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Short Communication

Liquid chromatographic analysis of leucine-enkephalin and its metabolites in homogenates of cultured human keratinocytes

PRAFUL K. SHAH* and RONALD T. BORCHARDT†‡

‡*Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045, USA*

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Introduction

Peptides and proteins are becoming an important new class of drugs, and advances in biotechnology promise to provide a large number of these compounds as potential drug candidates in the future [1, 2]. Their success as drugs depends, in part, on an understanding of their mechanism of action and their pathways of degradation. Equally important is the ability to deliver these agents to their site of action. Considering their poor bioavailability by conventional (e.g. oral) routes, transdermal delivery of peptides has become an attractive alternative. However, enzymatic hydrolysis of peptides in skin makes transdermal delivery potentially problematic. For example pathways for enkephalin metabolism in various tissues, including skin (P.K. Shah and R.T. Borchardt, unpublished data) have been shown to involve aminopeptidases, dipeptidases and carboxypeptidases (Fig. 1) [3].

The methods currently available to separate Leu-enkephalin and its metabolites are based on adsorption chromatography, ligand exchange chromatography, thin layer chromatography and liquid chromatography (LC) [3–10]. LC has been the method of choice for routine analysis of enkephalin and its metabolites because of its precision, reliability and con-

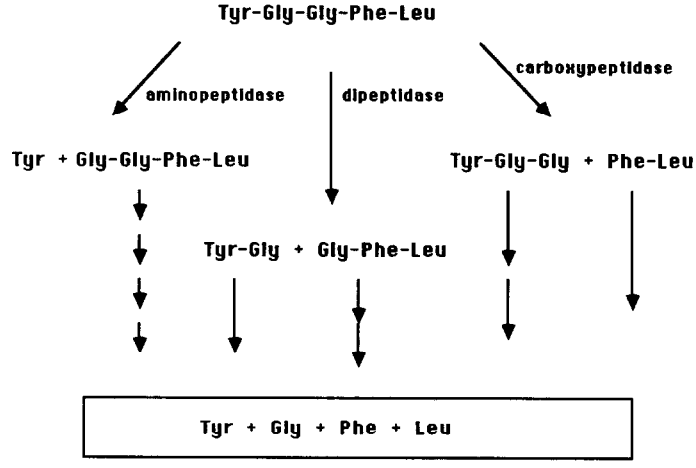

Figure 1
Enzymatic degradation pathways of Leu-enkephalin.

*Marion Merrell Dow Inc., Product Development Department, Kansas City, MO 64134, USA.
†Author to whom correspondence should be addressed.

venience. However, the reported LC methods did not separate all the major metabolites in a single run and the solvent gradient system often produced interfering "ghost" peaks. Although a number of detection methods are available to monitor the enkephalin metabolites, UV detection (214 nm) is the most convenient.

In this communication an improvement of the LC method of Kashi and Lee [3] is described, in which a one-step gradient solvent system replaced the gradient system for the separation of Leu-enkephalin and its metabolites. A novel application of the method to the peptide metabolism studies in cultured keratinocytes homogenates is investigated.

Experimental

Materials

Leu-enkephalin was obtained from ICN Biochemicals (Cleveland, OH). The enkephalin metabolites were obtained from Sigma Chemicals (St. Louis, MO). Cultured human keratinocytes and Keratinocyte Growth Media (KGM) were obtained from Clonetics Corporation (Palo Alto, CA). HaCaT cells were provided by Dr N.E. Fusenig, German Cancer Research Center (Heidelberg, FRG). Dulbecco's Modified Essential Medium (DMEM), Dulbecco's modified phosphate buffered saline (PBS) and fetal bovine serum were obtained from Hazelton Research Products (Lenexa, KS, USA).

All the chromatographic instruments, SCL-6A system controller, LC-6A pump and mixing chamber (2 ml), SIL-6A auto injector, SPD-6A variable wavelength UV detector and C-R6A integrator were manufactured by Shimadzu Corporation (Kyoto, Japan). The Ultrasphere ODS column (5 μm, 4.6 mm i.d. × 250 mm) was purchased from Altex Corporation (San Ramon, CA, USA). The guard column (RP-18) was purchased from Rainin Instrument Company (Woburn, MA, USA).

