Journal of Chromatography, 532 (1990) 249–259
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5455

Sensitive method for measuring hydrolysis of enkephalins in plasma, using high-performance liquid chromatography with electrochemical detection

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(First received April 19th, 1990, revised manuscript received June 18th, 1990)

ABSTRACT

This paper describes a simple and sensitive method for detection of [Leu]- and [Met]enkephalin and their N-terminal tyrosine-containing metabolic fragments (Tyr, Tyr-Gly, Tyr-Gly-Gly, and Tyr-Gly-Gly-Phe), using high-performance liquid chromatography with electrochemical detection. The method employs a carbon graphite working electrode with increased working electrode surface area (40 mm^2). The procedures were applied to assay of the activities of enkephalin-degrading enzymes in whole plasma collected from rats, mice, and chicks.

INTRODUCTION

Enkephalins and related substances have been detected by high-performance liquid chromatography (HPLC) with ultraviolet (UV), fluorimetric (FD), electrochemical (ED), and mass spectrometric (MS) detection, often coupled with radioimmunoassay (RIA) or radioreceptor assay (RRA) for quantification [1,2]. Although HPLC with MS, RIA or RRA has some advantages in terms of sensitivity or selectivity for detection of endogenous levels of some enkephalins, these methods can require considerable amounts of time and expensive equipment. In addition, with these methods it is technically difficult, if not impossible, to determine simultaneously the levels of endogenous enkephalins and their enzymatically formed metabolites.

By contrast, HPLC with UV, FD, or ED can be used for the simultaneous assay of enkephalins and their metabolites [3–6]. These methods have been suc-

0378-4347/90/$03.50

cesfully applied to characterization of the enzymatic hydrolysis of enkephalins in several biological samples, such as homogenate or membrane fractions of brain, cerebrospinal fluid, retina, and ileum [4,7–9]. HPLC–UV is the least sensitive of these three methods for assay of enkephalins. HPLC–FD is a recently introduced method that requires derivatization of the compounds of interest into fluorescent products [4]. However, HPLC–FD has lower sensitivity to free Tyr than to the other metabolic products of the enkephalins. On the other hand, HPLC–ED permits the sensitive and selective detection of free Tyr and N-terminal Tyr-containing peptides [5,10–12].

To improve the sensitivity and selectivity of the HPLC–ED method, various modifications of the detection system have been reported. These include using twin working electrodes [13,14], increasing the working electrode surface [15], modifying the working electrode composition [15], and altering the mobile phase [16]. In the present paper we describe a simple, inexpensive, rapid, and sensitive HPLC–ED method for detecting enkephalins and their N-terminal Tyr-containing fragments. This procedure uses a carbon graphite electrode with an increased working electrode surface area. We also report the application of this method to assay of the activities of enkephalin-degrading enzymes in plasma samples.

Hydrolysis of enkephalins in blood has not been characterized by any of the HPLC methods discussed above. However, we recently found that the activity of the enkephalin-degrading enzyme system in rat plasma changes during active avoidance training [17], suggesting that measurement of the activities of the individual enkephalin-hydrolyzing enzymes in plasma may be important to interpretation of our behavioral findings. Using thin-layer chromatographic (TLC) separation of radiolabelled [Leu]enkephalin (LE) from its N-tyrosine containing metabolites, in the absence and presence of inhibitors with high selectivity for various peptidases, we found that aminopeptidase M and angiotensin-converting enzyme together account for approximately 90% of the total hydrolysis of LE in rat plasma [18]. In the present study we compared the contributions of aminopeptidase and dipeptidyl carboxypeptidase activities to the hydrolysis of LE in rat, mouse, and chick plasma.

EXPERIMENTAL

Apparatus and chromatographic conditions

The apparatus consisted of a liquid chromatographic system (Yanagimoto Model L-2000, Kyoto, Japan) with a six-port injector (Rheodyne Model 7125, Berkeley, CA, U.S.A.) and an electrochemical detector (Eicom Model CE-100, Kyoto, Japan) with a thin-layer carbon graphite electrode (Eicom Model 6G). Using a 25-μm-thick gasket, the electrode surface area was adjusted to 40 mm^2 and the cell volume was adjusted to 1 μl.

