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USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE PREPARATION OF FLAVIN ADENINE DINUCLEOTIDE ANALYTE CONJUGATES

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SUMMARY

High-performance liquid chromatography (HPLC) was used for the study of the chemical synthesis of the flavin adenine dinucleotide analyte conjugates. A preparative HPLC method was also developed for the purification of the conjugates directly from the reaction mixture with minimum sample pretreatment. The preparations of flavin N^6-(N-6-aminohexyl)adenine dinucleotide and flavin adenine dinucleotide theophylline conjugate are given as examples to illustrate the importance of this method.

INTRODUCTION

Flavin adenine dinucleotide (FAD) analyte conjugate (Fig. 1) is a key component in the homogeneous apoenzyme reactivation immunoassay system (ARIS)[1–3], developed in our laboratories for the detection of low levels of clinically important analytes. In this homogeneous binding assay, a given amount of synthetic FAD analyte conjugate is allowed to compete with the analyte present in biological fluid for a fixed, limited number of binding sites on an analyte-specific antibody. The remaining free FAD analyte conjugate can then be detected by its reaction with an excess amount of apoglucose oxidase to produce an active glucose oxidase. The activity of the active glucose oxidase can be easily measured by its conversion of glucose to gluconolactone and hydrogen peroxide. Hydrogen peroxide can be detected by a variety of signal-generating oxidation reactions. Therefore, the amount of signal generated is directly proportional to the concentration of the analyte present in biological fluids.

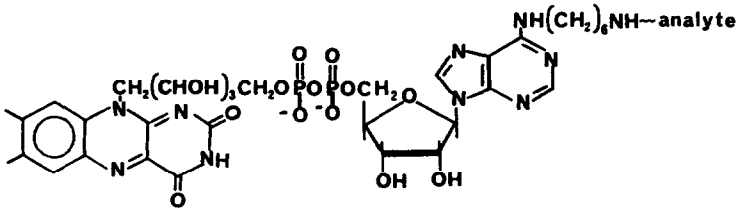

Fig. 1. Flavin adenine dinucleotide analyte conjugate.

