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Journal of Chromatography B, 689 (1997) 287–294

JOURNAL OF CHROMATOGRAPHY B

Determination of [D-Ala^2,D-Leu^5]enkephalin and the metabolites containing C-terminal D-leucine by high-performance liquid chromatography

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Received 12 April 1996; revised 22 July 1996; accepted 26 July 1996

Abstract

A high-performance liquid chromatographic procedure has been developed for the determination of [D-Ala^2,D-Leu^5]enkephalin (DADLE) and the fragments containing D-leucine in rat blood. The procedure was applied to the determination of blood levels of [^{3}H-D-Leu^5]DADLE and the C-terminal fragments after intravenous administration of [^{3}H-D-Leu^5]DADLE to a rat. Unlabelled DADLE and the C-terminal fragments were spiked as carriers to rat blood samples and the blood samples were extracted with 1% trifluoroacetic acid in methanol. The recoveries from rat blood were quantitative for all compounds. DADLE and the C-terminal four fragments were well separated on a reversed-phase column with gradient elution using a mobile phase composed of 0.14% $HClO_4$ and acetonitrile.

Keywords: [D-Ala^2,D-Leu^5]Enkephalin; D-Leucine

1. Introduction

[D-Ala^2,D-Leu^5]Enkephalin (Tyr-D-Ala-Gly-Phe-D-Leu; DADLE) is an enkephalin analog, which has selective δ-receptor binding activity [1]. Pharmacokinetic studies of DADLE have been performed by use of commercially available [^{3}H-Tyr^1]DADLE as the substrate [2,3]. However, this labelled peptide cannot be used to elucidate the detailed metabolic profile of DADLE since the Tyr–D-Ala bond is rapidly cleaved by aminopeptidase [3]. In view of the problem, we turned our attention to other sites accessible to radioactive labelling of DADLE, and synthesized a DADLE derivative selectively tritiated in the C-terminal D-Leu residue ([^{3}H-D-Leu^5]DADLE) [4]. The methods currently available to separate enkephalins and its metabolites are based on HPLC [3,5–11]. However, no separation of DADLE and the corresponding fragments containing D-Leu was previously reported. In an attempt to clarify the metabolic profile of DADLE using its [^{3}H-D-Leu^5]-derivative, the present studies have been conducted: (1) synthesis of the fragments containing D-Leu, (2) separation and determination of DADLE and the fragments containing D-Leu by use of HPLC equipped with a flow-through radioisotope detector and (3) extraction of DADLE and the fragments containing D-Leu from rat whole blood.

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PII S0378-4347(96)00354-4

2. Experimental

2.1. General

Melting points were determined on a Yamato (Tokyo, Japan) MP-1 melting point apparatus and were uncorrected. Optical rotations were determined with a Jasco (Tokyo, Japan) DIP-4 polarimeter using the sodium D line at 589 nm. The ^{1}H NMR-spectra were measured with a Bruker (Reinstetten, Germany) AM-400 spectrometer using tetramethylsilane or sodium 3-(trimethylsilyl)propanesulfonate as internal standard. Mass spectra were measured with a VG (Manchester, UK) Auto Spec mass spectrometer. The HPLC system consisted of a Waters (Milfold, MA, USA) M-600E multisolvent system, a Rheodyne (Cotati, CA, USA) 7125 injector, a Shimadzu (Kyoto, Japan) SPD-6A spectrophotomeric detector and an Aloka (Tokyo, Japan) synchronized accumulating radioisotope detector (SARD) [12]. The SARD was composed of five counting cells with a 1-ml cell volume and five pairs of photomultiplier tubes. Radioactivity was counted by an Aloka Model 1000 liquid scintillation counter (LSC).

2.2. Reagents

The ^{3}H-labelled [D-Ala^2,D-Leu^5]enkephalin ([^{3}H-D-Leu^5]DADLE; specific radioactivity, 4.95 TBq/mmol) was synthesized in this laboratory as previously described [4]. [D-Ala^2,D-Leu^5]Enkephalin (DADLE), D-leucine (dL), N-*tert.*-butyloxycarbonylphenylalanine (Boc-Phe), N-*tert.*-butyloxycarbonylglycine (Boc-Gly) and N-benzyloxycarbonyl-D-alanine (Z-D-Ala) were purchased from Peptide Institute (Osaka, Japan).

Acetonitrile was of chromatographic purity. Unless otherwise specified, all other chemicals and solvents were of analytical grade and were used without further purification. Tetrahydrofuran (THF) was dried by distillation from sodium under nitrogen with benzophenone as an indicator.

