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Fluorogenic derivatization of peptides with naphthalene-2,3-dicarboxaldehyde/cyanide: optimization of yield and application in the determination of leucine-enkephalin spiked human plasma samples

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Abstract: Initial attempts to derivatize the α-amino site of several tripeptides with naphthalene-2,3-dicarboxaldehyde/cyanide (NDA/CN) resulted in poor yields of the expected *N*-substituted 1-cyanobenz[f]isoindole (CBI) products. Examination of the CBI-formation mechanism, in conjunction with knowledge of the general structure–reactivity properties of the tripeptides, led to the recognition of a competing non-productive reaction pathway. Through the use of model reactions and the isolation and structural elucidation of a predicted side-product the viability of the competing pathway was confirmed. From an understanding of the key features of both the productive and non-productive reaction pathways, a rational approach for the optimization of CBI-derivative yield was proposed and confirmed experimentally. This information led, in turn, to the development of HPLC methodology suitable for the determination of leu-enkephalin spiked into human plasma; fluorescence detection was used in conjunction with leu-enkephalin amide as the internal standard. The method enabled leu-enkephalin to be determined at a concentration of 0.31 nmol ml^{-1} with an error of <4% for 25 pmol injected.

Keywords: *Naphthalene-2,3-dicarboxaldehyde; derivatization optimization of α-amino peptide groups; fluorescence detection; leu-enkephalin plasma determination.*

Introduction

In recent years many naturally occurring peptides exhibiting biological activity have been isolated and identified [1]. For example, leu-enkephalin (LE) and met-enkephalin (ME), two members of the opioid peptide family [2] were discovered by Hughes *et al.* in 1975 [3]. Initially, the determination of opioid peptides in biological samples such as brain tissue, cerebrospinal fluid or plasma was accomplished using direct RIA [4, 5]. In order to achieve greater specificity, HPLC was first used in conjunction with several off-line detection techniques. Later, on-line detection methods emerged in an effort to facilitate sample processing. The most frequently utilized off-line approaches have been bioassay [6], RIA [7] and mass spectrometry [8]. While these off-line techniques provide the required sensitivity and contribute to the selectivity, they are limited by the need for fraction collection and subsequent processing prior to quantitation. For example, extensive preparation is needed to conduct a bioassay, antibodies must be raised and labelled for RIA, and mass spectrometry involves the operation of a sophisticated instrument which may not be available routinely to many laboratories. Previously, several HPLC methods utilizing sensitive on-line detectors have been reported. In general, these approaches have been based on electrochemical [9, 10] or fluorescence detection [11–13], and with conventional chromatographic columns exhibit pmol detection limits.

Recently, the fluorogenic derivatization of primary amino acids with naphthalene-2,3-dicarboxaldehyde/cyanide (NDA/CN) to form efficiently fluorescent 1-cyanobenz[f]isoindole (CBI) derivatives has been described in these laboratories [14–16]. NDA/CN represents an

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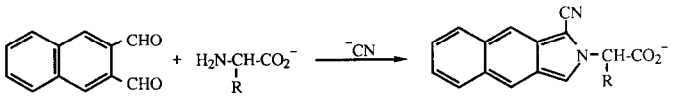


NDA CBI-derivative

extension of the *ortho*-phthalaldehyde (OPA) reagent [17–19], which when used in conjunction with HPLC has become one of the most widely utilized approaches for amino acid analysis [20]. In comparison to the products formed from derivatization with OPA, the NDA/CN products exhibit improved chemical stability [14], excitation maxima (420 and 440 nm) in the visible region [14], efficient chemiluminescence by the peroxyoxalate method [21], and efficient fluorescence quantum efficiency for amino acid and peptide derivatives [15]. Based on the known properties of CBI-derivatives, it appeared that NDA/CN would be a valuable pre-column reagent for the on-line HPLC detection of small peptides. However, in preliminary experiments the reaction yield previously observed for α-amino acids [14] was not realized for α-amino derivatization of small peptides. Therefore, the objectives of the present research were to investigate and optimize the NDA/CN derivatization of the α-amino moiety of small peptides and, utilizing this information, to establish methodology suitable for the liquid chromatographic determination of LE in human plasma.

Experimental

Apparatus

Spectrophotometric measurements were performed with a Perkin–Elmer Model 555 instrument equipped with a cell holder at 25.0 ± 0.1°C. Infrared, mass and ^{13}C NMR spectra were obtained on Beckman Acculab 3, Ribermag 10–10 and Varian XL 300 instruments, respectively. Melting points (uncorrected) were determined using an Electrothermal melting point apparatus. Liquid chromatography experiments were conducted on a modular HPLC system consisting of the following components: a LKB Model 2150 pump, a Rheodyne Model 7125 fixed loop injector (50 μL), and a Kratos Spectroflow 980 fluorescence monitor (tungsten–halogen source) or a Waters Associates Model 440 absorbance detector. The following HPLC columns were used in these investigations: ODS Hypersil (5 μm, 250 × 4.6 mm i.d.) for kinetic and product chracterization studies; and coupled ODS Hypersil columns (5 μm, 50 × 4.6 mm i.d. + 3 μm, 250 × 4.6 mm i.d.) for LE calibration experiments. These columns were packed using an upward slurry technique. The chromatograms were recorded with a Houston Instruments Omniscribe strip-chart recorder with quantitation being accomplished by manual peak height measurements. The pH-rate profile for CBI-(LE) formation was established using a Model 650-40 spectrofluorimeter (Perkin–Elmer) interfaced to a Model 3600 data station.

