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Use of reversed phase HP liquid chromatography to assay conversion of *N*-acylglycines to primary fatty acid amides by peptidylglycine-α-amidating monooxygenase

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Abstract

Primary fatty acid amides (R-CO-NH_2) and *N*-acylglycines (R-CO-NH-CH_2-COOH) are classes of compounds that have only recently been isolated and characterized from biological sources. Key questions remain regarding how these lipid amides are produced and degraded in biological systems. Relative to the fatty acids, little has been done to develop methods to separate and quantify the fatty acid amides and *N*-acylglycines. We describe reversed phase HPLC methods for the separation of C2–C12 primary fatty acid amides and *N*-acylglycines and also C12–C22 fatty acid amides. Separation within each class occurs primarily on the basis of simple interactions between the acyl chain and the chromatographic stationary phase, but the polar headgroups on these and related fatty acids and *N*-acylethanolamides modulate the absolute retention in reversed phase mode. We use these methods to measure the enzyme-mediated, two-step conversion of *N*-octanoylglycine to octanoamide.

Keywords: *N*-Acylglycines; Fatty acid amides; Peptidylglycine-α-amidating monooxygenase

1. Introduction

Fatty acid amides are an expanding family of mammalian, cell-signaling lipids. The members of this family include the *N*-acylethanolamines (NAEs, also called the *N*-(2-hydroxyethyl)-acylamides or *N*-acylethanolamides, the most famous member of which is anandamide) and the primary fatty acid amides (PFAMs) [1–4]. Of these two, the NAEs were the first to be isolated from a biological source [5] and have been widely studied [3,4]. The functions served by specific NAEs are understood and the pathways for the production and degradation of the NAEs in the brain have been elucidated. The PFAMs have been added only recently to the fatty acid amide family [6,7]. As a consequence, the body of knowledge concerning the PFAMs is not as extensive as that for the NAEs. The function of most of the PFAMs and the enzymes responsible for PFAM biosynthesis are unknown. In addition to their biological role, PFAMs are employed as slip additives in the plastics used to package foods [8,9].

Merkler et al. [10] proposed that *N*-acylglycines (NAGs) are also members of the fatty acid amide family, serving as biosynthetic precursors to the PFAMs. In vitro, oxidative cleavage of the NAGs by peptidylglycine α-amidating monooxygenase (PAM) yielded the corresponding PFAM and glyoxylate [10,11] (Fig. 1). The recent isolation and identification of *N*-arachidonoylglycine from bovine and rat brain [12] and tick salivary glands [13] supports the hypothesized role for PAM in PFAM biosynthesis. Fig. 1 also shows the metabolic fate of fatty acid amides [14].

Analytical methods for the detection of the neutral lipids, including NAEs, NAGs, and PFAMs, have largely employed gas chromatography, usually coupled with mass spectrometry (GC/MS) [6,7,15–22] or, especially for short-chain NAGs in urinary and blood samples in newborns, electro-

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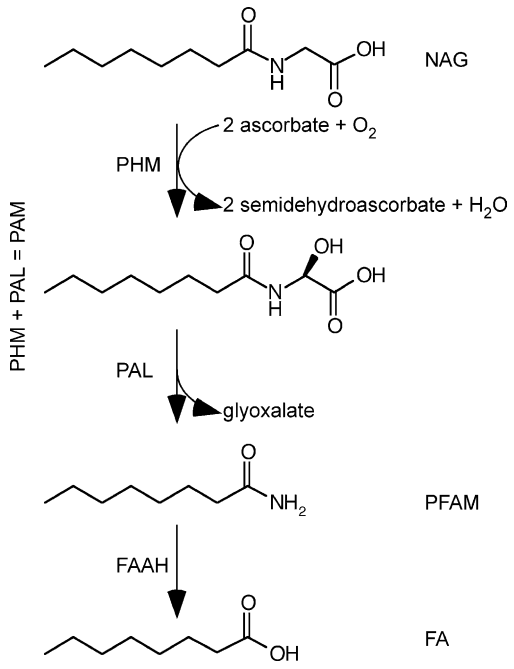