A hydrophilic scintillation cocktail was prepared by dissolving 100 g of naphthalene, 4.0 g of 2,5-diphenyloxazol and 0.4 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 l of a mixture of dioxane–toluene–ethyl cellosolve (75:15:10, v/v).

2.3. Synthesis of the C-terminal fragments of DADLE

2.3.1. Boc-Phe-D-Leu-OBzl (1)

A solution of D-Leu-OBzl·*p*-tosylsulfonate (3.9 g, 10 mmol) in 80 ml of dichloromethane was washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4 and concentrated to yield D-Leu-OBzl as free base. The oily D-Leu-OBzl was diluted with 10 ml of THF. To a solution of Boc-Phe (2.65 g, 10 mmol) in 25 ml of THF cooled to −15°C was added N-methylmorpholine (NMM) (1.1 ml, 10 mmol) followed by isobutyl chloroformate (IBCF) (1.32 ml, 10 mmol). After 10 min, the solution of D-Leu-OBzl in 10 ml of THF was added. The reaction mixture was stirred at −15°C for 30 min. The cold bath was removed and the mixture was allowed to warm to room temperature for 1 h. The mixture was concentrated and the residue was partitioned between ethyl acetate and water. The organic phase was washed with 5% $NaHCO_3$, water, 10% $KHSO_4$ and water, dried over Na_2SO_4 and concentrated to yield the crude product, which was subjected to silica gel chromatography (chloroform–ethyl acetate, 30:1). Fractions containing the pure product as revealed by TLC were combined and evaporated to yield compound **1** (3.71 g, 79%) as a white solid: m.p. 93.5–94.5°C; $[\alpha]_D$ +21.6° (c. 1.1, methanol); ^{1}H NMR ($CDCl_3$) δ 0.83 (3H, $C^{\delta}H_3$ of D-Leu), 0.85 (3H, $C^{\delta}H_3$ of D-Leu), 1.40 (9H, $(CH_3)_3C$), 1.35–1.56 (3H, $C^{\beta}H_2$ and $C^{\gamma}H$ of D-Leu), 3.05 (2H, $C^{\beta}H_2$ of Phe), 4.37 (1H, $C^{\alpha}H$ of D-Leu), 4.58 (1H, $C^{\alpha}H$ of Phe), 5.13 (2H, CH_2 of Bzl), 6.21 (1H, NH of Phe), 7.20–7.37 (10H, aromatic protons of Phe and Bzl); MS (EI) *m/z* 468 (M^+). C, H, N elemental analysis (%): Calculated for $C_{27}H_{36}N_2O_5$: C, 69.21; H, 7.74; N, 5.98. Found: C, 69.04; H, 7.86; N, 5.95.

2.3.2. Phe-D-Leu-OBzl·HCl (2)

Compound **1** (3.28 g, 7 mmol) was dissolved in 30 ml of 4 *M* HCl in dioxane and stirred at room temperature for 2 h. The solution was evaporated to dryness. The residue was redissolved in 30 ml of dioxane and evaporated to yield compound **2** (2.81 g, 99.0%) as a white solid: m.p. 178–179°C; $[\alpha]_D$ +74.7° (c. 1.0, methanol); ^{1}H NMR [$(CD_3)_2SO$] δ 0.73 (3H, $C^{\delta}H_3$ of D-Leu), 0.77 (3H, $C^{\delta}H_3$ of D-

Leu), 1.25 (1H, $C^{\gamma}H$ of D-Leu), 1.41 (1H, $C^{\beta}H_2$ of D-Leu), 3.07 (2H, $C^{\beta}H_2$ of Phe), 4.11 (1H, $C^{\alpha}H$ of Phe), 4.26 (1H, $C^{\alpha}H$ of D-Leu), 5.13 (2H, CH_2 of Bzl), 7.25–7.40 (10H, aromatic protons of Phe and Bzl), 8.38 (1H, NH of Phe), 8.93 (1H, NH of D-Leu); MS (FAB) *m/z* 369 ($[M+1]^+$), 233, 120. C, H, N elemental analysis (%): Calculated for $C_{22}H_{28}N_2O_3 \cdot HCl$: C, 65.25; H, 7.22; N, 6.92. Found: C, 65.21; H, 7.33; N, 6.88.