Cell culture and tissue homogenate

Keratinocytes obtained from Clonetics Corporation were cultured in serum-free KGM media according to the manufacturer's instructions. The cells were cultured for 8–10 days. HaCaT cells were cultured in DMEM (5% serum) media until a confluent monolayer was formed [11]. The cells were washed twice with PBS and then with 0.32 M sucrose solution. The cells were scraped in 0.32 M sucrose solution and homogenized in a Dounce homogenizer. The soluble and the membrane-bound enzymes were obtained according to the procedure described by Baranczyk-Kuzma and Audus [12].

Metabolism studies

Typically, 1 ml of the cell homogenate (~1 mg protein) and 1 ml of 200 μM or 2 mM Leu-enkephalin in PBS (pH 7.4) were combined at 37°C and incubated for various times. Aliquots (100 μl) were removed at various times and the reactions were quenched by the addition of 200 μl of ice-cold acetonitrile, which contained 50 μM tryptophan as an internal standard. The mixture was centrifuged (13,600 *g* for 15 min) to precipitate proteins. Acetonitrile was evaporated under a gentle stream of N_2 from the supernatant and a 10 μl sample of the residue was analysed by LC.

Liquid chromatography

The Ultrasphere ODS column was eluted with $NaClO_4$ (0.1 M) and H_3PO_4 (0.1%)–acetonitrile ($1.00 - x{:}x$, v/v), where $x = 0.07$ for 0–5 min and $x = 0.26$ for 5–21 minutes. After each run, the column was washed with acetonitrile–perchlorate/phosphate buffer (60:40%, v/v) for 5 min and re-equilibrated for 15 min with acetonitrile–perchlorate/phosphate buffer (7:93%, v/v). The flow rate was 1 ml min^{-1}, and Leu-enkephalin and the metabolites were detected at 214 nm.

Results and Discussion

To study Leu-enkephalin metabolism in the cultured human keratinocytes, the method of Kashi and Lee [3] was explored, which in our laboratory gave poor resolution of several metabolites. Therefore the method was modified for improved resolution. Leu-enkephalin and its metabolites were separated by LC and identified by their retention times. The intra-assay and interassay variations in the retention times for the internal standard Trp were less than 2% RSD ($n = 10$). Figure 2 is a typical chromatogram of Leu-enkephalin, its major metabolites and the internal standard Trp in a standard solution. The retention times (in min) of Leu-enkephalin, its hydrolytic fragments and Trp are as follows: Tyr-Gly-Gly (TGG) — 6.4; Tyr — 7.2; Tyr-Gly (TG) — 8.0; Leu — 9.9; Phe — 12.5; Trp — 14.3; Tyr-Gly-Gly-Phe