Fig. 1. A portion of the putative biosynthetic pathway for the production of primary fatty acid amides (PFAM) from *N*-acylglycines (NAG), illustrated with the production of octanoamide from *N*-octanoylglycine. Enzymes PHM (peptidyl α-hydroxylating monooxygenase) and PAL (peptidylamidoglycolate lyase) are domains of PAM that catalyze the separate reactions. FAAH is fatty acid amidohydrolase.

spray MS [23,24]. While GC/MS is an excellent technique for lipids, it is well complemented by liquid chromatography and mass spectrometry [25–29]. Liquid chromatography of NAEs and PFAMs has been used primarily for isolation of single compounds (e.g., oleamide) from biological samples, usually as part of multiple isolation steps [30–34], or for class screening [35]. Normal phase HPLC on spherical silica has been used to determine some amides in fatty nitrile mixtures [36]. A more systematic study of the liquid chromatography of PFAMs was accomplished to show specificity for oleamide [37].

This work details the development of methods to separate a series of *N*-acylglycines (C2–C12) and a series of PFAMs (C2–C22) using reverse phase HPLC with UV detection with an eye to determining their basic chromatographic properties as a series and relative to one another. This knowledge was put to use by creating a method to monitor the PAM-mediated conversion of the *N*-acylglycines to the PFAMs.

2. Experimental

2.1. Standards and reagents

All solvents for HPLC were HPLC grade from Fisher Scientific (Pittsburgh, PA). Butyramide was obtained from Eastman Organic Chemicals (Rochester, NY), hexanoamide was obtained from Aldrich Chemical Company (Milwaukee, WI), acetamide and capramide were obtained from Sigma Chemical Company (St. Louis, MO), and octanoamide and lauramide were obtained from TCI America (Portland, OR). The remaining fatty acid amides were synthesized from the corresponding fatty acids, which were obtained at high purity from either Aldrich or Acros/Fisher. *N*-Lauroylglycine and *N*-decanoylglycine were obtained from Nova Biochem (San Diego, CA), *N*-acetylglycine and *N*-octanoylglycine were obtained from Aldrich. Recombinant rat PAM was expressed in Chinese hamster ovary cells and purified as described in [38]. *N*-Acetyl-phenylalanylpyruvate (*N*-Ac-Phe-pyruvate, 2,4-diketo-5-acetamido-6-phenyl-hexanoic acid) was synthesized following a literature procedure [39].

2.2. Chromatographic system

The HPLC system used for the separation of NAGs and initial separations of PFAMs was a Hewlett Packard (Palo Alto, CA) 1100 Series liquid chromatograph equipped with a quaternary solvent delivery system, vacuum degasser, thermostatted column compartment, an auto injector, and a Hewlett Packard 1100 Series variable wavelength detector. A Hewlett Packard ChemStation controlled the system and detected the output signal. The HPLC system used for subsequent separation of PFAMs was a Waters 600 quaternary gradient system with manual injector, helium sparge degassing, and a Waters 2487 dual wavelength detector (Milford, MA) operated at 210 nm (PFAMs) or 214 nm (NAGs). Data was analyzed using Waters Millenium software.

The column for separation of NAGs and short-chain PFAMs was a Phenomenex Luna C8 column (250 mm × 4.6 mm i.d., 5 μm) fitted with a Phenomenex C18 Security Guard column (Torrance, CA). All injection volumes were 10 μL. The solvent system for the separation of NAGs consisted of (A) 50 mM sodium phosphate, pH 6.0 and (B) acetonitrile. The initial mobile phase was 95% A, 5% B with a linear gradient in 17 min to 25% A, 75% B. After 17 min, the mobile phase was returned to the initial mobile phase parameters for 6 min before the next sample injection. The flow rate was 0.8 mL/min. Separations of PFAMs (C2–C12) on the C8 column used a solvent system consisting of (A) deionized water and (B) acetonitrile. The initial mobile phase was 75% A, 25% B with a linear gradient of 15 min to 100% B followed by isocratic elution for 1 min at 100% B. After 16 min, the mobile phase was re-equilibrated to the initial mobile phase parameters for 6 min before another sample was injected. The flow rate was 0.8 mL/min. Detection was performed at 214 nm.