All separations were performed with an Ultrasphere ODS analytical column (average particle size 5 μm; 250 mm × 4.6 mm I.D.; Altex, Berkeley, CA,

U.S.A.). The column temperature was maintained at 25°C A short column containing octadecylsilane packing (10 mm × 4.5 mm I.D.) was used to protect the analytical column.

The detector potential was set at 1050 mV *versus* the Ag/AgCl reference electrode for assay of enkephalins (LE and [Met]enkephalin (ME), the internal standard) and Tyr-Gly-Gly-Phe, or at 950 mV for assay of shorter enkephalin metabolites (Tyr, Tyr-Gly, and Tyr-Gly-Gly) and the internal standard (Tyr-Ala).

Different mobile phases were used for assay of enkephalins and their metabolites. For separation of LE, ME, and Tyr-Gly-Gly-Phe, the mobile phase consisted of 0.05 *M* sodium citrate–citric acid buffer (pH 6.3) containing 19% acetonitrile, and the flow-rate was set at 1.2 ml/min. Tyr, Tyr-Gly, Tyr-Gly-Gly, and the Tyr-Ala internal standard were separated with a mobile phase containing 0 05 *M* sodium citrate–citric acid buffer (pH 6.3) and a flow-rate of 1.4 ml/min. Both mobile phases were sonicated under a vacuum prior to use in order to eliminate air bubbles that can interfere with the electrochemical detector.

Chemicals

LE and ME were obtained from Bachem (Torrance, CA, U.S.A.). Tyr, Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, Tyr-Ala, and bestatin were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile and HPLC-grade water were obtained from Fisher Scientific (Springfield, NJ, U.S.A.). All other chemicals for extraction and chromatography were purchased from Sigma and used without further purification.

Animals

Blood samples were collected from adult male Swiss–Webster mice (average weight 30 g; Harlan Sprague–Dawley, Indianapolis, IN, U.S.A.), adult male Sprague–Dawley rats (average weight 325 g; Harlan Sprague– Dawley), and two-day-old male Dekalb–Warren white leghorn chicks (Dekalb Hatchery, Petaluma, CA, U.S.A.). All animals were maintained under standard laboratory conditions prior to use.

Plasma hydrolysis of LE

Following decapitation, trunk blood was collected from each animal into heparinized polypropylene tubes. The plasma was rapidly separated by microcentrifugation and then incubated at 37°C for 10 min to permit metabolism of endogenous enkephalins. The plasma was then kept on ice until substrate addition as described below.

To measure plasma LE hydrolysis, the chilled plasma (138 μl) was incubated at 37°C for 10 min with 12 μl of either saline or bestatin (prepared in saline to achieve a final concentration of 500 μ*M*). LE (3.6 μl of 1.5 mg/ml LE prepared in saline) was then added to the reaction mixture to achieve a final substrate concentration of 64.8 μ*M*. After 0.5, 1, 2, 5, 10, or 20 min of incubation with LE, the

reaction was terminated by addition of 300 μl of a 5% trichloroacetic acid solution containing the appropriate internal standards (ME and Tyr-Ala for LE and metabolite assays, respectively). After microcentrifugation, 50 μl of the supernatant were injected into the HPLC system

Data analysis

Quantitative determinations of substrate and product levels were based on peak heights of the resulting chromatograms. Ratios of peak heights for substances of interest and the corresponding internal standards were compared. Working standards were assayed at regular intervals in order to verify the sensitivity of the detector.

The following recoveries were estimated for this procedure (all $n = 4$): LE, 87%; ME, 90%, Tyr, 83%; Tyr-Gly, 94%; Tyr-Gly-Gly, 94%; Tyr-Ala, 96%. The ratios of the recoveries between LE, Tyr, Tyr-Gly, or Tyr-Gly-Gly, and their corresponding internal standards (ME or Tyr-Ala), were 0.98 ± 0.01, 0.87 ± 0.02, 0.98 ± 0.03, and 0.98 ± 0.03 (all $n = 4$), indicating high recovery consistency.