*2.3.3. Phe-D-Leu (**3**: FdL)*

To a solution of compound **2** (0.42g, 1 mmol) in 10 ml of methanol was added 10% Pd/C (20 mg), and the mixture was stirred at room temperature under a hydrogen atmosphere for 2 h. The catalyst was removed by filtration and the filtrate was evaporated in vacuo. The oily residue was poured into water to yield dipeptide **3** (0.25 g, 87.7%) as a white solid: m.p. 237–238°C (decomp.); $[\alpha]_D$ +82.1° (c. 0.5, methanol); ^1H NMR (CD_3OD) δ 0.83 (3H, $C^{\delta}H_3$ of D-Leu), 0.87 (3H, $C^{\delta}H_3$ of D-Leu), 1.18 (1H, $C^{\gamma}H$ of D-Leu), 1.52 (1H, $C^{\beta}H_2$ of D-Leu), 3.16 (2H, $C^{\beta}H_2$ of Phe), 4.15 (1H, $C^{\alpha}H$ of D-Leu), 4.32 (1H, $C^{\alpha}H$ of Phe), 7.30–7.41 (5H, aromatic protons of Phe); MS (FAB) *m/z* 279 ($[M+1]^+$), 233, 132, 120. High-resolution MS (FAB). Calculated for $C_{15}H_{23}N_2O_3$ ($[M+H]^+$): 279.1709. Found: 279.1680. C, H, N elemental analysis (%): Calculated for $C_{15}H_{22}N_2O_3 \cdot 3/4H_2O$: C, 61.73; H, 8.11; N, 9.60. Found: C, 61.71; H, 7.93; N, 9.60.

*2.3.4. Boc-Gly-Phe-D-Leu-OBzl (**4**)*

To a solution of Boc-Gly (0.35 g, 2 mmol) and NMM (0.23 ml, 2 mmol) in 10 ml of THF was added IBCF (0.26 ml, 2 mmol) at −15°C with vigorous stirring. After additional stirring for 3 min, a solution of compound **2** (0.82 g, 2 mmol) and NMM (0.23 ml, 2 mmol) in 3 ml of dimethylformamide were added. The reaction mixture was stirred at −15°C for 30 min. The cold bath was removed and the mixture was allowed to warm to room temperature for 1 h. The mixture was concentrated and the residue was partitioned between ethyl acetate and water. The organic phase was washed with 5% $NaHCO_3$, water, 10% $KHSO_4$ and water, dried over Na_2SO_4 and concentrated to yield compound **4** (0.97 g, 92%) as a white solid: m.p. 143–145°C; $[\alpha]_D$ +12.4° (c. 1.0, methanol); ^1H NMR ($CDCl_3$) δ 0.82 (3H, $C^{\delta}H_3$ of D-Leu), 0.83 (3H, $C^{\delta}H_3$ of D-Leu), 1.42 (9H, $(CH_3)_3C$), 1.27–1.53 (3H, $C^{\beta}H_2$ and $C^{\gamma}H$ of D-Leu), 3.07 (2H, $C^{\beta}H_2$ of Phe), 3.71 (2H $C^{\alpha}H_2$ of Gly), 4.52 (1H, $C^{\alpha}H$ of D-Leu), 4.75 (1H, $C^{\alpha}H$ of Phe), 5.10 (2H, CH_2 of Bzl), 7.19–7.37 (10H, aromatic protons of Phe and Bzl); MS (FAB) *m/z* 526 ($[M+1]^+$). C, H, N elemental analysis (%): Calculated for $C_{29}H_{39}N_3O_6$: C, 66.27; H, 7.48; N, 7.99. Found: C, 66.20; H, 7.40; N, 8.04.

*2.3.5. Gly-Phe-D-Leu-OBzl·HCl (**5**)*

Compound **4** (1.58 g, 3 mmol) was dissolved in 30 ml of 4 *M* HCl in dioxane and stirred at room temperature for 2 h. The solution was evaporated to dryness. The residue was redissolved in 30 ml of dioxane and evaporated to yield compound **5** (1.38 g, 99.6%) as a white solid: m.p. 77–78°C; $[\alpha]_D$ +33.1° (c. 0.5, methanol); ^1H NMR ($(CD_3)_2SO$) δ 0.76 (3H, $C^{\delta}H_3$ of D-Leu), 0.82 (3H, $C^{\delta}H_3$ of D-Leu), 1.38–1.55 (3H, $C^{\beta}H_2$ and $C^{\gamma}H$ of D-Leu), 2.79 and 2.94 (2H, $C^{\beta}H_2$ of Phe), 3.48 (2H, $C^{\alpha}H_2$ of Gly), 4.27 (1H, $C^{\alpha}H$ of D-Leu), 4.70 (1H, $C^{\alpha}H$ of Phe), 5.10 (2H, CH_2 of Bzl), 7.16–7.39 (10H, aromatic protons of Phe and Bzl), 8.09 (3H, NH_3^+ of Gly), 8.64 (1H, NH of Phe), 8.74 (1H, NH of D-Leu); MS (FAB) *m/z* 426 ($[M+1]^+$), 369, 222, 205, 177, 120. High-resolution MS (FAB): Calculated for $C_{24}H_{31}N_3O_4$ ($[M+H]^+$): 426.2398. Found: 426.2385.