Isocratic separations of PFAMs used a Waters XTerra RP18 column (150 mm × 4.6 mm i.d., 5 μm; Milford, MA) operated at ambient temperature. Acetonitrile and deionized water for the mobile phase were filtered through 0.22 μm nylon membrane filters and degassed by helium sparge for 20 min at 100 mL/min followed by 30 mL/min throughout the experiment. Elution was performed isocratically at 1.0 mL/min with varying percentages of the two solvents.

Final separations of long-chain PFAMs used a Waters XTerra RP18 column (150 mm × 3.9 mm i.d., 5 μm) with (A) deionized water and (B) methanol. The gradient was 10% A and 90% B to 100% B with a linear gradient over 20 min followed by a 5 min hold at 100% B. PFAMs were injected individually to obtain retention times and then as a mixture for separation. This same system was used to separate mixtures of saturated and unsaturated PFAMs, but the gradient was run over 25 min.

2.3. PAM enzymatic conversion of N-acylglycines to amides

For the conversion of *N*-octanoylglycine to octanoamide, reactions were carried out at 37°C and were initiated by the addition of 43 μg PAM into 300 μL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 μg/mL bovine catalase, 1.0 μM $Cu(NO_3)_2$, 5.0 mM sodium ascorbate, and 5.0 mM *N*-octanoylglycine. Aliquots (30 μL) of the reaction mixture were added to an HPLC microvial containing 20 μL of 6% (v/v) trifluoroacetic acid to terminate the PAM reaction. The acidic aliquots were assayed for octanoamide formation by HPLC using the C8 column using the program given above for PFAMs but followed by a 2 min hold at 100% B. After 25 min, the column was re-equilibrated to the initial mobile phase conditions for 10 min to prepare for the next injection. Lauramide conversions were the same, but 53 μg of PAM was used, aliquots of the reaction mixture were 100 μL, incubation was for 3 h, and the separation used the NAG program for the C8 column, followed by an 8 min hold.

For the enzymatic conversion of *N*-octanoylglycine to octanoamide in the presence of inhibitor, the reaction was initiated by the addition of 42.5 μg PAM into 50 μM *N*-Ac-Phe-pyruvate (the PAL inhibitor), 5.0 mM *N*-octanoylglycine, 100 μL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 μg bovine catalase, 1.0 μM $Cu(NO_3)_2$, and 5.0 mM sodium ascorbate. The reaction was incubated at 37 °C for 2.5 h. After this time, an aliquot was removed and analyzed by HPLC using the HPLC conditions as listed above.

3. Results and discussion

3.1. Separation of N-acylglycines and primary fatty acid amides

Fig. 2 shows the separation of short-chain *N*-acylglycines and fatty acid amides. Note that the annotations refer to the number of carbons in the acyl chain, so the C2 NAG actually has four carbons (**C**H_3-**C**O-NH-CH_2-COOH). The separations are run under similar conditions on a Phenomenex C8 column of 14.75% C load, 5.5 μmol/m^2. Peak shapes were good, and there was enough separation that the gradient could be increased for shorter elution times. The baseline disturbances, particularly in the amide separations, were probably impurities from the synthetic process. Fig. 3 shows