Endogenous LE, ME, Tyr-Gly, and Tyr-Gly-Gly were not detectable with this assay system in plasma from any of the three species tested (detection limit 0.03 nmol/ml of plasma). High endogenous plasma concentrations of Tyr (50–100 nmol/ml of plasma) were detected in rat, mouse, and chick plasma in the absence of exogenous enkephalin addition. The amount of Tyr formed as a result of hydrolysis of exogenously added enkephalin was estimated by calculating the difference in the concentration of this substance following incubation of plasma in the presence *versus* the absence of added enkephalin.

RESULTS

Hydrodynamic voltammograms for the enkephalins, metabolites, and standards measured in this study are shown in Fig. 1. Electrochemical responses to LE, ME, and Tyr-Gly-Gly-Phe began at 625 mV and increased steadily to about 1100 mV. Responses to Tyr, Tyr-Gly, and Tyr-Gly-Gly began at 600 mV, increased steadily up to 900 mV, and then remained fairly stable up to 1050 mV. Further increases in the applied voltage resulted in lowered responses, such as might be expected from an interference by the background with the electrochemical response [6,11]. The electrochemical response to Tyr-Ala, the internal standard for the Tyr, Tyr-Gly, and Tyr-Gly-Gly separations, was the same as that to Tyr, Tyr-Gly, and Tyr-Gly-Gly, indicating that Tyr-Ala is an electrochemically appropriate internal standard for use in this HPLC assay system.

The retention times for LE, ME (used as an internal standard), and Tyr-Gly-Gly-Phe were influenced by the concentration of acetonitrile in the mobile phase (see Fig. 2A). Increasing the mobile phase acetonitrile concentration from 10 to 25% decreased the retention times for these peptides. Complete separation of LE,

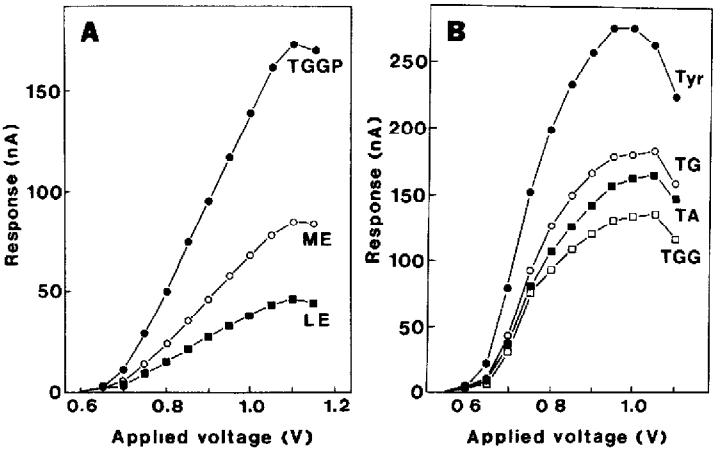

Fig 1 Hydrodynamic voltammograms for enkephalin-related substances, generated from the current response to 10 ng of Tyr or 20 ng of the other compounds (A) Voltammograms for LE, ME (the internal standard), and Tyr-Gly-Gly-Phe (TTGP) Mobile phase 0 05 *M* sodium citrate–citric acid (pH 6 3) containing 19% acetonitrile. (B) Voltammograms for Tyr, Tyr-Gly (TG), Tyr-Gly-Gly (TGG), and Tyr-Ala (TA, the internal standard) Mobile phase 0 05 *M* sodium citrate–citric acid (pH 6 3)

ME, and Tyr-Gly-Gly-Phe was achieved within 12 min with a mobile phase containing 19% acetonitrile.

As shown in Fig. 2B, the retention times for Tyr, Tyr-Gly, Tyr-Gly-Gly, and Tyr-Ala (used as an internal standard) on the reversed-phase column were influenced differentially by the pH of the mobile phase. The retention time for Tyr was