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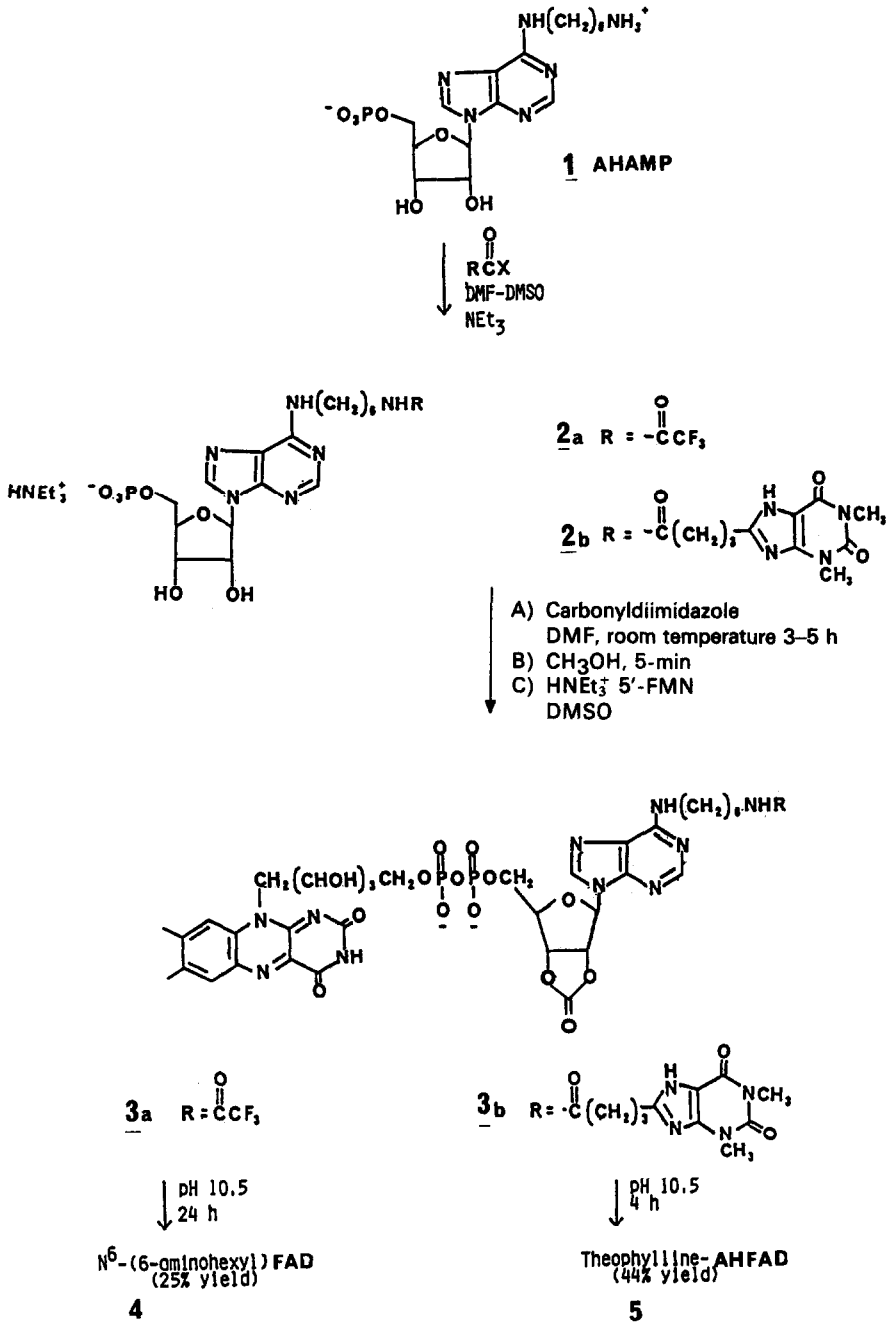

Fig. 2. Synthesis of flavin adenine dinucleotide analyte conjugates.

The advantages of the ARIS method are: (1) It is a homogeneous system, not requiring the isolation of free from antibody bound FAD analyte conjugate. The antibody bound FAD analyte conjugate does not activate the apoenzyme. In fact, all the components of the assay can be incorporated into a solid matrix, such as paper, and the assay can be performed by a dip-and-read procedure[4–6]. (2) It is specific. The rabbit antibody or the monoclonal antibody used in the assay is highly reactive with the analyte. Only very low cross reactivity with the analyte metabolites and other analyte-analogues possibly present in the sample is observed[5]. (3) It has a high sensitivity, because each molecule of FAD analyte conjugate is capable of activating an enzyme with a turnover rate of approximately 10000 moles min^{-1} $mole^{-1}$. This amplification results in the production of many molecules of hydrogen peroxide per molecule of analyte.

An integral component of the ARIS system is the synthetic FAD analyte conjugate. Each conjugate is selected to fulfill the following requirements: (1) The conjugate should retain its capacity to function as a FAD group for the rapid activation of the apoglucose oxidase. (2) The conjugate should also retain its ability to bind to the analyte-specific antibody. (3) The conjugate must also be able to compete effectively with free analyte for binding sites on the antibody in order to maximize assay sensitivity.

Since each analyte assay requires its own special FAD analyte conjugate, considerable effort had been spent to develop a general synthetic strategy for the conjugates[1]. It was found that for small, stable analytes, the conjugates were best prepared from the analyte derivatives of N^6-(6-aminohexyl)adenosine monophosphate (AHAMP) (1) according to the synthetic scheme outlined in Fig. 2. The preparation of the analyte derivatives of AHAMP usually proceeds in high yield, and they are purified by conventional purification procedures. However, the coupling of these analyte AMP conjugates with 5′-flavine monophosphates (5′-FMN) was found to give only moderate yields of FAD conjugates. The purification procedure of the FAD conjugates was also found to be very tedious and time-consuming. In this paper, we report the results of an high-performance liquid chromatography (HPLC) study to optimize the yield of the coupling reaction. In addition, we describe a preparative HPLC method for the purification of the FAD analyte conjugates directly from the reaction mixture with minimal pretreatment.