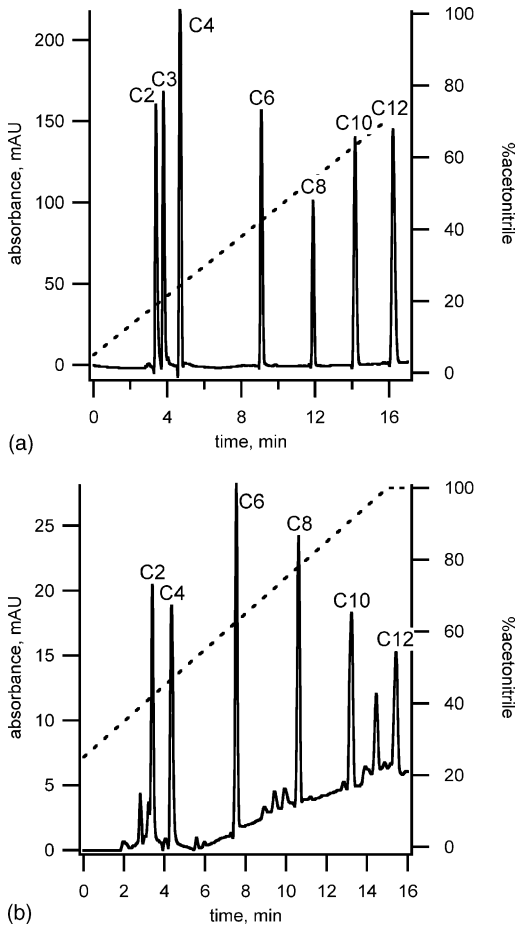

Fig. 2. Separation of short-chain lipid amides. Peaks are identified according to the number of carbons in the saturated acyl chain. (a) *N*-Acylglycines and (b) fatty acid amides. The gradient on the C8 column is shown graphically; the other mobile phase component is (a) 50 mM aqueous phosphate buffer and (b) deionized water.

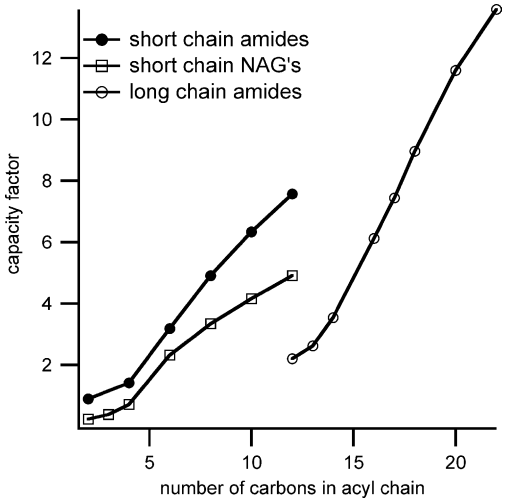

Fig. 3. Capacity factor of short-chain NAGs and PFAMs and long-chain fatty acid amides under gradient elution. Short-chain data was taken from Fig. 2 data. The long-chain amides are separated using a gradient on the Waters Xterra C18 column of 10–0% water in methanol over 20 min. The actual separation was very similar to those shown in Fig. 2 with respect to peak shape and efficiency, but the baseline was more pronounced.

the capacity factors from the separation shown in Fig. 2 and for a number of long-chain saturated PFAMs separated under a gradient that was similar to those used for the short chain compounds. The column in the latter case was a Waters Xterra C18 column of 15% C load and 2.2 μmol/m^2, and the gradient was in methanol rather than acetonitrile. The separation was begun at a higher level of the strong solvent than for the short-chain amides, and a slightly stronger solvent was used, in deference to the facts that the column was more hydrophobic, the compounds themselves were more hydrophobic, and solubility of saturated amides larger than about 16 carbons was very limited in solutions with any aqueous content. This plot showed that the intermolecular forces that dominated the separation at higher carbon numbers were directly related to number of carbons. The most plausible explanation was that London dispersion forces between the carbon chain and the stationary phase were essentially the only change as a function of carbon number for a given series of compounds.

A wide range of isocratic gradient conditions were applied, and it was clear that the separation was highly dependent on the exact amount of water in the starting mixture and on the shape of the gradient. For the Xterra C18 column, the gradient used for the data shown in Fig. 3 (10% water in methanol to 100% methanol over 20 min and a hold for 5 min) was close to optimum; changes of the gradient of 5–10 min in length and water content as high as 15% were sufficient to change the separation significantly.