Chemicals and reagents

The amino acids and peptides were obtained from Sigma Chemical Company and used as received. Benzaldehyde, obtained from Fisher Scientific, was distilled and stored in an amber vial under nitrogen prior to use. The NDA, provided by Professor R.G. Carlson (Chemistry Department, University of Kansas), was prepared by a standard procedure [22]. The water was de-ionized in mixed-bed ion-exchange columns and was subsequently distilled from an all-glass apparatus. All other chemicals were of the highest purity available and used as received. Human plasma was obtained from the Community Blood Center (Kansas City, USA).

Solutions

Buffers. The phosphate buffers were prepared in the following manner: pH 3.0 (0.05 M) potassium phosphate was prepared by dissolving the required quantity of potassium dihydrogen phosphate in water and titrating the resulting solution with concentrated phosphoric acid; pH 6.8 (0.5 M) potassium phosphate was prepared by dissolving equimolar amounts of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in water; pH 6.0, 6.5, 7.0, 7.5 and 8.0 (0.25 M) potassium phosphate was pre-

pared for the pH-rate profile experiments by blending equimolar solutions of the appropriate species. The pH 9.5 (0.1 M, μ = 0.5 M) sodium borate buffer was prepared by dissolving boric acid in water, adding the appropriate quantity of sodium chloride (boric acid pK_a = 8.96 at 25°C and μ = 0.5 M) and titrating to the required pH with sodium hydroxide (5 M).

Mobile phases. HPLC mobile phases were prepared by filtering the appropriate buffer solution through a 0.45-μm cellulose acetate membrane filter and then mixing with required amount of pre-filtered organic modifier and degassing prior to use.

Reagents and peptides. NDA stock solutions (5 mM) were prepared daily by dissolving the required amount of NDA in acetonitrile. Sodium cyanide stock solutions (10 mM) were prepared daily by dissolving the appropriate quantity of the salt in pH 6.8 potassium phosphate buffer. Peptide standard solutions were prepared by dissolving LE (0.5 mM) and leu-enkephalinamide (LEA) (0.1 mM) in pH 6.8 potassium phosphate buffer.

Kinetic investigations

Imine formation and stability. The formation of imine derivatives from reaction of benzaldehyde with an amino acid (gly) and several peptides (gly-gly, gly-gly-gly, and gly-pro-ala) was investigated spectrophotometrically (270 nm). These experiments were conducted by reacting benzaldehyde (1.0×10^{-4} M) with the amine (3.3×10^{-2} M) in pH 9.5 (0.1 M, μ = 0.5 M) sodium borate buffer at 25.0 ± 0.1°C.

CBI-derivative versus imidazolidinone side-product formation. The relative yield of CBI-derivative and imidazolidinone side-product (**6c**) formed from reaction of NDA with gly-gly-NH_2 was determined as a function of cyanide concentration. In order to observe both products it was necessary to utilize UV detection, thus necessitating the use of NDA as the limiting reactant. The reactants were initially present in pH 7.0 phosphate buffer at the following concentrations: gly-gly-NH_2, 1.0×10^{-3} M; NDA, 5.0×10^{-5} M and NaCN, $1.0–100.0 \times 10^{-4}$ M. The mobile phase comprised acetonitrile–phosphate buffer (pH 6.8, 50 mM) (28:72, v/v). To minimize analysis time, the flow rate was set at 1.0 ml min^{-1} for 5 min, then adjusted to 2.0 ml min^{-1} for 10 min.

CBI-(LE) pH-rate profile. The rate of formation of the CBI-(LE) derivative was established spectrofluorimetrically as a function of pH (6.0–8.0, in 0.5 unit increments) in potassium phosphate buffer at 25°C. The reaction was initiated by the addition of LE (60 μl, 2.5×10^{-4} M), sodium cyanide (30 μl 2.0×10^{-2} M) and NDA (30 μl, 1.0×10^{-2} M) to the potassium phosphate buffer (2.88 ml) in a quartz cuvette. The derivatization was monitored with 420 and 490 nm as the excitation and emission wavelengths, respectively. The rate constants were obtained by evaluation of the resulting data with a nonlinear curve-fitting program.

Preparations

In situ *generation of the gly-gly-NH_2 imidazolidinone.* The product was generated *in situ* by adding a solution of NDA (50 μ, 5.0×10^{-3} M) and gly-gly-NH_2 (50 μl, 0.1 M) to a sufficient quantity of pH 9.0 borate buffer to result in a 1.0 ml reaction volume. The reaction was conducted at ambient temperature and the progress monitored by HPLC using a mobile phase comprising acetonitrile–phosphate buffer (pH 3.0, 50 mM) (20:80, v/v) with a 1.0 ml min^{-1} flow rate.