*2.3.6. Gly-Phe-D-Leu (**6**: GFdL)*

To a solution of compound **5** (0.46g, 1 mmol) in 10 ml of methanol was added 10% Pd/C (20 mg), and the mixture was stirred at room temperature under a hydrogen atmosphere for 2 h. The catalyst was removed by filtration and the filtrate was evaporated in vacuo. The oily residue was poured into water to yield tripeptide **6** (0.37 g, 100%) as a white solid: m.p. 210–211°C (decomp.); $[\alpha]_D$ +25.3° (c. 1.0, methanol); ^1H NMR (CD_3OD) δ 0.81 (3H, $C^{\delta}H_3$ of D-Leu), 0.85 (3H, $C^{\delta}H_3$ of D-Leu), 1.28 (1H, $C^{\gamma}H$ of D-Leu), 1.49 (1H, $C^{\beta}H_2$ of D-Leu), 2.95 and 3.07 (2H, $C^{\beta}H_2$ of Phe), 3.67 (2H, $C^{\alpha}H2$ of Gly), 4.32 (1H, $C^{\alpha}H$ of Phe), 4.70 (1H, $C^{\alpha}H$ of D-Leu), 7.18–7.29 (5H, aromatic protons of Phe); MS (FAB) *m/z* 336 ($[M+1]^+$), 279, 205, 177, 120.

C, H, N elemental analysis (%): Calculated for $C_{17}H_{25}N_3O_4 \cdot 1/2H_2O$: C, 59.29; H, 7.61; N, 12.20. Found: C, 59.04; H, 7.33; N, 12.11.

2.3.7. Z-D-Ala-Gly-Phe-D-Leu-OBzl (7)

To a solution of Z-D-Ala (0.34 g, 1.5 mmol) and NMM (0.16 ml, 1.5 mmol) in 10 ml of THF was added IBCF (0.2 ml, 1.5 mmol) at −15°C with vigorous stirring. After additional stirring for 3 min, a solution of compound **5** (0.69 g, 2 mmol) and NMM (0.16 ml, 2 mmol) in 3 ml of THF were added. The reaction mixture was stirred at −15°C for 30 min. The cold bath was removed and the mixture was allowed to warm to room temperature for 1 h. The mixture was concentrated and the residue was partitioned between ethyl acetate and water. The organic phase was washed with 5% $NaHCO_3$, water, 10% $KHSO_4$ and water, dried with Na_2SO_4 and concentrated to yield the crude products, which was subjected to silica gel chromatography (chloroform–methanol, 20:1). Fractions containing the pure product as revealed by TLC were combined and evaporated to yield compound **7** (0.71 g, 75%) as a white solid: m.p. 160.5–161.5°C; $[\alpha]_D$ +24.6° (c. 0.5, methanol); ^{1}H NMR ($CDCl_3$) δ 0.80 (3H, $C^{\delta}H_3$ of D-Leu), 0.82 (3H, $C^{\delta}H_3$ of D-Leu), 1.33 (3H, CH_3 of D-Ala), 1.37–1.55 (3H, $C^{\beta}H_2$ and $C^{\gamma}H$ of D-Leu), 3.03 and 3.11 (2H, $C^{\beta}H_2$ of Phe), 3.92 (2H $C^{\alpha}H_2$ of Gly), 4.38 (1H), 4.55 (1H), 4.87 (1H), 5.02–5.12 (4H, CH_2 of Bzl and CH_2 of Z), 7.14–7.33 (15H, aromatic protons of Phe, Bzl and Z); MS (FAB) *m/z* 631 ($[M+1]^+$). C, H, N elemental analysis (%): Calculated for $C_{35}H_{42}N_4O_7$: C, 66.65; H, 6.71; N, 8.88. Found: C, 66.65; H, 6.81; N, 8.87.

