SYNTHESIS AND DISTRIBUTION OF HCl · Pro-Phe-[^{14}C]-Gly-NH_2 IN MICE

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UDC 547.963:615.015.1

The hypothalamic hormone melanostatin (Pro-Leu-Gly-NH_2 · NH_2) has various actions on the central nervous system [2], which explains the continuing interest in it from both the chemical and pharmacological points of view. However, among the many synthetic analogs of this tripeptide, only compounds with high biological activity are known [6, 8]. We have previously shown that substitution of the position 2 of the Leu molecule for the similar Phe results in an increase in psychotropic activity [3, 5]. In order to study the distribution of the compound in the body, we have now synthesized and investigated HCl · Pro-Phe-[^{14}C]-Gly-NH_2 (I).

EXPERIMENTAL (CHEMICAL)

Peptide I was synthesized using 2-[^{14}C]-glycine with activity of 1.63 Ci/mole. The radiochromatographic purification and specific activity of I and its intermediates in the synthesis were determined by zonal thin layer chromatography (TLC). Detection of radiolabeled materials was carried out using an LKB Rack-Beta 1219 liquid beta scintillation counter with internal and external standardization of samples. The counting efficiency was not less than 95%. The composition of the scintillant mix was: 2,3-diphenyloxazone: 1,4-bis(2,5-phenyloxazolyl)benzene:toluene:ethanol = 4:0.1:609:243. Results were processed by computer using an Olivetti M-29, Wallac. Reagent purity was monitored by TLC on Silufol (Kavalier, Czechoslovakia) plates using the following solvent systems: A) benzene:acetone:acetic acid 100:50:1; B) butanol:acetic acid:water 4:1:1, C) ethanol:water 4:1. Chromatography plates were developed with chlorotoluidine and ninhydrin reagents. Amino acid analysis was carried out using a Microtechno T-339 (Czechoslovakia) amino acid analyzer, with peptide hydrolysis in 6 M HCl at 110°C for 24 h. Determinations of amino acid compositions corresponded with the amino acid composition of the peptide. The proportions of substances reacted were determined using a Perkin–Elmer 241 MC spectrophotometer. Peptide synthesis was carried out using amino acids and their L-configuration derivatives from Merck (Switzerland) and Reanal (Hungary). Amino acid abbreviations were as recommended by IUPAC-IUB [7]. Other abbreviations were as follows: $(BOC)_2O$ is di-tert-butylpyrocarbonate; t-BuOH is tert-butyl alcohol; DCCl is dicyclohexylcarbodiimide; Et_3N is triethylamine; i-BuOCOCl is isobutylchloroformiate; 15-c-5 is 15-crown-5; BtOH is 1-hydroxybenzotriazole.

BOC-[^{14}C]-Gly-OH (II)

$NaHCO_3$ (0.065 g in 0.65 ml of water) and $(BOC)_2O$ (0.37 g, 1.69 mmoles, in 1.3 ml of t-BuOH) were added to a solution of 0.1 g (1.3 mmoles) of H-[^{14}C]-Gly-OH in 1.3 ml (1.3 mmoles) of 1 M NaOH. The reaction volume was made up to 5 ml with t-BuOH and was stirred for 2 h at room temperature. The volume was then made up to 25 ml with water, washed with 25 ml of hexane, acidified to pH 3 and 1 M HCl, and extracted with ethyl acetate (3 × 25 ml). The ethyl acetate solution was dried over $MgSO_4$ and the solvent was evaporated in vacuo, and the product was redissolved in hexane. The yield was 0.195 g (86.2%) of II, and the melting temperature was 93-94°C, R_f was 0.49 in solvent system A, and the specific radioactivity was 0.63 Ci/mole.

A. B. Bogatskii Physicochemical Institute, Ukrainian Academy of Sciences, Odessa. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 26, Nos. 11-12, pp. 16-18, November-December, 1992. Original article submitted January 30, 1992.