Isolation and characterization of the gly-gly-NH_2 imidazolidinone. The product was prepared by dissolving gly-gly-NH_2 (167.6 mg, 1 mmol) in a solution of sodium hydroxide (20 ml, 0.1 M) and then adding NDA (184 mg, 1 mmol) which had been previously dissolved in dioxane (80 ml). The reaction was shielded from light and stirred at ambient temperature for 48 h. After this time, approximately 90% of the solvent was removed *in vacuo* with gentle heating. Subsequent cooling led to crystallization of the product. The solid was collected, sequentially washed with water and ethyl acetate, then dried to provide a crystalline product in 26% yield: dec, 220°C; IR (KBr) 3350, 1680, 1660, 1500, 1425 cm^{-1}; UV in pH 9.0 borate buffer, (λ, ϵ) 226 nm, 25,500 and 280 nm, 1800; HRMS (EI) calcd for $C_{16}H_{15}N_3O_3$ 297.11124, found 297.11204; MS (EI), *m/z* (% relative intensity) 297 (4.1), 279 (2.9), 239 (57.0), 196 (17.6), 182 (17.6), 168 (25.1), 139 (18.9), 44 (100); ^{13}C NMR (DMSO-d_6, 300 MHz) d 172.9, 169.0, 141,4,

134,4, 133.9, 133.0, 128.4, 128.3, 126.6, 126.3, 122.9, 122.7, 94.0, 80.4, 54.5, 42.3.

Calibration

Mobile phases. For both the aqueous solution and human plasma calibration experiments, the HPLC mobile phase comprised acetonitrile–tetrahydrofuran–phosphate buffer (pH 3.0, 50 mM) (45:4:51, v/v/v).

Aqueous solution. The derivatization reaction was initiated by adding aliquots of sodium cyanide stock solution (12.5 μl) and NDA stock solution (50 μl) to the pH 6.8 phosphate buffered solutions (200 μl) containing LE and LEA. The resulting concentrations of these reactants in the derivatization solution were as follows: NDA, 1.0×10^{-3} M; sodium cyanide, 5.0×10^{-4} M; LEA, 2.0×10^{-6} M; and LE 1.0×10^{-7} to 1.0×10^{-5} M. After a reaction time of 3 min (ambient temperature), a 50-μl aliquot of the reaction mixture was analysed by HPLC.

Plasma. Plasma working standards (4.0 ml) containing LE and LEA were prepared by the addition of stock solutions of these peptides to human plasma (these additions comprised ≤4% of the total volume). These working standards contained LE and LEA at concentrations of 3.125×10^{-7} to 6.25×10^{-6} and 1.25×10^{-6} M, respectively. Immediately after preparation, each of the working standards was subjected to the following procedure: addition of an acidic solution of *N,N*-dimethylglycine (750 μl, 1% w/v in 1.0 M HCl); addition of perchloric acid (250 μl, 70%), followed by centrifugation at 2500 rpm for 15 min; and collection of the supernatant in 500 μl aliquots which were stored frozen (−24°C) until the time of analysis. Each sample was then thawed and the entire amount applied to an octyl-silica solid-phase extraction cartridge (Baker-10 SPE-C_8, J.T. Baker Chemical Co.) which had been pre-conditioned with methanol (6.0 ml) and water (6.0 ml). The loaded cartridge was washed with water (15 ml) and the enkephalins were eluted with methanol (1.5 ml). The solvent was removed by heating at 50°C under a gentle stream of nitrogen. The derivatization was conducted by dissolving the enkephalin containing residues in pH 6.8 potassium phosphate buffer (200 μl) followed by the addition of NDA stock solution (25 μl) and sodium cyanide stock solution (25 μl). This resulted in the following reactant concentrations: NDA, 1.0×10^{-4} M; sodium cyanide, 5.0×10^{-4} M; LEA, 2.0×10^{-6} M; and LE, 5.0×10^{-7} to 1.0×10^{-5} M (assuming 100% recovery and a concentration factor of 1.6 during sample work-up). After reaction for 3 min at ambient temperature, a 50-μl aliquot was analysed by HPLC.

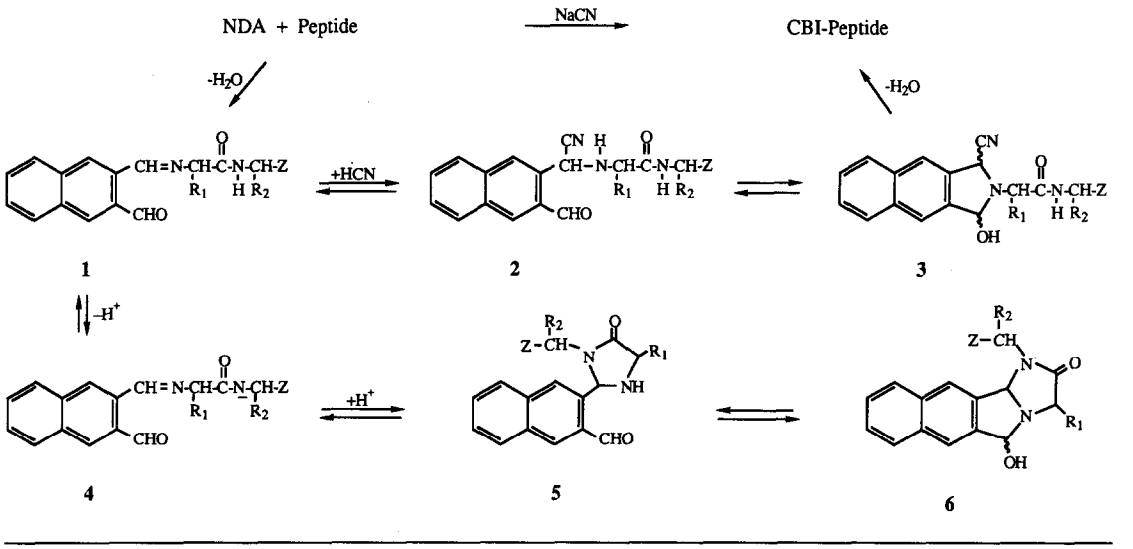
Results and Discussion

Background and initial observations

The derivatization of alanine with NDA/CN has been investigated in detail by de Montigny *et al.* [14], who found that in the presence of excess derivatization reagent, the pH-rate profile for CBI-(ala) formation was bell-shaped. The reaction yield was constant from pH 8–11 [23] and the maximal rate occurred from pH 9.1–9.5 [14]. Based on these results, the derivatization reaction was buffered at pH 9.1–9.5 when the reagent was used in the trace determination of amino acids [14].