EXPERIMENTAL

Materials and equipment

All organic solvents used in the synthesis were dried over molecular sieves. N^6-6-N(trifluoroacetamido)hexyladenosine 5′-monophosphate (TFA-AHAMP)[2] (2a), N^6-[theophylline-8-(4-butylamidohexyl)]adenosine 5′-monophosphate (Theo-AHAMP)[3] (2b) and 5′-FMN[7] were prepared or purified as described previously. Chromatographic solvents were of HPLC-grade. Both analytical and preparative HPLC were performed with the following instruments: IBM LC/5933 liquid chromatograph (IBM, Danburg, CT, U.S.A.), Beckman 165 variable UV–VIS detector (Beckman, Irvine, CA, U.S.A.), and Hewlett-Packard 3390A recorder integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.). A Whatman reversed-phase Partisil ODS-3 (C_{18}, 10 μm, 25 cm × 4.6 mm I.D.) column (Whatman, Clifton, NJ, U.S.A.)

was used for all analytical measurements. Whatman reversed-phase Partisil ODS-3 M-9 (C_{18}, 10 μm, 50 cm × 9 mm I.D.) or M-20 (50 cm × 20 mm I.D.) columns were used for the preparative work.

Kinetic Studies of the formation of conjugates

TFA-AHAMP (2a) (100 μmoles) was dissolved in 0.5 ml of dimethylformamide (DMF). The solvent was removed by evaporation. The dry residue was then dissolved in 0.5 ml of DMF. The solution was treated with 1,1'-carbonyldiimidazole (CDI, 500 μmoles, 81 mg) and then stirred at room temperature (RT) for 4 h. Methanol (0.5 ml) was added and the solution was again stirred for 5 min. After removal of all solvents by evaporation, the residue was dissolved in 0.5 ml DMF. Different concentrations of 5'-FMN was dissolved in 0.5 ml dimethyl sulfoxide (DMSO) (in concentrations of 1.5, 2.0, 2.5 or 3.0 equivalent of TFA-AHAMP). The solvents were removed by evaporation and the residue, dissolved in 0.5 ml of DMSO, was added to the phosphorylimidazolide solution in DMF. The resulting solution was incubated at either 20°C, 30°C, or 37°C. At different time intervals, 2 μl of the reaction mixture was removed and diluted with 200 μl of potassium phosphate buffer (0.02 *M*, pH = 5.5). A 5 to 10-μl aliquot of these samples was analyzed by the analytical column with a mobile phase of phosphate buffer–acetonitrile (7:3) at a flow-rate of 1 ml/min. The unreacted 5'-FMN had a retention time of 2.77 min, and the product (TFA-AHFAD-2',3'-carbonate, 3a) had a retention time of 3.91 min. The molar extinction coefficient at 450 nm was the same for both 5'-FMN and the product. The peak integration at 450 nm was then used to determine the degree of conversion of 5'-FMN and TFA-AHAMP to the product. Kinetic results are shown in Figs 3 and 4.

