] or halogenoacyl[14–16] labelling agents, such as 4-bromomethyl-7-methoxycoumarin[11] or 1-bromoacetylpyrene[16] have been used for the esterification of carboxylic acids. However, these reagents have seldom been employed for the derivatization of amino acids because they require anhydrous media and react also with the amino groups to give complex products.

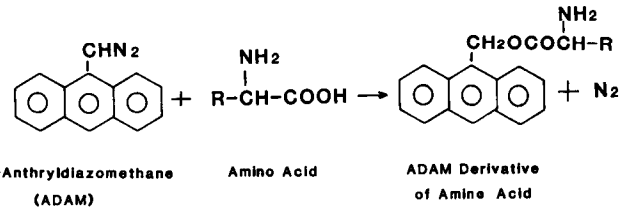

Fig. 1. Esterification of amino acids with ADAM.

ADAM has been proved to be highly reactive with carboxyl groups so that most carboxylic acids can be esterified at room temperature. Protection of the amino groups against alkylation seemed unnecessary under such mild reaction conditions. ADAM was therefore applied to the selective labelling of carboxyl groups in amino acids. Fig. 1 shows the proposed reaction course.

However, the yields of the ADAM derivatives of amino acids were unexpectedly poor. In order to improve the yields, the reaction conditions were reinvestigated using alanine. The effects of organic amines, hydrochloric acid, non-ionic surfactants, arene- and alkanesulphonates and SDS were tested. The amount of alanine derivative formed in each reaction was monitored by HPLC. Hydrochloric acid, alkane- and arenesulphonates and SDS improved the yields, whereas amines and non-ionic surfactants reduced the yields. However, hydrochloric acid was effective only in a very narrow range of concentration. The effects of the sulphonates and SDS were approximately the same. SDS was adopted as the additive as it is readily available.

The influence of the SDS concentration and organic solvents used as the reaction media on the yield of the alanine derivative were investigated. The peak height reached a plateau at SDS concentrations of more than 0.4%. The use of ethanol, isopropanol and acetone gave almost identical yields which were higher than those obtained by the use of other solvents. However, ethanol gave an unknown peak close to the peak of alanine, and acetone showed a large when the UV detector was used. Therefore, isopropanol was routinely used as the solvent for the reaction.

The optimum temperature for the reaction of ADAM with amino acids was found to be 50°C. The relative peak height at 60°C was lower than that at 50°C on account of the decomposition of ADAM. On the other hand, the reaction proceeds only slowly at room temperature and should be allowed to continue for more than 16 h.

Even under the improved reaction conditions, involving the addition of SDS and elevated temperature, amino groups were not affected by ADAM. Tests using

TABLE I

EFFECT OF SOLVENTS ON FLUORESCENCE INTENSITY

Solvent	*Fluorescence intensity*
Acetonitrile	1.0
Acetonitrile–phosphoric acid (1000:1)	2.5
Acetonitrile–water (9:11)	1.3
Acetonitrile–1% SDS–phosphoric acid (450:550:1)	1.7

TABLE II

HPLC CONDITIONS FOR SEPARATION OF AMINO ACIDS

Column	TSK gel LS-410, 150 × 4 mm I.D. ODS, particle size 5 μm
Column temperature	50°C
Mobile phase	Acetonitrile–1% SDS–phosphoric acid (450:550:1)
Flow-rate	1.0 ml/min
Detector	Fluorescence: excitation, 255 nm; emission, 412 nm. UV: 250 nm

ninhydrin and OPTA confirmed that the amino groups of the products remained intact.

The excitation and emission spectra of the ADAM derivative of alanine were measured in various HPLC mobile phases. The spectra were similar to those of other carboxylic acids[1–5] such as fatty acids.

Table I shows the relative fluorescence intensity of the ADAM derivative of alanine in various solvent systems compared with that in acetonitrile. The addition of phosphoric acid was found to enhance the fluorescence intensity. A comparatively high intensity was observed for alanine dissolved in the mobile phase, acetonitrile–1% SDS–phosphoric acid (450:550:1), and the highest intensity was observed in acetonitrile–phosphoric acid (1000:1).

The conditions for chromatographic separation of the ADAM derivatives of amino acids are summarized in Table II. A typical chromatogram of the ADAM derivatives of glycine, alanine and γ-amino-*n*-butyric acid is shown in Fig. 2. Since the ADAM derivatives are relatively polar owing to their free amino groups, their

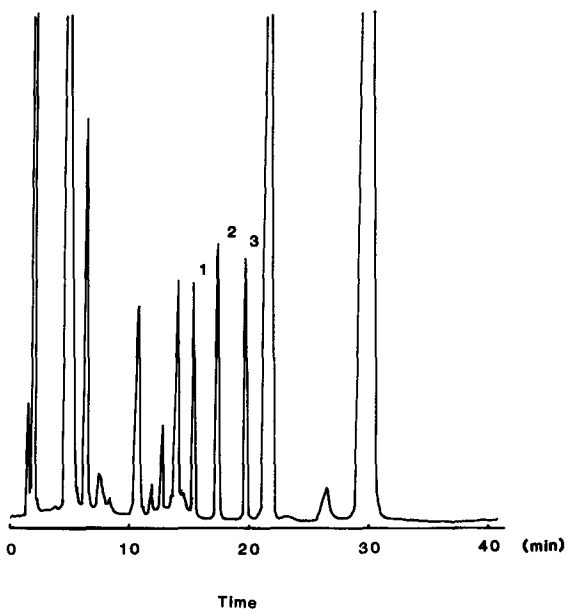

Fig. 2. Chromatogram of the ADAM derivatives of glycine (1), alanine (2) and γ-amino-*n*-butyric acid (3). Each sample solution contained 10 ng of amino acid.

retention times were very close to those of impurities and hydrolysed products of ADAM, and, therefore, they could hardly be separated. Addition of SDS into the mobile phase was found to be effective for the separation of the amino acid esters by increasing their retention times due to the formation of ion pairs. The retention times of impurities and hydrolysed products of ADAM were not so influenced.

A calibration curve for alanine using SDS as the additive was linear in the range 0.02–10 ng, and passed through the origin ($y = 2.539\ x - 0.109$, $r = 0.999$). The detection limit for alanine was about 20 pg per injection (signal-to-noise ratio = 4). The derivatization procedure described here was satisfactorily reproducible for the examined amino acids.

The present study provided a simple and reliable method for fluorescent labelling of the carboxyl groups of amino acids with ADAM. This result seems to extend the use of this reagent to the labelling of carboxylic acids having polar groups.

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