0091-150X/92/1112-0803$12.50

TABLE 1. Contents (in cpm · 10^3/min/2 ml) of Total Radioactive Material in Mouse Organs and Tissues after s.c. Dosage with HCl · Pro-Phe-[^{14}C]-Gly-HN_2

Organ or tissue	Time after dosage							
	10 min	30 min	1 h	2 h	4 h	6 h	8 h	24 h
Plasma	3,60±0,61	11,51±1,34	4,40±0,52	2,63±0,23	3,46±0,66	3,00±0,72	2,58±0,49	2,27±0,37
Brain	0,83±0,12	1,47±0,32	1,82±0,22	1,86±0,51	0,54±0,10	0,41±0,13	0,36±0,10	0,41±0,09
Heart	3,23±0,81	2,98±0,42	2,73±0,24	2,34±0,59	0,92±0,13	0,52±0,15	0,51±0,11	1,91±0,12
Lungs	5,27±0,90	6,05±1,10	3,18±0,38	3,58±1,20	1,16±0,15	1,04±0,27	0,58±0,12	0,58±0,16
Liver	15,82±0,75	20,11±3,56	18,85±1,16	24,78±1,76	5,20±0,91	3,65±0,57	4,85±0,14	1,21±0,15
Kidneys	25,60±0,73	21,69±1,65	18,14±2,75	18,58±2,40	4,12±0,70	3,41±0,77	2,31±0,70	0,56±0,14
Spleen	5,61±1,00	10,88±2,81	9,01±1,22	7,97±0,43	3,07±0,38	3,07±0,40	1,81±0,57	1,60±0,73
Fatty tissue	1,51±0,43	2,49±0,39	1,83±0,24	1,94±0,42	0,52±0,12	0,36±0,16	0,52±0,12	0,36±0,10
Muscle	2,15±0,39	3,90±0,42	3,34±0,12	1,17±0,20	0,21±0,08	0,18±0,06	0,25±0,09	0,29±0,03

TABLE 2. Contents (in cpm · 10^3/min/2 ml) of Total Radioactive Material in Mouse Organs and Tissues after i.v. Dosage with HCl · Pro-Phe-[^{14}C]-Gly-NH_2

Organ or tissue	Time after dosage							
	10 min	30 min	1 h	2 h	4 h	6 h	8 h	24 h
Plasma	5,52±0,53	5,71±0,81	4,75±0,20	2,95±0,29	5,23±0,84	4,40±0,31	4,76±0,32	2,00±0,34
Brain	2,04±0,50	0,85±0,32	0,84±0,31	0,49±0,12	0,58±0,13	0,45±0,15	0,44±0,08	0,41±0,07
Heart	5,50±0,80	1,49±0,46	1,27±0,36	1,29±0,29	0,83±0,06	0,66±0,08	0,71±0,11	0,57±0,12
Lungs	9,40±0,63	3,53±0,36	1,83±0,44	4,02±0,85	1,96±0,29	0,89±0,18	0,48±0,05	0,49±0,03
Liver	30,89±2,09	65,77±1,26	69,91±1,28	48,90±1,02	6,05±0,29	3,96±0,37	3,72±0,26	2,45±0,21
Kidneys	50,61±3,07	9,02±0,77	7,76±0,15	5,06±0,84	5,60±0,84	5,60±0,95	5,27±0,95	2,57±0,72
Spleen	10,87±0,83	4,17±0,57	2,03±0,62	0,56±0,09	2,16±0,72	2,41±0,82	1,28±0,09	0,48±0,10
Fatty tissue	3,14±0,33	0,88±0,30	0,69±0,08	0,68±0,10	0,32±0,08	0,29±0,01	0,26±0,03	0,19±0,01
Muscle	2,72±0,57	0,23±0,04	0,25±0,02	0,17±0,05	0,15±0,01	0,29±0,09	0,18±0,05	0,15±0,04

HCl · H-[^{14}C]-Gly-NH_2 (III)

Chloroform (4 ml), dimethylformamide (1 ml), BtOH (0.162 g, 1.2 mmoles), and DCCl (0.237 g) were added to 0.195 g (1.1 moles) of II; the reaction mix was stirred for 30 min at room temperature. NH_4OH (0.11 ml, 1.43 mmoles, in a 25% solution) was then added, and the reaction was again stirred, for 3 h. The solvents were evaporated in vacuo, and the residue was dissolved in 15 ml of water and washed with ethyl acetate (4 × 15 ml). The ethyl acetate solution was dried over $MgSO_4$ and evaporated in vacuo. The product was treated with 3 ml of 4 M HCl in dioxane. The yield was 0.119 g (98.2%) of III; the melting temperature was 201-203°C, the R_f was 0.22 in solvent system B, and the specific radioactivity was 0.38 Ci/mole.