Preparation of N^6-(6-aminohexyl)flavin adenine dinucleotide (AHFAD) (4)

TFA-AHAMP (2a) (574 mg, 1.0 mmole) was dissolved in 5 ml of DMF. The

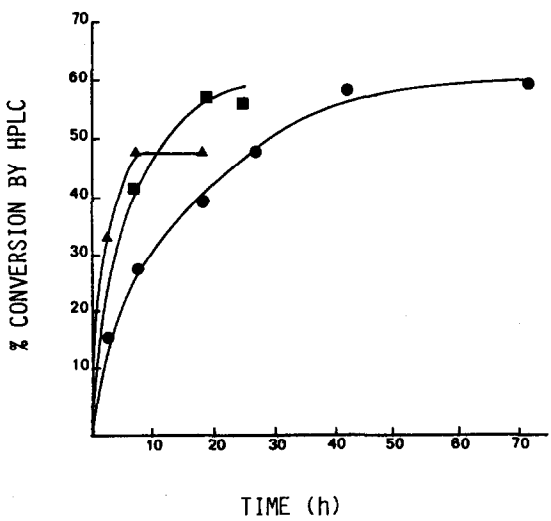

Fig. 3. Effect of temperature on the rate of formation of N^6-(trifluoroacetamidohexyl)FAD-2',3'-carbonate by phosphonylimidazolide activation. ●, 20°C; ■, 30°C; ▲, 37°C.

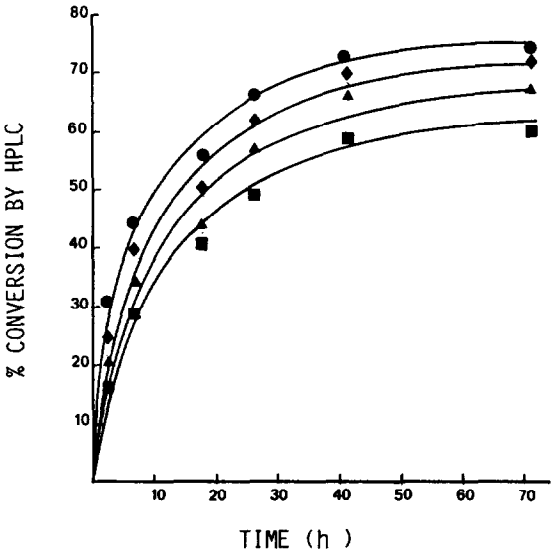

Fig. 4. Effect of 5′-FMN concentration on the rate of formation of N^6-(trifluoroacetamidohexyl)FAD-2′,3′-carbonate. ■, 1.5 equivalents; ▲, 2.0 equivalents; ◆, 2.5 equivalents; ●, 3.0 equivalents.

solvent and moisture were removed by rotary evaporation under reduced pressure (oil pump, 30°C bath). The residue was then dissolved in 5 ml of DMF. CDI (810 mg, 5 mmole) was then added and the resulting reaction mixture was capped and stirred at RT for 3 h. The excess CDI was destroyed by the addition of 5 ml of methanol and stirring at room temperature for 5 min. The solvents were then removed very carefully by rotary evaporation under reduced pressure (oil pump, 30°C bath). The residue was then redissolved in 5 ml of DMF.

5′-FMN (triethylammonium salt, 900 mg, 1.5 mmole) was dissolved in 5 ml of DMSO. The solvent and moisture were removed by rotary evaporation under reduced pressure (oil pump, 35–40°C bath). The residue was dissolved in 3 ml of DMSO and then added into the DMF reaction mixture. Two 1-ml portions of DMSO were used to rinse the 5′-FMN and were added to the reaction mixture. The resulting reaction mixture was then incubated at 30°C for 24 h. All solvents were removed under reduced pressure. The residue was dissolved in 10 ml of distilled water. The solution was filtered through a 0.45-μm membrane filter, and the filtrate was separated into two equal portions. Each portion was purified by HPLC without further delay.

The sample was applied to a Whatman reversed-phase ODS-3 M-9 HPLC column. Distilled water was used to wash the column, removing the imidazole and most of the nucleotides (Fig. 5). The column was then washed with potassium phosphate buffer (0.02 *M*, pH = 5.5)–acetonitrile (9:1) to remove the remaining nucleotides. The desired product, recovered as TFA-AHFAD-2′,3′-carbonate (3a) was eluted from the column with water–acetonitrile (75:25) and the eluate was concentrated to a small volume.