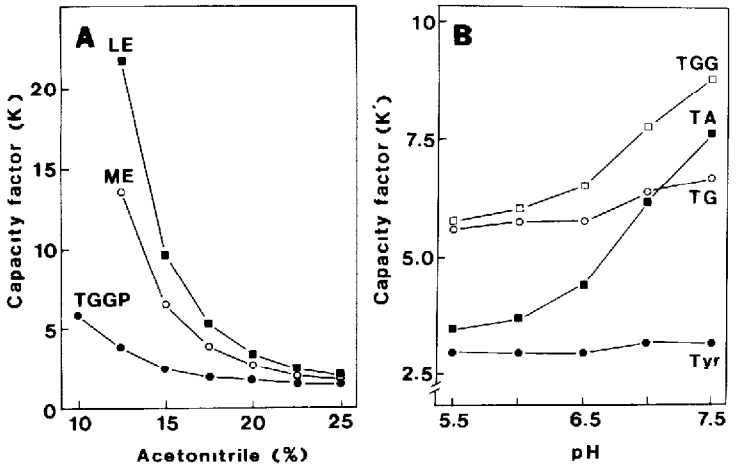

Fig. 2. Effects of acetonitrile and pH on the retention times for enkephalin-related substances (A) Changes in LE, ME, and Tyr-Gly-Gly-Phe (TGGP) retention times with changes in mobile phase acetonitrile concentration (B) Changes in Tyr, Tyr-Gly (TG), Tyr-Gly-Gly (TGG), and Tyr-Ala (TA) retention times with changes in mobile phase pH

nearly constant between pH 5.5 and 7.5, while the retention times for Tyr-Gly, Tyr-Gly-Gly, and Tyr-Ala increased with increasing pH of the mobile phase. Tyr-Ala retention time was particularly sensitive to pH changes. Separation of Tyr, Tyr-Gly, Tyr-Gly-Gly, and Tyr-Ala was achieved within 12 min when the mobile phase pH was between 6.0 and 6.5. LE and ME were not detectable under these separation conditions because of their longer retention times.

Previous investigations of the enzymatic hydrolysis of enkephalins in brain homogenates or membrane fractions [4,5,7] included acetonitrile and/or methanol, but no internal standard, in the mobile phase used to separate these substances. However, high endogenous Tyr concentrations were found to interfere with the complete separation of Tyr, Tyr-Gly, Tyr-Gly-Gly, and Tyr-Ala in plasma in the presence of these mobile phases.

The linearity of the detector response to enkephalins and their metabolites was evaluated by measuring the heights of the peaks produced by a wide range of peptide concentrations (see Fig. 3). Regression analysis of the responses to LE, ME, and Tyr-Gly-Gly-Phe, when injected in amounts between 60 pg and 20 ng, gave correlation coefficients of 0.997–0.999 (all $p < 0.001$) between peak height and amount of peptide injected. Detector responses to Tyr, Tyr-Gly, Tyr-Gly-Gly, and Tyr-Ala, when injected in amounts between 60 pg and 20 ng, had correlation coefficients of 0.999 (all $p < 0.001$) with peptide concentration. Responses to all substances tested remained linear up to at least 500 ng ($r = 0.997$–0.999, all $p < 0.001$). Based on these findings, a simple comparison of peak heights was used for calculation of the concentrations of enkephalins and metabolites. Using a signal-to-noise ratio of 5:1, the following detection limits for this

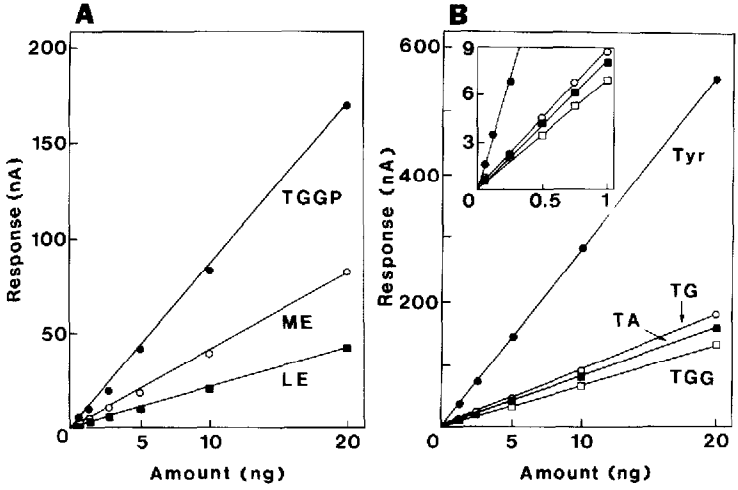

Fig 3 Linearity of the detector responses to changes in concentrations of enkephalins and their metabolites (A) Detector responses to LE, ME, and Tyr-Gly-Gly-Phe (TGGP). (B) Detector responses to Tyr, Tyr-Gly (TG), Tyr-Gly-Gly (TGG), and Tyr-Ala (TA) Inset: detail of responses to concentrations up to 1 ng.