BOC-Pro-Phe-OH (IV)

Solution A was prepared by dissolving 1.652 g (10 mmoles) of H-Phe-OH in 10 ml I M KOH, and adding 2.2 ml (10 mmoles) of 15-c-5 in 10 ml of dimethylformamide; water was evaporated in vacuo at ≤40°C, and the residue was cooled to −15°C. Solution B was prepared by dissolving 2.15 g (10 mmoles) of BOC-Pro-OH in 10 ml of dimethylformamide and cooling it to −15°C; 1.4 ml (10 mmoles) of Et_3N and 1.3 ml (10 mmoles) of i-BuOCOCl were then added. After 1 min, solutions A and B were mixed together, and kept for 30 min at room temperature. The reaction mix was then filtered, and 0.6 ml (10 mmoles) of acetic acid, 20 ml of saturated aqueous NaCl, and 50 ml of ethyl acetate were added. The ethyl acetate solution was separated and sequentially washed with 1 M HCl (3 × 50 ml), saturated aqueous NaCl (2 × 50 ml), 5% $NaHCO_3$ (2 × 50 ml), and saturated aqueous NaCl (2 × 50 ml). The ethyl acetate solution was then dried over $MgSO_4$ and evaporated in vacuo (at ≤40°C), and the residue was redissolved in hexane. The yield was 3.19 g (88.0%) of IV; the melting temperature was 149-151°C, $[\alpha]_{578}^{20}$ was 37.9° (c 0.8, MeOH), R_f was 0.46 in solvent system A, and the molecular formula was $C_{19}H_{26}N_2O$.

HCl · Pro-Phe-[^{14}C]-Gly-NH_2 (I)

Solution A was prepared by dissolving 2.81 g (5 mmoles) of IV in 5 ml of 1 M KOH and adding 1.1 ml (5 mmoles) of 15-c-5 and 10 ml of dimethylformamide. Water was evaporated in vacuo (at ≤40°C), and the residue was cooled to −15°C.

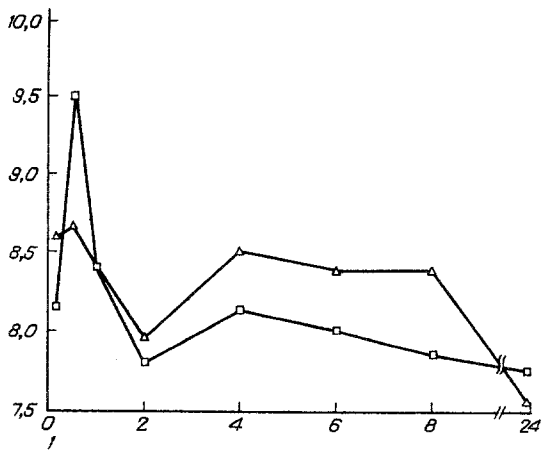

Fig. 1. Changes in the content of radioactive material in mouse plasma after i.v. (a) and s.c. (b) dosage with I. Here and in Fig. 2: the abscissa show time in h, and the ordinate shows the ratio of concentrations in brain and plasma (see text).

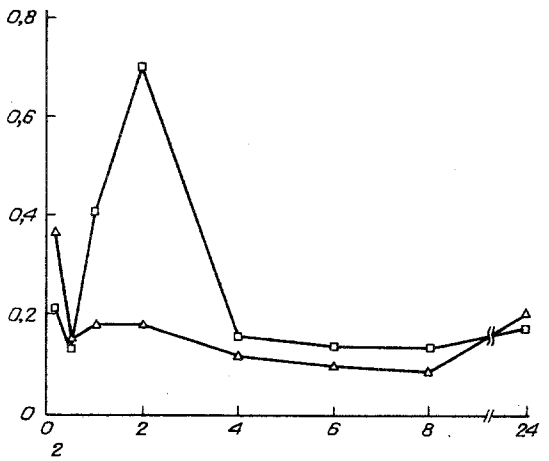

Fig. 2. Ratios of total radioactive material in mouse brain and blood after i.v. (a) and s.c. (b) dosage with I.