After the products from both portions were combined, distilled water was added to bring the final volume to 10 ml. The pH of the solution was adjusted to

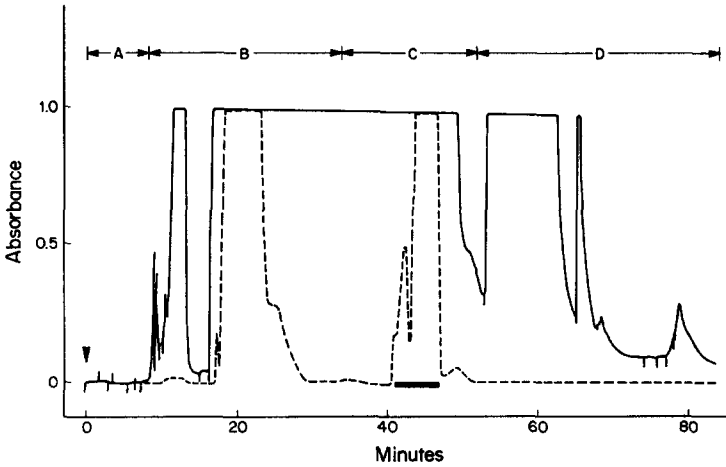

Fig. 5. Purification of TFA-AHFAD-2',3'-carbonate. Whatman Partisil ODS-3, dimensions M-9, 10-μm column. Sample, one-half of a reaction mixture of 1.0 mmole TFA-AHAMP and 1.5 mmole of 5'-FMN; flow-rate, 4.0 ml/min. Mobile phase A = water, 8 min; B = potassium phosphate buffer–acetonitrile (9:1), 26 min; C = water–acetonitrile (75:25), 18 min; D = water–acetonitrile (1:1) 33 min; detector: solid line = 230 nm, dashed line = 520 nm. Product was collected from the column in the area designated by the solid bar.

10.5 with 1.0 *N* potassium hydroxide and stirred for 24 h at room temperature, maintaining the pH at 10.5 with the aid of potassium hydroxide. The pH was then adjusted to 6.5 with 2.0 *N* hydrochloric acid and the solution was filtered through a 0.45-μm filter. The filtrate was purified on a Whatman reversed-phase ODS-3, M-20 HPLC column, which was washed with distilled water and then eluted with water–acetonitrille (92.5:7.5) (Fig. 6). Fractions containing the product were combined and freeze-dried to give a yellow powder (250 μmoles, 25% yield) of AHFAD as potassium salt.

Preparation of N^6-[Theophylline-8-(4-Butylamidohexyl)]flavin adenine dinucleotide (Theo-AHFAD) (5)

Theo-AHAMP (2b) (163 mg, 0.2 mmole) was dissolved in 1 ml of DMF. The solvent and moisture were removed by rotary evaporation under reduced pressure (oil pump, 30°C bath) and the residue was dissolved in 1 ml of DMF. CDI (160 mg, 1 mmole) was then added, and the resulting reaction mixture was capped and stirred at room temperature for 3 h. The excess CDI was destroyed by the addition of 1 ml of methanol and stirring at room temperature for 5 min. The solvents were carefully removed by under reduced pressure and the residue was dissolved in 1 ml of DMF.

5'-FMN (triethylammonium salt, 180 mg, 0.3 mmole) was dissolved in 1 ml of DMSO. The solvent and moisture were removed by evaporation under reduced pressure. The residue was dissolved in 0.6 ml of DMSO, and then added to the DMF reaction mixture. Two 0.2-ml portions of DMSO were used to rinse the 5'-FMN and were added to the reaction mixture. The resulting reaction mixture was incubated at 30°C for 24 h. All solvents were then removed under reduced pressure. The residue was distilled in 4 ml of distilled water, and the solution was filtered through a