2.3.8. D-Ala-Gly-Phe-D-Leu (8: dAGFdL)

To a solution of compound **7** (0.5 g, 0.8 mmol) in a mixture of methanol (10 ml) and water (2 ml) was added 10% Pd/C (20 mg), and the mixture was stirred at room temperature under a hydrogen atmosphere for 1.5 h. To the mixture was added 15 ml of 1.5% acetic acid and 10% Pd/C (20 mg), followed by hydrogenation for another 1.5 h. The catalyst was removed by filtration and washed with water. The combined filtrate and washings were adjusted to pH 6 with concentrated ammonia and evaporated to yield a white solid, which was recrystallized from methanol–water to yield tetrapeptide **8** (0.22 g, 69.5%) as a white solid: m.p. 223–234°C (decomp.); $[\alpha]_D$ +16.7° (c. 0.2, 1 *M* HCl); ^{1}H NMR (1 *M* DCl) δ 0.74 (3H, $C^{\delta}H_3$ of D-Leu), 0.81 (3H, $C^{\delta}H_3$ of D-Leu), 1.48–1.57 (5H, $^{\beta}H_2$ of D-Leu and $C^{\beta}H_3$ of D-Ala), 1.74 (1H,$C^{\gamma}H$ of D-Leu), 2.92 and 3.07 (2H, $C^{\beta}H_2$ of Phe), 3.99 (2H, $C^{\alpha}H_2$ of Gly), 4.15–4.22 (2H, $C^{\alpha}H$ of D-Leu and $C^{\alpha}H$ of D-Ala), 4.66 (1H, $C^{\alpha}H$ of Phe), 7.12–7.45 (5H, aromatic protons of Phe); MS (FAB) *m/z* 407 ($[M+1]^+$), 336, 279, 129, 120. C, H, N elemental analysis (%): Calculated for $C_{20}H_{30}N_4O_5$: C, 59.09; H, 7.44; N, 13.79. Found: C, 59.26; H, 7.69; N, 13.86.

2.4. HPLC analysis of DADLE and the metabolites containing D-Leu

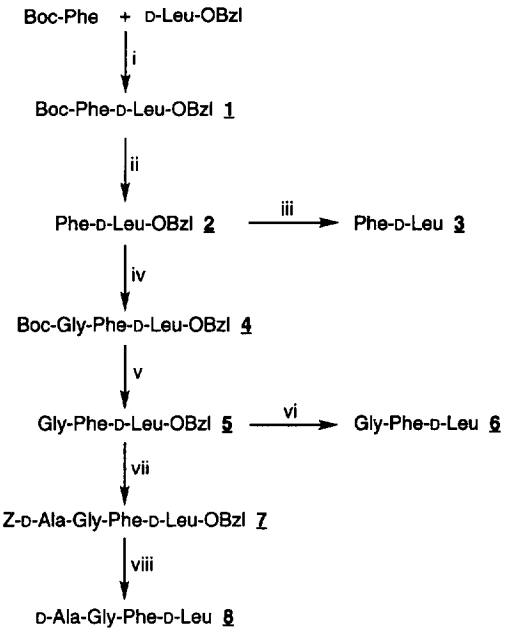
Separation was performed on a LiChrospher RP-18 column (250×4.0 mm I.D., 5 μm particle size). Mobile phases were composed of (A) 0.14% $HClO_4$ and (B) 0.14% $HClO_4$ and acetonitrile (50:50, v/v). The flow-rate was 1.0 ml/min. Elution was carried out with a gradient from 0 to 20% solvent B over 3 min, from 20 to 43% over 2 min, from 43 to 45% over 30 min and 45 to 58% over 5 min, isocratic at 58% over 7 min, followed by a gradient from 58 to 70% over 5 min and final isocratic elution at 70% B for 15 min. The column effluent was monitored by UV absorption at 214 and 200 nm, respectively, and/or the SARD flow-through radioisotope detector. When coupled to the SARD detector, the effluent from the UV detector was mixed with the hydrophilic scintillation cocktail in the ratio 1:9 (v/v), and thus the total flow-rate measured at the SARD outlet was 10 ml/min.

2.5. Counting efficiency

Aliquots of 100 Bq of [^{3}H-D-Leu^5]DADLE dissolved in 9 ml of the hydrophilic scintillation cocktail and 1 ml of the respective mobile phases were counted by LSC. The following mobile phases composed of 0.14% $HClO_4$ and acetonitrile were used: (A) 90:10; (B) 80:20; (C) 75:25; (D) 70:30 (v/v). The counting efficiency in each mobile phase was calculated by an external channel-ratio method.