The separation of PFAMs and NAGs can be discussed with analogy to reversed-phase separation of free fatty acids. There is a considerable body of literature on the separation of fatty acids, though, realistically, only separations done with more modern columns are relevant. The reader is referred to several recent reviews [26,40,41]. It is nearly impossible to compare individual gradients on different columns, but it is clear from literature surveys that a general rule for separation of medium-to-long chain fatty acids (C10–C22) is to use a gradient of about 85% methanol to 100% methanol in acidified water or low-pH buffer (for ion suppression). Stronger solvents can be used for so-called very long chain fatty acids [27]. With such gradients, it is possible to separate an impressive array of saturated and unsaturated fatty acids [26,27,40,41], though closely related isomers, especially of conjugated species, may need relatively high capacity factors [42,43]. Given the sensitivity to gradient conditions, the water content and gradient shape can be tweaked to achieve most separations of critical pairs, though perhaps not throughout the full range of chain length. Fig. 4 shows the separation of such a critical pair, palmitamide (C16:0) and oleamide (C18:1), along with stearamide. This separation was done at a 20% lower gradient slope than the separations represented in Fig. 3, but resolution was baseline ($R = 1.49$).

Given the relative separation of the saturated NAGs and PFAMs demonstrated in Fig. 2, it was possible to develop separations that exploited headgroup polarity differences for an assay for PAM (see Fig. 1). The amides required a stronger gradient to elute them in a time frame similar to that of the NAGs. The additional polarity imparted by the CH_2-COOH headgroup on the NAGs subtracted effectively about two carbons from the corresponding amide; that is, a C8 amide and a C10 NAG should elute at approximately the same time in a mixture or when run with the same gradient. The net effect was approximately the same as that of a double bond, leading to the same potential "critical pair" issues if attempting to separate a mixture of PFAMs and NAGs. The same was approximately true of fatty amides and fatty acids, with the acids eluting later. Fig. 5 shows a sep-

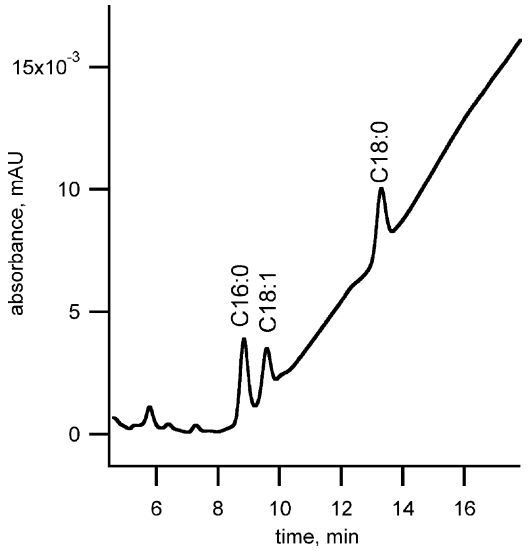

Fig. 4. Separation of a fatty acid amide critical pair on the Waters Xterra C18 column with a gradient of 10–0% water in methanol over 25 min. Peak designations refer to number of carbons in the chain and the degree of unsaturation; C16:0 is palmitamide, C18:1 is oleamide, and C18:0 is stearamide.

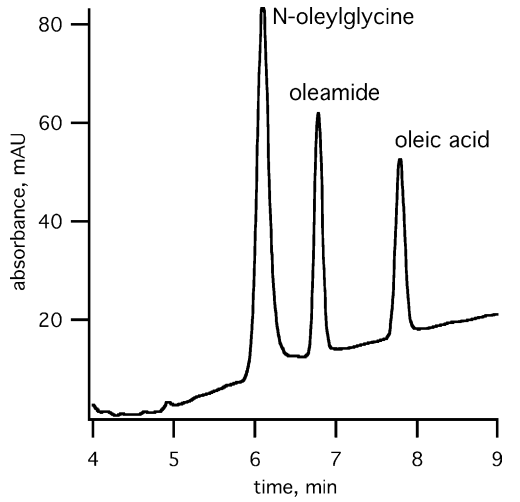

Fig. 5. Separation of C18 analogs. Separation on a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 μm) with a gradient of 95:5 to 50:50 acetonitrile:methanol over 10 min at 0.8 mL/min. All compounds at 5 mM; 10 μL injection.