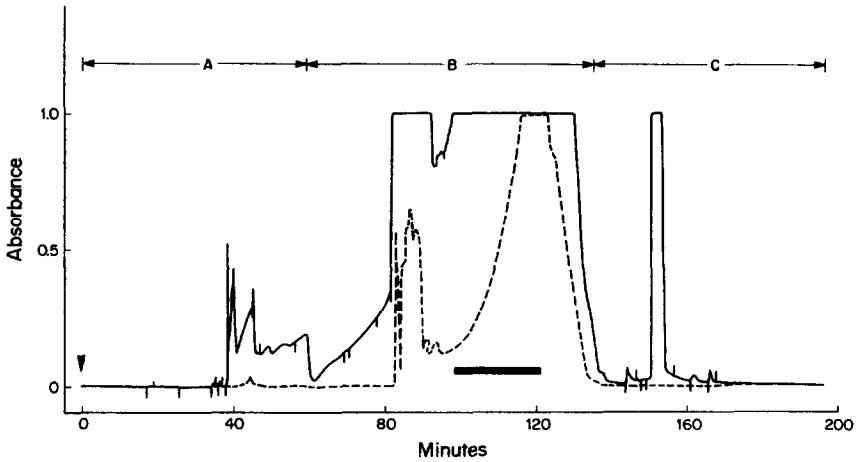

Fig. 6. Purification of AHFAD. Whatman Partisil ODS-3, M-20 dimensions, 10-μm column. Sample: reaction mixture after deblocking (about 500 μmoles); flow-rate, 10 ml/min. Mobile phase A = water, 58 min; B = water–acetonitrile (92.5:7.5), 77 min; C = water–acetonitrile (1:1), 60 min. Detector: solid line = 230 nm, dashed line = 520 nm. Product was collected from the column in the area designated by the solid bar.

0.45-μm membrane filter and the filtrate was purified by HPLC without further delay.

The sample was applied to a Whatman reversed-phase ODS-3 M-9 HPLC column. Distilled water was used to wash the column in order to remove the imidazole and most of the nucleotides (Fig. 7). The column was then washed with potassium phosphate buffer (0.02 *M*, pH = 5.5)–acetonitrile (85:15) to remove the remaining nucleotides. The desired product, recovered as Theo-AHFAD-2′,3′-car-

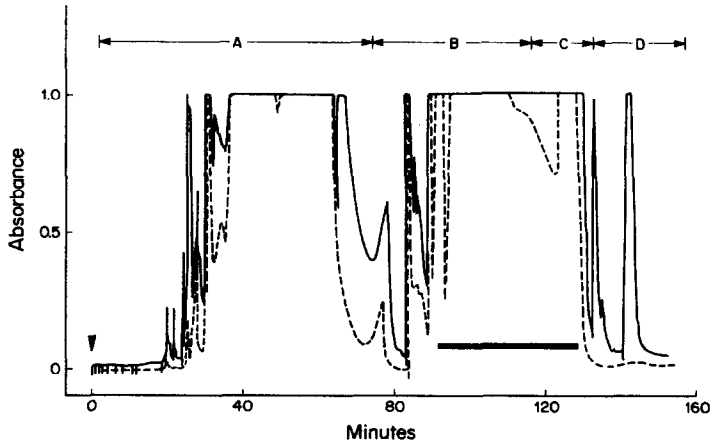

Fig. 7. Purification of Theo-AHFAD-2′,3′-carbonate by HPLC. Whatman Partisil ODS-3, 10 μm, M-9 dimensions column. Sample: reaction product from 200 μmole of Theo-AHAMP and 300 μmoles of 5′-FMN; flow-rate, 4.0 ml/min; mobile phase: A = water, 72 min; B = potassium phosphate buffer acetonitrile (85:15), 42 min; C = water–acetonitrile (75:25), 17 min; D = water–acetonitrile (1:1), 25 min; detector: solid line = 290 nm, dashed line = 500 nm. Product was collected from the column in the area designated by the solid bar.