2.6. Sample preparation

A solution of [^{3}H-D-Leu^5]DADLE (1.33 μg/kg) in saline was injected into a male Wister rat through the right tail vein. Blood samples (80 μl) were taken from the left tail vein at 1, 3, 5, 10, 15, 30, 45, 60, 120 and 240 min after administration. Immediately after collection, the sample was denatured by addition of 400 μl of 1% trifluoroacetic acid (TFA) in methanol containing the non-radioactive DADLE, dAGFdL, GFdL, FdL and dL (each 8 nmol) as carriers. After vortex-mixing for a few seconds, the sample was centrifuged for 5 min at 14 000 *g*. The supernatant was separated and the residue was washed, 3 times, with 400 μl of 1% TFA in methanol. The supernatant and washings were combined and evaporated under a stream of nitrogen. To the residue was added 200 μl of 0.14% $HClO_4$–acetonitrile (9:1, v/v). The samples were filtered through a membrane filter (pore size 0.45 μm) and the filtrates were subjected to HPLC analysis. Another aliquot of the filtrates was mixed with 10 ml of the hydrophilic scintillation cocktail and the radioactivity of the mixture was counted by LSC.



Scheme 1. Reagents: (i) Et_3N, isobutyl chloroformate; (ii) 4 *M* HCl/dioxane; (iii) H_2, 10% Pd/carbon; (iv) Et_3N, isobutyl chloroformate followed by Boc-Gly; (v) 4 *M* HCl/dioxane; (vi) H_2, 10% Pd/carbon; (vii) Et_3N, isobutyl chloroformate followed by Z-D-Ala; (viii) H_2, 10% Pd/carbon.

3. Results and discussion

The C-terminal fragments of DADLE were prepared using a stepwise solution-phase synthesis (Scheme 1). Coupling reactions were performed by intermediate synthesis of the mixed anhydrides using isobutyl chloroformate. The removal of *tert.*-butyloxycarbonyl (Boc) group by HCl in dioxane proceeded quantitatively. The cleavage of the benzyloxycarbonyl (Z) group and benzyl (Bzl) group by catalytic hydrogenation in the presence of Pd/C was also accomplished with good yield.

The separation of DADLE and the C-terminal fragments was performed on a reversed-phase column with gradient elution using the mobile phase composed of 0.14% $HClO_4$ and acetonitrile. Fig. 1 (upper part) shows a typical chromatogram of FdL, GFdL, dAGFdL and DADLE. The retention times are: FdL, 55.8 min; dAGFdL, 57.1 min; GFdL, 57.9 min and DADLE, 64.2 min. dL was not detectable at λ=214 nm, but was detectable at λ=200 nm and the retention time was 8.4 min.

On radioisotope detection, variation of the mobile phase composition under gradient elution may cause chemical quenching and then lower counting efficiencies are achieved. The influence of the mobile phase on the counting efficiency was investigated by measuring authentic [^{3}H-D-Leu^5]DADLE (100 Bq) in a solution containing different mobile phase compositions. Table 1 shows the counting efficiency on LSC. The counting efficiencies were almost constant. The result indicated that the counting efficiency on the radioisotope detector was constant under the gradient elution conditions, because a linear relationship existed between the counting efficiency on the radioisotope detector and the counting efficiency by use of 'off-line' LSC [13].

The limit of detection was investigated by measuring authentic [^{3}H-D-Leu^5]DADLE in the range 3 to 10 Bq (Fig. 2). When the limit of detection is defined as the amount of labelled peptide that will provide a signal-to-noise ratio of 2, the limit of detection for [^{3}H-D-Leu^5]DADLE is about 5 Bq, which corresponds to 1 fmol since the specific radioactivity is

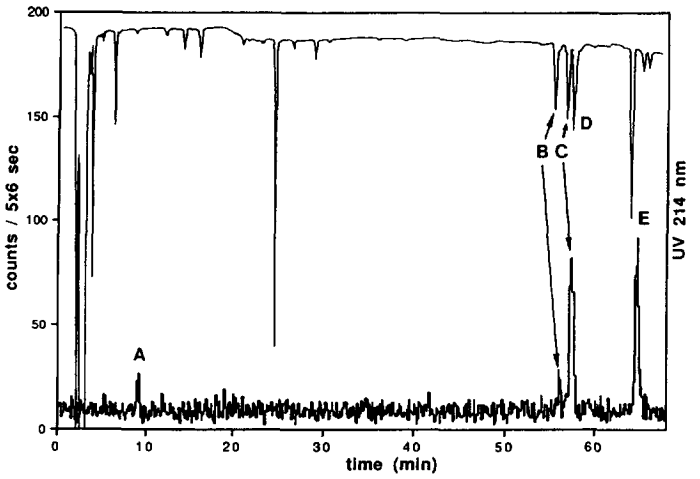


Fig. 1. HPLC tracing of [^{3}H-D-Leu^5]DADLE and the metabolites in rat whole blood at 15 min after intravenous administration of [^{3}H-D-Leu^5]DADLE (1.33 μg/kg). Conditions: column, LiChrospher RP-18 (250×4.0 mm I.D., 5 μm particle size); mobile phase, 0.14% $HClO_4$–acetonitrile; flow-rate, 1.0 ml/min; total flow-rate, 10 ml/min; λ=214 nm. Gradient conditions are given in the experimental section. (A)=D-Leu (dL), (B)=Phe-D-Leu (FdL), (C)=D-Ala-Gly-Phe-D-Leu (dAGFdL), (D)=Gly-Phe-D-Leu (GFdL), (E)=Tyr-D-Ala-Gly-Phe-D-Leu (DADLE).