separation were determined: LE, 250 pg; ME, 125 pg; Tyr-Gly-Gly-Phe, 75 pg; Tyr, 5 pg; Tyr-Gly, 10 pg; Tyr-Gly-Gly, 12.5 pg; and Tyr-Ala, 10 pg.

Typical chromatograms obtained following the enzymatic hydrolysis of LE in mouse plasma are shown in Fig. 4. The retention time for LE was 10 min when it was separated with a mobile phase containing 19% acetonitrile. Tyr-Gly-Gly-Phe, which would result from carboxypeptidase activity against LE, was not detected in this system because of interference from the front peak. Tyr-Gly-Gly-Phe also was not detected when the acetonitrile concentration was lowered to 12% to facilitate separation of the Tyr-Gly-Gly-Phe and front peaks, suggesting that Tyr-Gly-Gly-Phe is not a major enkephalin metabolite in mouse plasma.

Fig. 5 shows typical chromatograms for Tyr, Tyr-Gly, and Tyr-Gly-Gly measured in mouse plasma following a 10-min incubation with LE. Accumulation of these products following incubation with LE indicates that enzymes are available in mouse plasma to hydrolyze the Tyr-Gly, the Gly-Gly, and the Gly-Phe bonds of enkephalins.

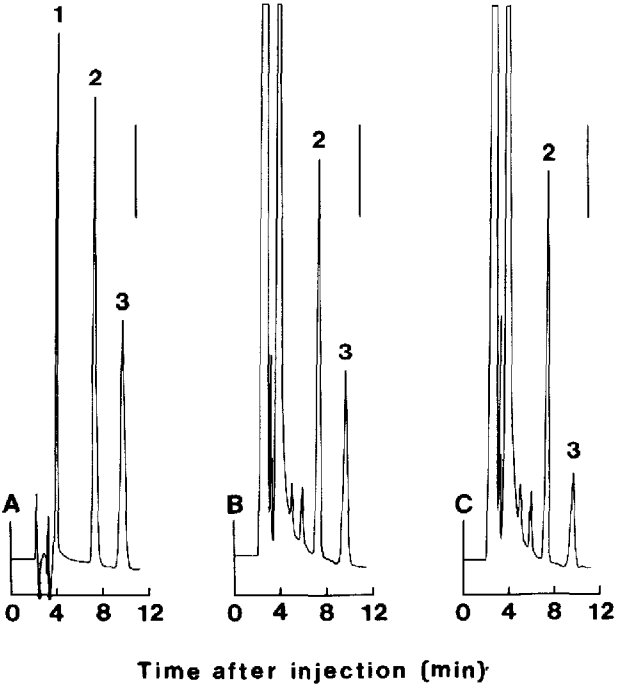

Fig 4. Representative chromatograms for enkephalin-related substances (A) Chromatogram for standard mixture containing 500 ng each of LE and ME, and 250 ng of Tyr-Gly-Gly-Phe. (B) "Blank" chromatogram for mouse plasma denaturated with 5% trichloroacetic acid containing ME (internal standard), followed by addition of LE (final concentration 64.8 μM). (C) Chromatogram for mouse plasma following 10 min of incubation with LE (64.8 μM final concentration) at 37°C Peaks: 1 = Tyr-Gly-Gly-Phe, 2 = ME (internal standard); 3 = LE. The vertical bars indicate a 400-nA detector response