bonate (3b) was eluted from the column with water–acetonitrile (75:25) and the eluate was concentrated to a small volume. Distilled water was added to bring the final volume to 10 ml and the pH of the solution was adjusted to 10.5 with 1.0 *N* potassium hydroxide solution. The solution was then stirred for 4 h at room temperature while the pH was maintained at 10.5 with the aid of potassium hydroxide. The pH was then adjusted to 6.5 with 2.0 *N* hydrochloric acid and the solution was filtered through a 0.45-μm filter. The filtrate was purified on a Whatman reversed-phase ODS-3, M-9 HPLC column by washing with distilled water and then eluting with potassium phosphate buffer–acetonitrile (85:15) (Fig. 8). Fractions containing the product were combined and freeze-dried to give a yellow powder (88 μmoles, 44% yield) of Theo-AHFAD as potassium salt.

RESULTS AND DISCUSSION

N^6-(6-Aminohexyl)FAD conjugates were prepared from N^6-(6-aminohexyl)-AMP derivatives according to the synthetic scheme outlined in Fig. 2. The AMP was first allowed to react with 1,1′-carbonyldiimidazole (CDI)[8] to give quantitatively the AMP-imidazolide 2′,3′-carbonate[9], which was then allowed to react with 5′-flavin mononucleotide (5′-FMN) to give the corresponding FAD derivative. Analytical HPLC was found to be very useful for establishing the optimum conditions of the formation of the pyrophosphate bond between the 5′-FMN and the imidazolide. The formation of the N^6-(6-trifluoroacetamidohexy)FAD was used as a model system for the study. The major by-products of this reaction appeared to be due to decomposition and intramolecular coupling of the AMP-imidazolide 2′,3′-carbonate. These by-products have UV absorption below 300 nm only. Hence, in the reaction mixture, only the 5′-FMN and the desired FAD product have absorption in the visible region. Since 5′-FMN and FAD product have a similar molar extinction coefficient at 450 nm, the degree of conversion of the 5′-FMN to the product can be easily monitored by the corresponding peak area integration at 450 nm without any interference from the by-products. The decarbonylation of the product during the determination was kept to the minimum by using a slightly acidic solution as the dilution buffer as well as the mobile phase. It was also found that the formation of the pyrophosphate bond was quite temperature-sensitive. At 20°C, the pyrophosphate formation was slow, reaching maximum conversion of about 60% (from AMP) at 60–70 h (Fig. 3). As expected, the formation of the product was accelerated at high temperature. At 30°C, the reaction reached its maximum conversion after 24 hours. No difference in maximum conversion was detected between 20°C and 30°C. At 37°C, an even faster reaction rate was observed. However, at this temperature the net maximum conversion was found to be less than that at lower temperature. This may be explained by the observation of a rapid decrease in product concentration upon incubation at 37°C, presumably due to an acceleration in the rate of product decomposition. From this data, it was determined that the optimum reaction conditions for the formation of the pyrophosphate were at 30°C and 24 h. The effect of the concentration of 5′-FMN was also studied. Increasing the concentration of the 5′-FMN increased the conversion only moderately. A 15% increase of the maximum conversion was observed when the concentration of the 5′-FMN was increased from 0.15 *M* to 0.3 *M* (Fig. 4). The concentration of 0.15 *M* was therefore selected for most of the preparative work.

Kinetic study of the pyrophosphate bond formation was also performed with other FAD analyte conjugates than the two described here. Similar temperature and concentration effects were also observed for most of those reactions. However, the maximum conversion was found to be strongly dependent on the nature of the analyte moiety of the conjugate.

HPLC was also found to be the method of choice for the purification of the FAD conjugates. Only two steps were required to purify the product, and sample pretreatment was minimal. Since a large difference in retention times was observed among the unreacted starting materials, by-products, and the FAD-2',3'-carbonate, it was important to isolate the FAD-2',3'-carbonate in the first purification step in the case of some of the FAD conjugates. The carbonate and other protecting groups on the FAD derivatives were removed by alkaline treatment. The final product was then isolated in the second purification step.