Table 1
Counting efficiency

Mobile phase composition (0.14% $HClO_4$–acetonitrile, v/v)	Counting efficiency (mean±S.D., n=3) (%)
90:10	49.8±0.5
80:20	50.3±0.7
75:25	50.4±0.2
70:30	51.8±0.6

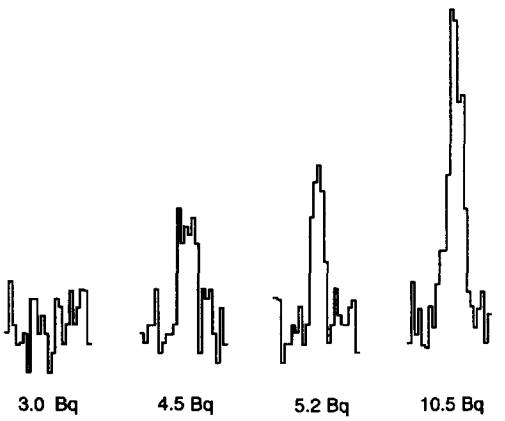


Fig. 2. Limit of detection. Data obtained by injecting [^{3}H-D-Leu^5]DADLE in the range of 3 to 10 Bq.

4.95 TBq/mmol. The limit of detection is three orders of magnitude lower than that obtained by measuring unlabelled DADLE with UV detection.

Special attention was paid to avoid production of artifacts via metabolism during sample preparation for subsequent HPLC. It is a prerequisite for the recovery of trace amounts of [^{3}H-D-Leu^5]DADLE and the C-terminal fragments from blood samples to add unlabelled DADLE and C-terminal fragments as carriers. These non-labelled peptides should also serve as masking agents for residual silanol groups on a reversed-phase column in order to suppress undesired silanophilic interactions [14]. In addition, it will be feasible to spike a blood sample with a few nmol of unlabelled DADLE and the corresponding C-terminal fragments, which then can be used for identification of the corresponding radioactive metabolites of [^{3}H-D-Leu^5]DADLE. Therefore, unlabelled DADLE, dAGFdL, GFdL, FdL and dL (each 8 nmol) were added to blood samples as reference standards. Furthermore, an appropriate extraction solvent had to be chosen in the present study. Our preliminary study demonstrated that Leu-enkephalin was extracted quantitatively from rat whole blood with five volumes of 1% trifluoroacetic acid (TFA) in methanol. The extraction solvent completely denatured hydrolyzing enzymes. [^{3}H-D-Leu^5]DADLE

(1.37 kBq, 279 fmol) added to pooled blank rat whole blood was extracted with five volumes of 1% TFA in methanol. The absolute extraction recovery of [^{3}H-D-Leu^5]DADLE from spiked rat blood was 99.3±0.8% (mean±S.D., n=3). Radio-HPLC analysis of the extract showed no degradation products, which indicated that DADLE was stable under the applied extraction conditions. The absolute recoveries of the C-terminal fragments were estimated by comparing the UV peak areas obtained from direct injection of known quantities of the analytes with those obtained from the injection of extracts of blood samples spiked with the corresponding unlabelled standards. The results depicted in Table 2 revealed that 1% TFA in methanol proved to be efficient for complete recovery of DADLE and its C-terminal fragments.

For assessment of the separation efficiency of the present HPLC method, the determination of [^{3}H-D-Leu^5]DADLE metabolites in rat whole blood after intravenous administration of [^{3}H-D-Leu^5]DADLE (1.33 μg/kg) was performed. Fig. 1 (lower part) illustrates a representative radio-chromatogram of [^{3}H-D-Leu^5]DADLE and its metabolites obtained from a blood sample at 15 min after peptide administration. The delay time between UV and radioisotope detection was estimated by injecting [^{3}H-D-Leu^5]DADLE spiked with the corresponding nonlabelled derivatives, and was found to be 1 min. The retention time values of DADLE and the C-terminal fragments were used to identify the radioactive metabolites: (A), dL; (B), FdL; (C), dAGFdL; (E), DADLE. Fig. 3 shows the concentrations of [^{3}H-D-Leu^5]DADLE and the C-terminal fragments vs. time curve in a rat. The disappearance of [^{3}H-D-Leu^5]DADLE in blood was very rapid. By 45 min, there was no detectable unchanged [^{3}H-D-Leu^5]DADLE. The appearance of dAGFdL was very quickly detected in the first blood sample taken 1 min after peptide