In the present experiments, with several small peptides (gly-gly, ala-ala, gly-gly-gly and ala-ala-ala), the reaction medium was similarly buffered to pH 9.5 and CBI-derivative formation was monitored by HPLC with fluorescence detection. At equivalent concentrations, each dipeptide formed a CBI-derivative whose relative fluorescence response was similar to that previously observed for alanine. In contrast, a substantially reduced fluorescence response (approximately 5–10-fold) was observed for the tripeptides gly-gly-gly and ala-ala-ala. The diminished response observed for these tripeptides could have resulted from either a compromised chemical yield or poor fluorescence quantum efficiency. However, the later possibility was precluded because CBI-(gly), CBI-(gly-gly) and CBI-(gly-gly-gly) have been isolated in pure form and the ϕ_f value determined to be 0.6, 0.5 and 0.5, respectively [15]. Thus compared with the dipeptides (gly-gly and ala-ala), the chemical conversion of the tripeptides (gly-gly-gly and ala-ala-ala) to the corresponding CBI-derivatives must have been substantially less efficient and further research was directed towards gaining an understanding of this undesirable result.

The mechanism previously advanced [14] for CBI-derivative formation is shown in Fig. 1. In this multistep sequence, the key trans-



#	Peptide	R_1	R_2	Z	CBI Formation	6 Formation
a	gly-gly	H	H	CO_2H	+	-
b	gly-gly-gly	H	H	$CONHCH_2CO_2H$	+	+
c	gly-gly-NH_2	H	H	$CONH_2$	+	+
d	ala-ala	CH_3	CH_3	CO_2H	+	-
e	ala-ala-ala	CH_3	CH_3	$CONHCH(CH_3)CO_2H$	+	+

Figure 1
Reaction pathways illustrating the mechanisms for CBI-derivative formation and imidazolidinone side-product formation. The observed propensity for the various peptides to form each type of product is summarized.

formations include, the initial reaction of a primary amine with one aldehyde moiety of NDA to form an imine (**1**), the addition of the elements of HCN to form an α-aminonitrile (**2**), an intramolecular cyclization to form the cabinolamine (**3**), and finally dehydration to complete CBI-derivative formation. Based on this mechanism, once intermediate **2** has formed, it is difficult to rationalize differences in the chemical reactivity of dipeptides compared with tripeptides in the subsequent reactions required for CBI-derivative formation. Therefore, further experimentation was focused on understanding differences in the formation and reactivity of intermediate **1** as a function of peptide primary structure.

Model systems for determination of peptide imine reactivity

Ideally, one would like to study the formation and reactivity of **1** as an isolated chemical event. However, the reaction of *ortho*-aromatic-dicarboxaldehydes with primary amines is known to result in the formation of a complex mixture of products [24], thus precluding the unambiguous direct investigation of the reaction of NDA with various peptides. Alternatively, reaction of monofunctional aromatic aldehydes with primary amines is a reaction amenable to investigation, and by using benzaldehyde as a model aromatic aldehyde, imine formation and reactivity with the series, gly, gly-gly and gly-gly-gly was undertaken. These experiments were conducted in buffered aqueous media (pH 9.5) and product formation monitored by absorbance measurements at 270 nm [25]. Interestingly, while gly and gly-gly underwent reaction with benzaldehyde to form stable imines (Fig. 2), gly-gly-gly appeared to initially form an imine, which was subsequently transformed to a chemical species having decreased absorbance at 270 nm.

Previous workers have noted that peptides containing the α-amino group in conjunction with a secondary amide at the *N*-terminus, i.e. the α-amino-*sec*-amido moiety (H_2N-CHR-CONH-R), undergo reaction with aliphatic ketones [26] and aldehydes [27] to form disubstituted imidazolidinones (Fig. 3). Such transformations specifically shown in Fig. 3 for gly-gly and gly-gly-gly, appear to occur by first forming an imine (**8**) followed by amide ionization (**9**). Subsequently, the amidate-ion

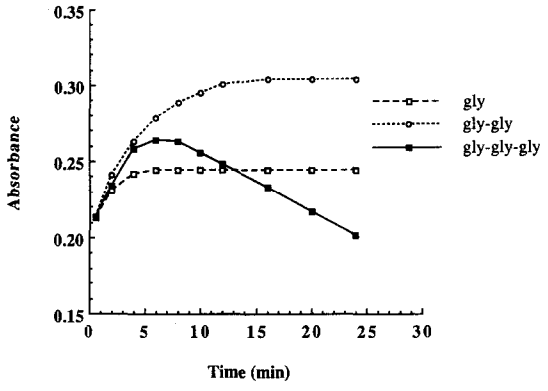
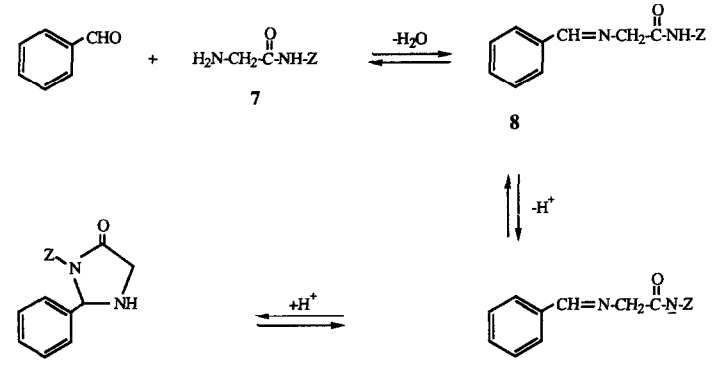