In the preparation of the N^6-(6-aminohexyl)FAD from a reaction mixture from 500 μmoles (287 mg) of TFA-AHAMP and 750 μmoles (450 mg) of 5'-FMN, the M-9 column was used for the first purification step (Fig. 5). A M-20 column was then used for the second purification of about 500 μmoles of crude product after alkali treatment to remove the carbonate and the TFA groups (Fig. 6). A total of 250 μmoles (310 mg, 25% yield) of pure AHFAD was recovered. The isolated yield was lower than the observed HPLC conversion, due to both the incomplete removal of the N-TFA protecting group and the partial decomposition of product during the required alkali treatment.

For the preparation of the Theo-AHFAD, the M-9 column was used in the first purification step for a reaction mixture of 200 μmoles (162 mg) of Theo-AHAMP and 300 μmoles (180 mg) of 5'-FMN (Fig. 7). After the crude product was treated with alkali to remove the carbonate (Fig. 8), the same column was used for the second purification step. A total of 88 μmoles (44% yield) of pure Theo-AHFAD was re-

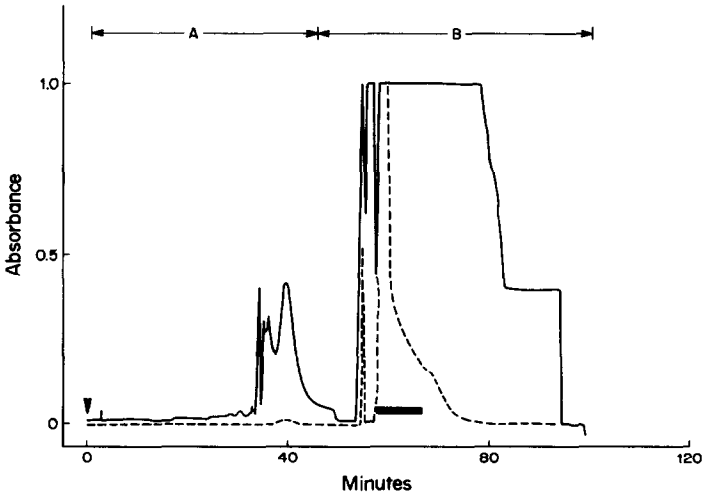

Fig. 8. Purification of Theo-AHFAD. Whatman Partisil ODS-3, 10 μm, M-9 dimensions column. Sample: reaction mixture after alkaline treatment (about 50 μmoles); flow-rate, 4 ml/min; mobile phase A = water, 45 min; B = potassium phosphate buffer–acetonitrile (85:15), 55 min; detector: solid line = 290 nm, dashed line = 510 nm. Product was collected from the column in the area designated by the solid bar.

covered. Comparison of this purification procedure to that initially reported[2] shows the new method to be considerably faster and more reproducible.

The FAD conjugates purified by this method were found homogeneous by analytical HPLC having a purity of >99% (at both 210 and 450 nm). The compounds were characterized by UV–VIS, ^{1}H, ^{13}C and ^{31}P NMR spectra. The correct molecular ions were also observed with high resolution FAB mass spectra. In biochemical assays, it was found that the FAD analyate conjugates were consistently equal to or better than the conjugates which were purified by the original isolation method[2].

AHFAD has found use as a labeling reagent for the preparation of proteins and other FAD analyte conjugates in which the analyte is unstable, *e.g.* penicillin[10]. The conjugate Theo-AHFAD is being used as a key component in the manufacture of the theophylline immunostrip test[5,6] for the Ames Seralyzer®.

By using our general preparation and purification method, other FAD analyte-conjugates were prepared for the development of assays for analytes in serum comprising several therapeutic classes of compounds, such as the antiarrythmics, anticonvulsants, and antidepressants. In addition, FAD conjugates of hormones and proteins of clinical importance, such as thyroxine, triiodothyronine, human chorionic gonadotropin and immunoglobulin G, have also been prepared.

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