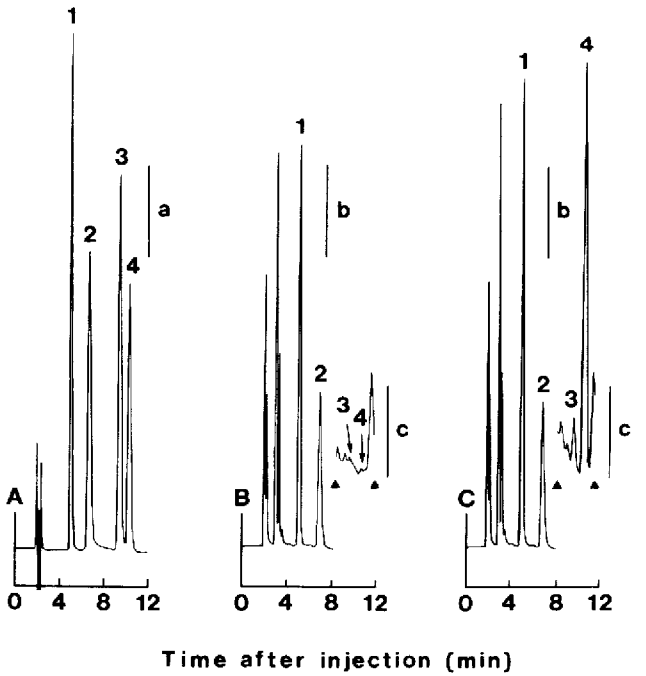

Fig 5 Representative chromatograms for enkephalin metabolites (A) Chromatogram for standard mixture containing 100 ng each of Tyr-Gly, Tyr-Gly-Gly, and Tyr-Ala (internal standard) and 50 ng of Tyr. (B) "Blank" chromatogram for mouse plasma denatured with 5% trichloroacetic acid containing the Tyr-Ala internal standard, followed by addition of LE (final concentration 64.8 μM) (C) Chromatogram for mouse plasma following 10 min of incubation with LE (64 8 μM final concentration) at 37°C. Detector sensitivity was increased by 12 5 times during the period between the two triangles Peaks: 1 = Tyr, 2 = Tyr-Ala (internal standard); 3 = Tyr-Gly, 4 = Tyr-Gly-Gly. The vertical bars indicate a 200-nA (A), 1000-nA (B), or 80-nA (C) detector response.

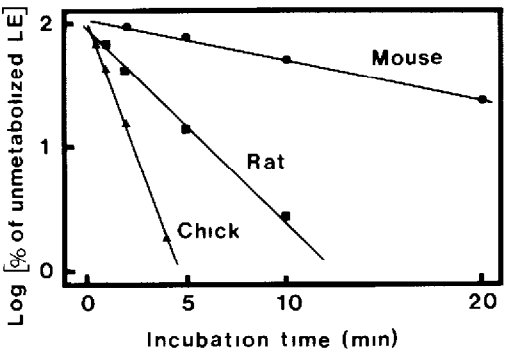

Fig 6 Time course for the disappearance of LE (initial substrate concentration 64.8 μM) from mouse, rat, and chick plasma. Values are means for four animals The regression coefficients for linearity were −0.999 for all species The half-life of LE was estimated to be 9.0 min in the mouse, 1.7 min in the rat, and 0 7 min in the chick

TABLE I

ENZYMATIC FORMATION OF Tyr, Tyr-Gly, AND Tyr-Gly-Gly FROM LE IN MOUSE, RAT, AND CHICK PLASMA

Hydrolysis was measured 5 min after substrate addition (64.8 μM), in the absence or presence of bestatin (500 μM) Values are means ± S D (n = 5); N D = not detectable

Species	Inhibitor	Product (nmol/ml of plasma)		
		Tyr	Tyr-Gly	Tyr-Gly-Gly
Mouse	None	19 7 ± 4 8	0 33 ± 0 05	2 90 ± 2.16
	Bestatin	1.3 ± 1 4	0 73 ± 0 14	8.41 ± 4.50
Rat	None	59 7 ± 6 0	0.46 ± 0.09	0.83 ± 0 14
	Bestatin	3 4 ± 3.1	1 56 ± 0.25	3 24 ± 0.18
Chick	None	68 7 ± 2 9	N.D.	0 11 ± 0 05
	Bestatin	5 3 ± 4.7	N D.	1 82 ± 0.24