Figure 2
Results of the imine formation and stability studies. The reaction was conducted at ambient temperature by dissolving the various peptides and benzaldehyde in sodium borate buffer (pH 9.5, 0.1 M, μ = 0.5 M) and spectrophotometrically (270 nm) monitoring the formation of the imine bond.

ments necessary for imidazolindinone formation, i.e. each peptide possessed an α-amino-*sec*-amido moiety, the question arose as to why gly-gly formed a stable imine upon reaction with benzaldehyde while gly-gly-gly formed an unstable imine. Superficially, gly-gly and gly-gly-gly would appear to possess quite similar physico-chemical properties. However, it is instructive to examine the ionization constants of the α-amino group for the following series: gly, pK_a 9.8; gly-gly, pK_a 8.3; and gly-gly-gly, pK_a 7.9 [28]. Immediately one notes that as the number of peptide linkages increases, the pK_a value of the α-amino group decreases. The peptide bond has a powerful ability to enhance the acidity of neighbouring groups [29], apparently due to an inductive effect, and thus is responsible for the observed decline of the α-amino group pK_a



7	Peptide	Z	Imine (8) Formation	Imine Stability
a	gly-gly	$CH_2CO_2^-$	+	+
b	gly-gly-gly	$CH_2CONHCH_2CO_2^-$	+	–

Figure 3
The mechanism of imidazolidinone formation from reaction of benzaldehyde with various peptides. The ability to sequentially form an imidazolidinone in pH 9.5 media via further transformation of the various imines is summarized for each peptide investigated.

undergoes nucleophilic addition on the neighbouring imine linkage to complete formation of the imidazolidinone (**10**) [26, 27]. Based on this precedent, it appeared likely that the observed instability of the imine derived from gly-gly-gly was due to further reaction resulting in the formation of the corresponding imidazolidinone (Fig. 3).

Since all of the peptides utilized in the initial experiments fulfilled the structural requirevalue as additional glycine residues are added to the monomeric unit. Considering this effect, re-examination of the gly-gly versus the gly-gly-gly structures revealed that, with respect to the *N*-terminal peptide bond, the dipeptide possesses a β-carboxylate substituent while the tripeptide has a β-peptide linkage (Fig. 3, **7a** versus **7b**). Due to the presence of the neighbouring peptide bond, the *N*-terminal peptide bond of the tripeptide will exhibit an N—H

bond of substantially enhanced acidity compared with the dipeptide. This tripeptide structural feature facilitates ionization of the *N*-terminal peptide bond [29] (Fig. 3, **9**) and the resulting amidate ion can nucleophilically attack the neighbouring imine linkage, thus resulting in formation of imidazolidinone (Fig. 3, **10**).

While this difference in the physico-chemical properties of dipeptides, compared with those of tripeptides, appears to explain the presently observed difference in imine stability for gly-gly as compared with gly-gly-gly, further support for this conclusion was obtained from experiments with the tripeptide ala-pro-gly. Since amide ionization, and subsequent imidazolidinone formation, is precluded for peptides possessing a proline residue in the second position of a peptide's primary structure, one would predict that ala-pro-gly should undergo reaction with benzaldehyde to form a stable imine. Repetition of the imine formation experiments utilizing ala-pro-gly confirmed these expectations, thus lending additional support to the chemical sequence postulated to be responsible for the initially observed reduction in CBI-derivative yield.

Identification of a NDA-peptide side-product

Based on the physico-chemical relationships and results presented above, dipeptide amides, in general, would also be expected to undergo facile reaction with aromatic aldehydes to form the corresponding substituted imidazolidinones. Therefore, in a basic solution containing gly-gly-NH_2, NDA and cyanide, the dipeptide amide could be predicted to react with NDA to form the imidazolidinone product (**5c**) shown in Fig. 1. To substantiate that this reaction sequence was the basis for the compromised yield of the CBI-derivative for gly-gly-gly and ala-ala-ala, further experiments were performed. Firstly, the product resulting from reaction of NDA and gly-gly-NH_2 in basic solution was isolated and characterized. Exact mass determination indicated an empirical formula consistent with the structures (Fig. 1, **5c** or **6c**), which were supported by IR and UV spectra. Additionally, the furthest downfield resonance observed in the ^{13}C NMR spectrum occurred at $\delta = 172.9$, and since the carbonyl carbons of aromatic aldehydes are usually observed at $\delta \geq 190.0$ [30], it appeared that the remaining carboxaldehyde functionality of NDA had also undergone reaction. These findings suggest that the initial imidazolidinone (**5**) participated in ring-chain tautomerism (**5c** and **6c**), and that in solution this equilibrium is dominated by the cyclic form (**6c**). Secondly, **6c** was generated *in situ* by reaction of NDA and gly-gly-NH_2 in pH 9.1 aqueous solution. When the reaction was monitored by HPLC, a single product was observed, which had the same retention time as authentic **6c**. Together, the results of these experiments support the postulation that imidazolidinone formation was responsible for the diminished CBI-derivative yield observed when one attempts to label gly-gly-gly and ala-ala-ala with NDA/CN under conditions previously found optimal for simple amino acids.