aration of *N*-oleyl analogs. This separation showed clearly that, for a given chain length, head group polarity differences were easily large enough to be exploited for a general assay for PAM. Thin-layer chromatography on silica gel and NP-HPLC have confirmed this polarity ranking [35,44]. Aside from implications for separations of general mixtures of lipids, this finding made it possible to use a simple HPLC method for the assay of PAM activity (see below).

3.2. Quantitation of amides

In order to apply these methods to enzyme assays, the method must be quantitative and reproducible. Calibration curves were generated using *N*-octanoylglycine and octanoamide from 1 to 150 nmol injected (0.1 to 15 mM) and were linear in peak height and peak area versus mass injected for both the *N*-acylglycine (for height: $y = 56.38 \pm 16.2 + 3.99 \pm 0.21x$, $r^2 = 0.986$, for area: $y = 138.81 \pm 75.4 + 47.18 \pm 0.98x$, $r^2 = 0.998$) and the PFAMs (for height: $y = 0.021 \pm 0.996 + 1.37 \pm 0.019x$, $r^2 = 0.999$, for area: $y = 0.20 \pm 6.77 + 8.58 \pm 0.13x$, $r^2 = 0.999$). All plots have seven data points and were generated from three injections at each concentration. Precision of peak area ranged from 3% R.S.D. at 5 nmol injected to less than 1% R.S.D. above 10 nmol for PFAMs; precision was slightly worse for NAGs. Detection limits were somewhat difficult to determine in the usual way because of excessive low frequency noise in the baseline, but estimates placed detection limits at about 100 μM (1 nmol). These parameters were quite adequate for kinetic assays of PAM under physiological conditions. The use of methanol unfortunately caused baseline change and a higher background late in the separation. This problem is solvable with other detector types, such as evaporative light scattering.

3.3. Application to the assay of PAM

Because reverse phase HPLC can exploit selectivity based on differences in polarity of the reactants in the PAM reaction (above), HPLC can be used in a relatively simple assay of PAM reactivity towards a variety of lipid substrates. Fig. 6 shows typical HPLC assays of the PAM reaction in