The kinetics of LE hydrolysis following its addition (64.8 μM final concentration) to mouse, rat, and chick plasma, are depicted in Fig. 6 The disappearance of LE from the plasma of all three species followed first-order kinetics. The half-life of LE in plasma *in vitro* was determined to be 9.0 min in the mouse, 1.7 min in the rat, and 0.7 min in the chick. Table I compares the formation of Tyr, Tyr-Gly, and Tyr-Gly-Gly from LE in mouse, rat, and chick plasma, in the presence *versus* the absence of bestatin, an inhibitor with high selectivity for aminopeptidase M. Less Tyr resulted from the hydrolysis of LE in mouse than in rat or chick plasma, while Tyr-Gly-Gly formation was greater in the mouse than in the other two species Small amounts of Tyr-Gly were detected in mouse and rat plasma, but Tyr-Gly was not detectable in chick plasma. These data indicate that the types of enzymes participating in enkephalin hydrolysis are similar in the two rodent species, while a somewhat different pattern of enzyme activities is present in chick plasma.

DISCUSSION

We report here a simple and sensitive procedure for separation and detection of enkephalins and their N-terminal Tyr-containing metabolic fragments. The assay is based on an HPLC system utilizing an electrochemical detector with a carbon graphite electrode. To increase sensitivity and selectivity, other investigators have used coulometric detectors [19] or twin working electrodes [13,14], or have increased the surface area of the working electrode [15]. For example, Ehrenstrom [15] recently demonstrated that the surface area of the working electrode affects

the magnitude of the detector response, and that the material of which the working electrode is made affects the background current. However, while a carbon paste-oil electrode leads to low background current, this type of electrode is not adequate for use with some mobile phases containing high concentrations of organic solvents such as acetonitrile, because it shortens the lifetime of the electrochemical material. In the present study we found that a carbon graphite electrode is useful with mobile phases containing acetonitrile. We found that using a detector with a carbon graphite electrode (electrode surface area 40 mm^2) produced an approximate twenty-fold increase in responses to Tyr compared to responses produced by detectors using a glassy carbon working electrode (electrode surface area 5.6 mm^2; [12]). In addition, the responses to enkephalin and its metabolites obtained in the present study are markedly greater than those obtained by previously reported HPLC–ED techniques [5,6,10,11]. This finding suggests that a carbon graphite electrode is useful for sensitive detection of N-terminal Tyr-containing peptides.

In the present study we applied an HPLC–ED separation using a carbon graphite electrode to assay of the activities of enkephalin-degrading enzymes in whole plasma *in vitro*, as measured by the accumulation of N-terminal Tyr-containing metabolic products. This assay allowed us to assess the relative contributions of aminopeptidases, dipeptidyl aminopeptidases, and dipeptidyl carboxypeptidases to the hydrolysis of LE in mouse, rat, and chick plasma.

The data reported here indicate that the rate of hydrolysis of LE is lower in the mouse than it is in rat or chick plasma. Similarly, Venturelli *et al.* [20] demonstrated that the half-life of LE in whole and fractionated mouse plasma is markedly longer than that in whole and fractionated plasma taken from other animal species (rat, rabbit, chicken, and guinea pig), as measured with TLC or HPLC separation of radiolabelled LE from its Tyr-containing metabolites. Our findings, based on interspecies differences in formation of Tyr, Tyr-Gly, and Tyr-Gly-Gly, also indicate that dipeptidyl carboxypeptidase activity plays a greater role in mouse than in rat or chick plasma, while aminopeptidase activity plays a greater role in chick than in rat or mouse plasma. Detailed characterization of the enkephalin-hydrolyzing peptidases active in plasma from each species are reported elsewhere [21,22].

In conclusion, the HPLC–ED method described here is applicable to the sensitive determination of the enzymatic hydrolysis of enkephalin in whole plasma *in vitro*. We anticipate this method also will be applicable to assay of the enzymatic hydrolysis of enkephalins in other biological samples and to assay of other peptides containing an N-terminal Tyr moiety.

ACKNOWLEDGEMENTS

This work was supported by National Institute of Drug Abuse, No. DA04195 and No. DA04795. We thank Edward L. Bennett for discussions during the conduct of this research and the preparation of this report

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