Optimization of the derivatization reaction

Once the preceding information was available, further research was directed toward the improvement of CBI-derivative yield for peptides activated to imidazolidone formation. The initially formed imine (Fig. 1, **1**) can partition kinetically between reaction pathways involving external-nucleophilic addition (Fig. 1, **1** to **2**), resulting in CBI-derivative formation, or internal nucleophile attack (Fig. 1, **1** to **5**) forming the imidazolidinone side-product. Examination of the reaction mechanisms for these competing transformations revealed that a simple approach was potentially available to optimize CBI-derivative yield. Imidazolidinone formation is apparently initiated by specific-base catalysis (Fig. 1, **1** to **4**). In contrast, investigations of α-aminonitrile formation (Fig. 1, **1** to **2**) have shown that the reaction velocity is proportional to the concentrations of imine, hydronium ion and cyanide ion [31, 32]. Mechanistically, the transition state has been interpreted to consist of hydronium ion hydrogen atom associated with the imine lone-pair and a second hydronium ion hydrogen atom associated with cyanide ion [31]. Overall, this results in an enhancement of imine-bond polarization, enhancing the susceptibility of the imine to addition by cyanide ion. For the competing reactions of present concern, if these mechanistic features are indeed correct, then by simply conducting the derivatization reaction at a more acidic pH, one could selectively suppress the events leading to imidazolidinone formation while enhancing the rate of α-aminonitrile formation; at the lower pH values, the diminished concentration of the external nucleophile, cyanide

ion, could be compensated by simply increasing the total concentration of this reactant.

To test this concept, a series of experiments were conducted in which the activated dipeptide amide, gly-gly-NH_2 and NDA were placed in aqueous solutions (pH 7.0) containing various concentrations of cyanide. In these experiments, NDA was utilized as the limiting reactant, thereby allowing for the simultaneous determination (UV detection) of the relative quantities of both CBI-derivative and imidazolidinone side-product. Figure 4 shows that, at low concentrations of cyanide, the imine was not efficiently trapped by the external nucleophile, leading to an increased yield of the imidazolidinone side-product, while with increasing cyanide concentration, the formation of the corresponding CBI-derivative was maximized.

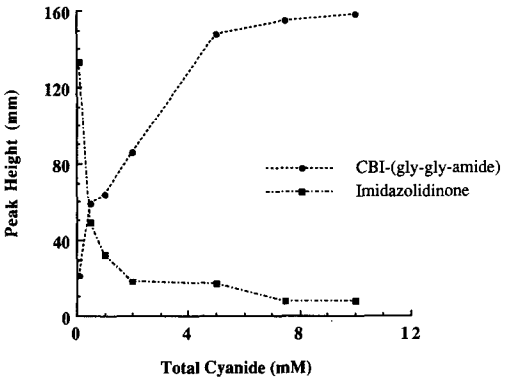


Figure 4
The observed peak height responses for CBI-(gly-gly-NH_2) and the imidazolidinone side-product as a function of total cyanide concentration. The reaction was conducted in pH 7.0 phosphate buffer with NDA and gly-gly-NH_2 initially present at concentrations of 0.05 and 1.0 mM, respectively. The products formed were determined by HPLC with an ODS Hypersil support (5 μm, 250 × 4.6 mm i.d.) using a mobile phase of acetonitrile–(pH 6.8) phosphate buffer (28:72, v/v). The flow rate was 1.0 ml min^{-1} for 5 min then 2.0 ml min^{-1} for 10 min.

In a final group of kinetic experiments, the pH-rate profile for reaction of LE with NDA/CN to form CBI-(LE) was established. Like the results previously noted for alanine, a bell-shaped profile resulted (Fig. 5); however, with LE the maximal rate was observed at approximately pH 7. In the presence of a sufficient quantity of cyanide, it is plausible that the rate-determining-step in CBI-derivative formation is formation of the intermediate imine (Fig. 1, **1**). Kinetically, imine formation has been investigated extensively,

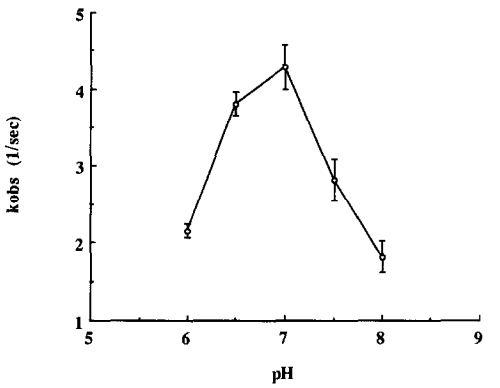


Figure 5
The pH-rate profile for CBI-(LE) formation. The profile was established by conducting the reactions in phosphate buffered media (0.25 M) at ambient temperature and spectrofluorimetrically monitoring CBI product formation (excitation at 420 nm and emission at 490 nm). The initial concentrations of the reactants were as follows: LE, 5.0 × 10^{-6} M; NDA, 1.0 × 10^{-4} M; and sodium cyanide, 2.0 × 10^{-4} M.

and is known to produce a characteristic bell-shaped pH profile whose maximum is dependent on the pK_a value of the amine [33]. In general, oligopeptides have α-amino pK_a values of approximately 7–8 [28], which is a reasonable value for LE. Apparently, this difference in pK_a value for the peptide LE versus the amino acid alanine was responsible for shifting the pH-rate profile maxima from pH 9.5 to 7.0. Thus, in the specific case of α-amino peptide derivatization, not only does conducting the reaction at neutral pH values result in optimal product yield, but it also results in the most rapid conversion of the α-amino functional group to the corresponding CBI-derivatives.