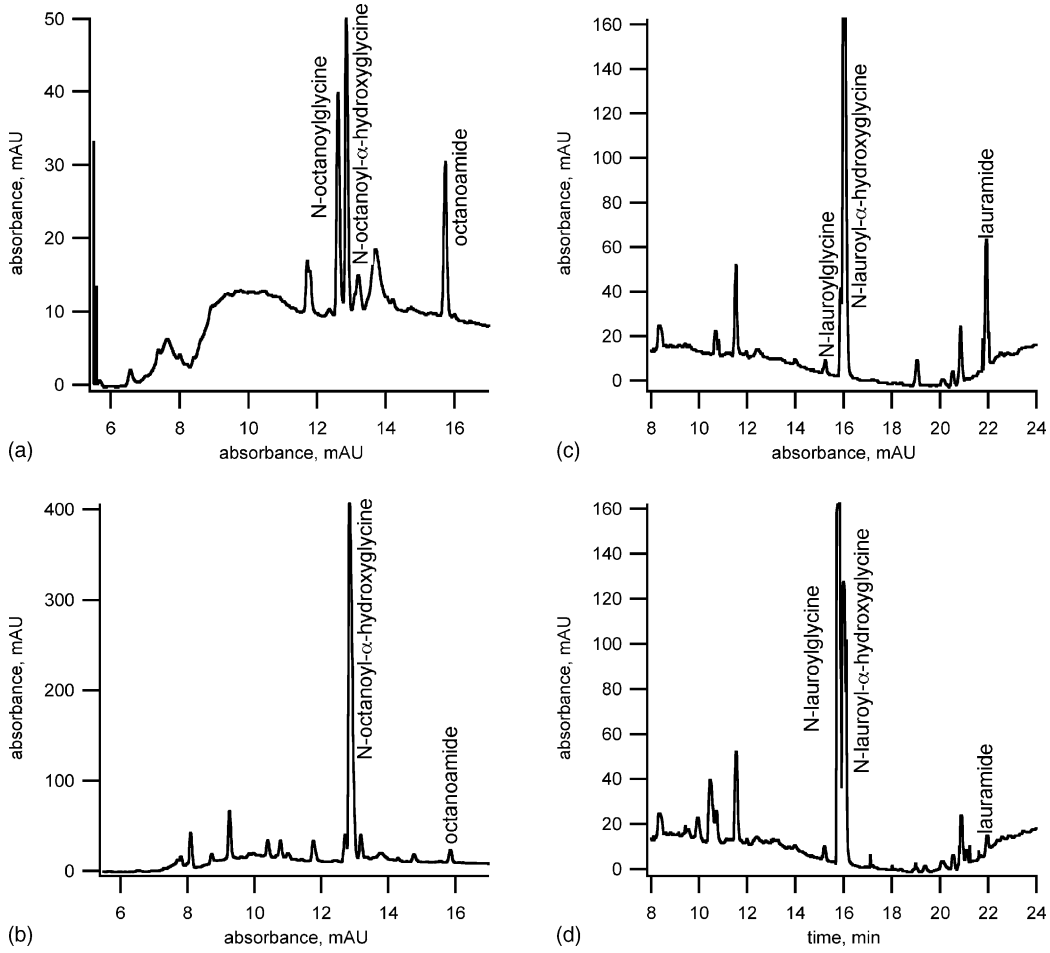

Fig. 6. PAM (PAL domain) assay by HPLC showing reactants, intermediates, and products for C8 (a, b) and C12 (c, d). (a, c) Separation of acidified reaction aliquot taken at 2.5 h of reaction under conditions specified in the text. (b, d) Separation of acidified reaction aliquot taken at 2.5 h (b) and 3 h (d) of reaction in the presence of a PAL inhibitor. See Section 2 details.

progress. Fig. 6a and c show snapshots of the reaction for C8 and C12. Clearly, all three species (substrate, intermediate, and product) were quantifiable. The background was relatively low and stable, considering the very simple sample preparation scheme. Reaction species were separated from interferents, so the peaks were readily quantifiable.The sample preparation scheme would probably have to be modified for use in vivo assays. Fig. 6b and d show the reaction with inhibition of the PAL domain of PAM; even after 2.5–3 h of reaction, the same as in Fig. 6a and c, very little amide was formed. These results demonstrated that, even with the relatively poor sensitivity of the detector used here, there was enough signal-to-noise ratio to discern the small amount of amide that was formed.

4. Conclusion

The separation of fatty acid amides and *N*-acylglycines by HPLC has been explored in the context of similar separations of related lipids. Aside from the potentially general utility as a method for determining these compounds, we showed that specific differences in polarity of fatty headgroups resulting from the action of PAM on NAGs can be exploited for a simple HPLC assay for the enzyme. The results also suggest that other substrates for PAM could be assayed in this way with only a change in gradient conditions or column to provide reasonable overall capacity factors. The assay developed herein for fatty substrates provides a straightforward, rapid assay of PAM activity, the sensitivity of which could be increased by the use of an evaporative light scattering detector or radiolabeled substrates and a radiochemical detector. Better sample preparation will be required for biological samples; a solid-phase extraction method can be developed from similar methods [45] and thin-layer chromatography results [44]. In addition, these HPLC methods have the potential to complement GC/MS methods for analysis of amides and, more generally, in lipid profiling applications that involve primary and secondary amides.

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