Table 2
Recovery of DADLE and the metabolites containing D-Leu from rat whole blood

	Recovery (mean±S.D., n=3) (%)
DADLE	94.5±2.4
dAGFdL	89.9±9.6
GFdL	94.7±3.3
FdL	103.4±5.5

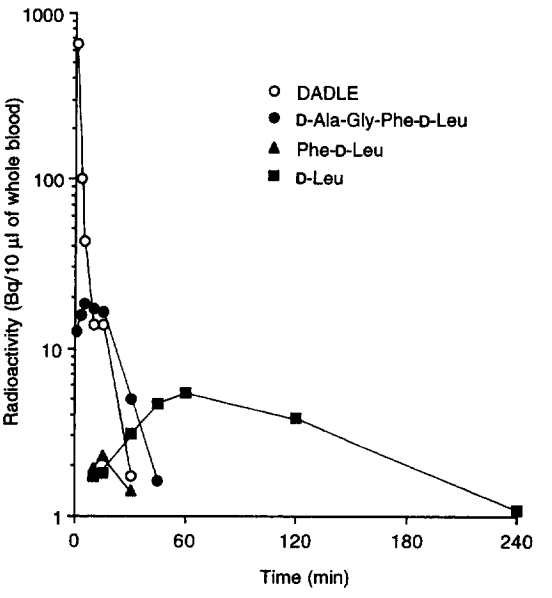


Fig. 3. Blood concentration–time profiles of [^{3}H-D-Leu^5]DADLE and the C-terminal metabolites in a rat after intravenous administration of [^{3}H-D-Leu^5]DADLE (1.33 μg/kg).

administration. These findings seemed to indicate that degradation of [^{3}H-D-Leu^5]DADLE occurred predominantly by removal of the tyrosine residue at the N-terminal end to yield dAGFdL. The tetrapeptide (dAGFdL) was then further hydrolyzed to FdL, which rapidly degraded at the Phe–D-Leu bond to yield dL. GFdL was not observed in all samples. The pharmacokinetics of DADLE will be discussed in detail elsewhere.

The present method provides a sensitive and reliable method for determining blood levels of [^{3}H-D-Leu^5]DADLE and the C-terminal metabolites. Thus, the method is applicable to the monitoring of pharmacokinetic and metabolic profiles of DADLE.

References

[1] S. Udenfriend and J. Meienhofer (Editors), The Peptides, Vol. 6, Academic Press, Orlando, FL, 1984, p. 147.
[2] G. Schulteis, S.B. Weinberger and J.L. Martinez Jr., Peptides, 10 (1989) 913.
[3] K.S.E. Su, K.M. Campanale, L.G. Mendelsohn, G.A. Kerchner and C.L. Gries, J. Pharm. Sci., 74 (1985) 394.
[4] H. Hasegawa, Y. Shinohara and S. Baba, J. Chem. Soc., Perkin Trans., 1 (1990) 2641.

[5] F.B. Craves, P.Y. Law, C.A. Hunt and H.H. Loh, J. Pharmacol. Exp. Ther., 206 (1978) 492.
[6] M. Nakano, M. Kai, M. Ohno and Y. Ohkura, J. Chromatogr., 411 (1987) 305.
[7] M. Ohno, M. Kai and Y. Ohkura, J. Chromatogr., 421 (1987) 245.
[8] H. Lentzen and R. Simon, J. Chromatogr., 389 (1987) 444.
[9] P.K. Shah and R.T. Borchardt, J. Pharm. Biomed. Anal., 8 (1990) 457.
[10] S. Shibanoki, S.B. Weinberger, K. Ishikawa and J.L. Martinetz Jr., J. Chromatogr., 532 (1990) 249.
[11] M.A. Hussain, S.M. Rowe, A.B. Shenvi and B.J. Aungst, Drug Metab. Dispos., 18 (1990) 288.
[12] S. Baba, M. Horie and K. Watanabe, J. Chromatogr., 244 (1982) 57.
[13] S. Baba, Y. Suzuki, Y. Sasaki and M. Horie, J. Chromatogr., 392 (1987) 157.
[14] D. Liu and D.M. Desiderio, J. Chromatogr., 422 (1987) 61.