Methodology for the determination of LE in human plasma

Human plasma samples containing LE and the internal standard LEA were prepared; however, the integrity of the analytes in this matrix was of concern. Other workers [4, 34] have noted that these peptides are particularly susceptible to hydrolysis by aminopeptidases in human plasma. Additionally, these workers reported that the hydrolytic process can be circumvented by the rapid addition to plasma of aprotinin and *N,N*-dimethylglycine (DMG) dissolved in 1.0 M HCl. In the present investigations, it was found that plasma samples of LE and LEA were stabilized simply by the addition of acidic DMG; the use of aprotinin was discontinued, preliminary experiments

indicating that aprotinin introduced unwanted chromatographic interference.

To establish methodology suitable for the determination of LE in human plasma, the development of a suitable sample pretreatment was required. The approach taken was to deproteinize the plasma sample by perchloric acid precipitation, recover the peptide containing supernatant, load the supernatant onto an octyl-silica solid-phase extraction cartridge (SPEC), wash extensively with water (this was found to be particularly important in providing a chromatogram free of interfering peaks), and elute the analytes from the SPEC with methanol. The methanolic eluent was evaporated to provide a residue containing LE and LEA, which was redissolved in phosphate buffer (pH 6.8, 0.5 M) and derivatized with NDA/CN (2:1 molar ratio). A reaction time of 3 min at ambient temperature, was sufficient to form the corresponding CBI-derivatives in yields similar to those previously attained with α-amino acids [14].

A mobile phase of acetonitrile–tetrahydrofuran–phosphate buffer (pH 3.0, 50 mM) (45:4:51, v/v/v) was utilized to separate CBI-(LE) and CBI-(LEA) from plasma constituents in <10 min (Fig. 6).

Of note chromatographically was the observation that the presence of tetrahydrofuran sharpened the peaks of interest while maintaining resolution of the CBI-derivatives from other peaks related to the biological sample matrix (Fig. **6c**, **6d**). Additionally, the effect of mobile-phase pH on retention and resolution was briefly investigated. At pH values >3.0, the CBI-(LE) peak exhibited a diminished retention resulting in coelution with the internal standard CBI-(LEA).

Calibration

The derivatization of a series of aqueous and plasma standards was performed in order to establish calibration plots for LE in both matrices. LEA was included as the internal standard. Each calibration point was generated by averaging the results of two determinations in which the individual peak height values differed by <5%. From the data generated, calibration plots could be constructed on the basis of either LE peak heights (PH) or the LE/LEA peak height ratios (PHR). Linear regression analysis resulted in the following equations: (a) for the aqueous standards; PH (mm) = 42.747·[LE (nmol ml^{-1})] + 4.548 (mm), r = 0.998 and; PHR = 0.629·[LE (nmol