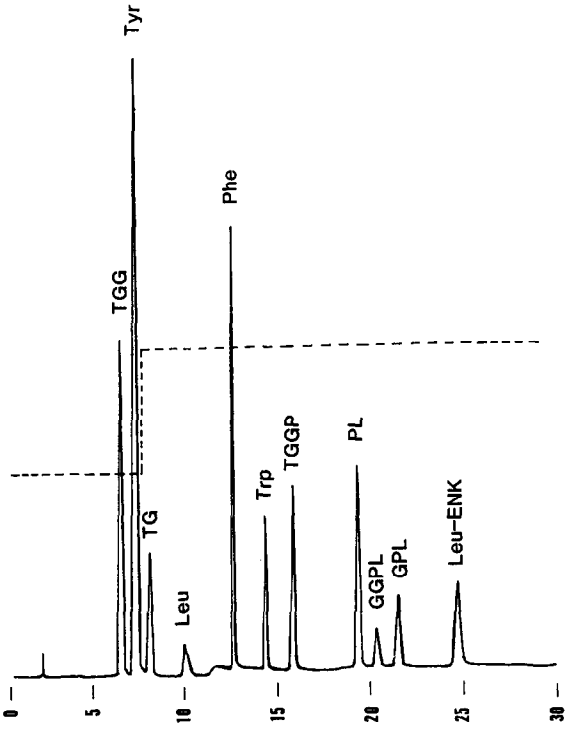

Figure 2
Chromatogram of a mixture of Leu-enkephalin, its metabolites and the internal standard, Trp. The standard mixture was separated on Ultrasphere ODS column and detected at 214 nm. Acetonitrile concentration was kept constant at 7% for the first 5 min and then raised to 26% for 21 min. The dashed line shows acetonitrile concentration at the column inlet. Key: Tyr-Gly-Gly (TGG), Tyr-Gly (TG), Try-Gly-Gly-Phe (TGGP), Phe-Leu (PL), Gly-Gly-Phe-Leu (GGPL), Gly-Phe-Leu (GPL) and Leu-enkephalin (Leu-Enk). Approximate concentrations of analytes ranged from 0.1 to 2 mM, and the injection volume was 20 μl.

(TGGP) — 15.7; Phe-Leu (PL) — 19.3; Gly-Gly-Phe-Leu (GGPL) — 20.3; Gly-Phe-Leu (GPL) — 21.4; and Leu-enkephalin (Leu-Enk) — 24.6. Leu-enkephalin and its metabolites were quantified using standard curves and gave a linear response over the range of 100–2000 ng (peak area = −282.6 + 40.72 (ng Leu-enkephalin), r^2 = 1.000). The limit of detection (signal to noise ratio of 3) for Leu-enkephalin, determined experimentally, was 11 ng. The recovery of Leu-enkephalin and the internal standard, Trp, after protein precipitation in acetonitrile was identical. The delay time between the solvent mixer and the column inlet was 2.6 min.

Figure 3 shows typical chromatograms of the Leu-enkephalin metabolites produced in the homogenates of the cultured Clonetics cells. Control experiments showed that the enzyme and Leu-enkephalin solutions were chemically stable for at least 2 h. Leu-enkephalin (initial concentration = 100 μM) hydrolysis followed Michaelis–Menten kinetics and proceeded with calculated (V_{max}/K_m) first-order rate constants of 1.06 min^{-1} in the Clonetics cell homogenate and 1.11 min^{-1} in the HaCaT cell homogenate.

To visualize the metabolites' profile more accurately, Leu-enkephalin metabolism at initial concentration of 1 mM was studied. Figure 4 shows the concentration–time profiles of Tyr, TG, Phe and GGPL in the homogenate of HaCaT cells. Similar results were obtained with the Clonetics cells (data not shown). In the cellular homogenate, the concentration of Leu-enkephalin decreased, [Tyr] and [Phe] increased to 1 mM, the intermediate [GGPL] increased to a maximum of 0.7 mM after 35 min and then decreased, while [TG] reached maximum at 0.03 mM after 35 min and then decreased.

The appearance of Tyr and TG peaks in the keratinocytes homogenate indicates the presence of aminopeptidases and a dipeptidyl peptidase. Metabolism by aminopeptidases has been shown to be the major pathway for enkephalin degradation in other tissue homogenates [3]. The preponderance of Tyr relative to TG and TGG suggests that aminopeptidases are the main peptidases acting on the Leu-enkephalin in cultured keratinocytes homogenate. In separate studies, it was shown that inhibition of the aminopeptidases substantially decreases the rate of the hydrolysis of Leu-enkephalin in homogenates of cultured keratinocytes (data not shown). The delayed appearance of Phe (Fig. 4) suggests the presence of a carboxyl peptidase or a dicarboxyl peptidase in the homogenate.