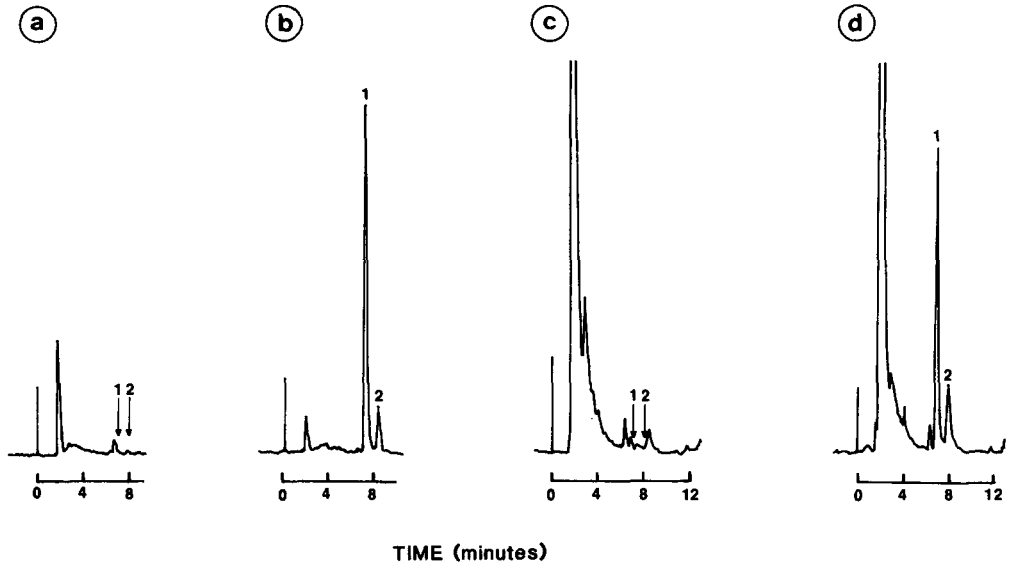


Figure 6
Typical chromatograms generated from the NDA/CN derivatization of aqueous and plasma samples containing LE and the internal standard LEA to form (1) CBI-(LEA) and (2) CBI-(LE). The chromatograms are of (a) an aqueous blank; (b) an aqueous sample containing LE, 0.25 nmol ml^{-1} and LEA, 2.0 nmol ml^{-1}; (c) a plasma blank; and (d) a plasma sample containing LE, 0.31 nmol ml^{-1} and LEA, 1.25 nmol mol^{-1}. The chromatographic support consisted of coupled ODS Hypersil columns (Nos 1–5 μm, 50 × 4.6 mm i.d. and Nos 2–3 μm, 250 × 4.6 mm i.d.). A mobile phase comprising acetonitrile–tetrahydrofuran–potassium phosphate buffer (pH 3.0, 50 mM) (45:4:51, v/v/v) operated at ambient temperature was utilized at a flow rate of 1.0 ml min^{-1} for these separations.

ml^{-1})] + 0.032, r = 0.9998 and (b) for the plasma standards; PH (mm) = 42.747·[LE (nmol ml^{-1})] + 4.548 (mm), r = 0.998 and; PHR = 0.629·[LE (nmol ml^{-1})] + 0.032, r = 0.9998.

The accuracy of the two different calibration approaches was established by using the appropriate regression equation to calculate the concentration of LE in the standards as compared with that originally known to be present. For each matrix, accuracy is improved significantly by inclusion of the internal standard LEA (Tables 1 and 2).

After allowing for the concentration factor for the plasma assay methodology (1.6), the overall recovery of LE and LEA from plasma can be estimated. Based on point-by-point comparisons for equivalent standard solutions, the mean recoveries of LE and LEA were 72 and 81%, respectively.

Conclusions and Future Directions

Based on the product identity and kinetic results presented, it appears that the rationale offered for optimization of α-amino peptide derivatization was correct and by careful control of pH and cyanide concentration one can utilize NDA/CN for the efficient fluorigenic labelling of the α-amino group of peptides. Appropriate methodology, based on acid mediated deproteinization coupled with a solid-phase extraction step, has been established for the preparation of plasma samples prior to derivatization. However, there is concern that when more sensitive detection

Table 1
Comparison of errors associated with absolute versus the internal standard method of calibration for aqueous LE samples

LE aqueous standards* (nmol ml^{-1})	Calculated concentrations (nmol ml^{-1})†			
	Peak height (LE)		Peak height ratio (LE/LEA)	
Prepared	Observed	% Error	Observed	% Error
0.10	0.080	−20.0	0.108	8.0
0.25	0.230	−8.0	0.258	3.2
0.50	0.469	−6.2	0.474	−5.2
1.00	0.896	−10.4	0.904	−9.6
2.00	2.101	5.1	2.101	5.1
5.00	5.164	3.3	5.029	0.6
10.00	9.910	−10.9	9.976	−0.2

*The standard solutions were prepared in pH 6.8 phosphate buffer as described in the Experimental section. LEA was present in each standard at a concentration of 2.0 nmol ml^{-1}.

†LE and LEA were measured as millimeters of peak height response. Using the appropriate linear equation (R&D, calibration subsection) the observed concentration of LE was calculated. The % error values were determined using the following formula: % Error = (observed concentration − prepared concentration)/(prepared concentration) × 100. The chromatographic conditions and parameters are given in Fig. 6.

Table 2
Comparison of errors associated with absolute versus the internal standard method of calibration for LE spiked plasma standards

LE plasma standards* (nmol ml^{-1})	Calculated concentrations (nmol ml^{-1})†			
	Peak height (LE)		Peak height ratio (LE/LEA)	
Prepared	Observed	% Error	Observed	% Error
0.31	0.221	−29.2	0.303	−3.1
0.63	0.584	−6.6	0.615	−1.6
1.25	1.297	3.8	1.303	4.2
3.13	3.297	5.5	3.077	−1.5
6.25	6.163	−1.4	6.265	0.2

*The spiked plasma standards were prepared as described in the Experimental section. LEA was present in each standard at a concentration of 1.25 nmol ml^{-1}.

†LE and LEA were measured as millimeters of peak height response. Using the appropriate linear equation (R&D, calibration subsection) the observed concentration of LE was calculated. The % error values were determined using the following formula: % Error = (observed concentration − prepared concentration)/(prepared concentration) × 100. The chromatographic conditions and parameters are given in Fig. 6.

methods, such as laser-induced fluorescence or chemiluminescence, are utilized for the determination of peptides present at substantially lower concentrations, that the current sample handling approach may result in lower analyte recoveries because of adsorption to glass or the precipitated plasma proteins. Additionally, while sufficient cyanide is required to trap the imine intermediate, thus ensuring efficient CBI-derivative formation, little information is available on the optimal ratio of NDA/CN as a function of analyte structure, amine pK_a, and amine type, e.g. α-amino or ϵ-amino moieties present on the *N*-terminus or lysine residues of peptides, respectively. Finally, bis-CBI-derivatives are known to exhibit a poor fluorescence quantum yield [15], but due to the significantly differing structural environments and pK_a values of α-amino versus ϵ-amino sites, the possibility exists that the selective formation of an efficiently fluorescent mono-CBI-derivative can be achieved by kinetic control. Such a result would likely prove to be analytically useful for analytes bearing these two types of primary amines. Currently, all of these issues are being actively investigated in these laboratories and will be reported in forthcoming publications.

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