In conclusion, this paper describes a simple LC system using a single step–gradient solvent system that separates Leu-enkephalin and its major metabolites. The isocratic solvent system avoids the problem of interfering ghost peaks. The method offers additional simplicity since all the separations occur in a single run. Although UV detection provided sufficient sensitivity for this study, it may be possible to enhance sensitivity by fluorescence detection with post-column derivatization. The LC system described in this article has potential utility in analysing Leu-enkephalin metabolites in other tissues. For example this analytical method has also been used to study Leu-enkephalin metabolism in cultured brain endo-

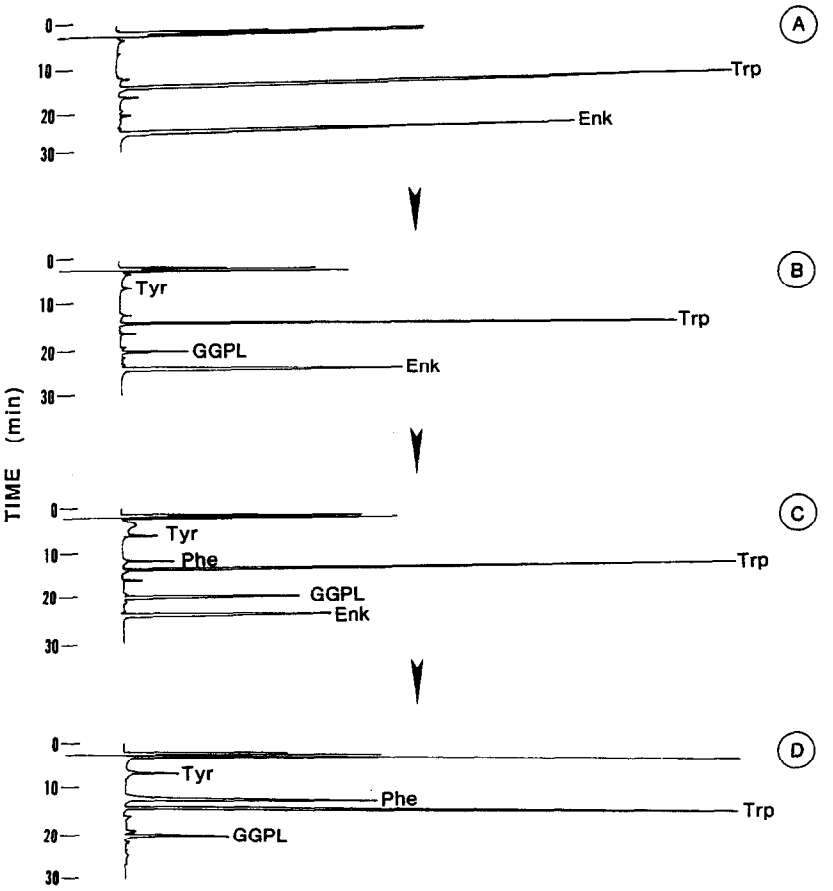

Figure 3
Leu-enkephalin metabolism in cultured Clonetics cell homogenate. Leu-enkephalin (100 μM) was incubated at 37°C for 0 min (Panel A), 2 min (Panel B), 5 min (Panel C) or 15 min (Panel D) as described in the Experimental section. Aliquots were removed and analysed by LC as described in Fig. 2.

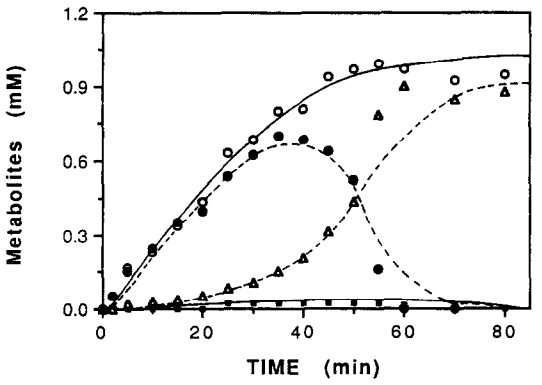

Figure 4
Concentration–time profiles for the major metabolites produced in a homogenate of HaCaT cells. Leu-enkephalin (1 mM) was incubated at 37°C in a homogenate of HaCaT cells, aliquots were removed and analysed by LC (Fig. 2) for its metabolites. ○, Tyr; ■, Tyr-Gly; △, Phe and ●, Gly-Gly-Phe-Leu.

thelial cells (S.E. Thompson and K.L. Audus, University of Kansas, personal communication).

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