Solution B was prepared by mixing 0.55 g (5 mmoles) of III with 5 ml of dimethylformamide and 0.7 ml (5 mmoles) of Et_3N. After 20 min, the precipitate was removed by centrifugation, and the supernatant was cooled to −15°C. i-BuCOCl (0.68 ml, 5 mmoles) was added to solution A, and solutions A and B were mixed together 1 min later, and incubated at room temperature for 30 min. The reaction mix was then supplemented with 20 ml of saturated aqueous NaCl and 50 ml ethyl acetate. The ethyl acetate layer was collected, and was sequentially washed with 1 M HCl (3 × 50 ml), saturated aqueous NaCl (2 × 50 ml), 5% $NaHCO_3$ (2 × 50 ml), and saturated aqueous NaCl (2 × 50 ml). The solution was dried over $MgSO_4$ and evaporated in vacuo. The residue was treated with 3 ml of 4 M HCl in dioxane. After 20 min the solvent was evaporated in vacuo, and the produce was redissolved in hexane. The yield was 1.43 g (80.2%) of I; the melting temperature was 156-157°C $[\alpha]_{589}^{22}$ was 18.0° (c 5.0, MeOH), R_f was 0.57 in solvent system B, and the specific radioactivity was 0.13 Ci/mole. Amino acid analysis gave Pro:Phe:Gly = 1.1:1.0:1.04.

TABLE 3. Kinetic Parameters of the Slow Phase (4-24 h) of Elimination from Mouse Plasma of the ^{14}C-Peptide

Kinetic parameters	Route of dosage	S.c.
C_0+mc	5,620+0,16	3,330+0,13
$K+mk$	0,037+0,003	0,017+0,002
S_{0-24}	179786,4	131224,1

Notes. C_0) Initial concentration (cpm · 10^3/min/ml) of ^{14}C-products in the slow phase of elimination from plasma; K) rate constant of elimination in the slow phase (r^{-1}); S_{0-24}) area under the pharmacokinetic curve of the plasma concentration of I. The absolute bioavailability was 0.73.

EXPERIMENTAL (BIOLOGICAL)

Mice (20 g) received I (2.5 mg/kg) in isotonic saline s.c and i.v. total radioactive material was determined at 0.17-24 h in plasma, organs and tissues, using analytical methods described elsewhere [1]. Results were processed using the algorithm described in [4].

RESULTS AND DISCUSSION

Determination of the total content of radioactive I and its metabolites in mice after s.c. dosage revealed higher liver and kidney levels at 10 min-2 h (Table 1). This was followed at 4-24 h by a significant reduction in the amounts of radioactive substance in these organs. Almost identical results were obtained with cardiac muscle, lung tissue, and spleen, where the quantity of substance at different time points were significantly lower than in the liver and kidneys.

The maximum content in plasma and in most organs and tissue occurred 30 min after dosage. However, the content remained the same at other time points. The results in Table 1 show that the brain, fatty tissue, and skeletal muscle are sites with the lowest levels of compound I.

A rather different result was obtained for the tissue and organic distribution of I after i.v. dosage (Table 2). Peak concentrations occurred in most organs and tissues (excluding the liver) in the first 10 min. There was an especially sharp decrease in concentration in the kidneys, where the quantity decreased more than fivefold at 30 min. Another characteristic feature of the distribution of I after i.v. dosage was the insignificant accumulation of I in fatty tissue and muscle at 0.5-24 h, as compared with concentrations after s.c. dosage.

Figure 1 shows changes in the concentration (on a logarithmic scale) of ^{14}C-products in mouse plasma after i.v. and s.c. dosage with I. The absolute bioavailability of I was determined from the ratio of the area under the curve of its plasma concentration over the period S_0 — 24 h. The area under the curve after 24 h was not used in these experiments because the concentration at these times was very low.

As shown in Fig. 1, dosage by both routes gave a slow phase of elimination of radioactive material from the plasma over the time period 4-24 h. The pharmacokinetic parameters of this phase are given in Table 3, which show that the absolute biological availability of the peptide after s.c. dosage was quite high in mice, with a value of 0.73.

In order to study the permeability of the blood–brain barrier for the peptide, we investigated the uptake of I into its target site (brain) from the external milieu (Fig. 2). The brain:blood concentration ratio was very low after both i.v. and s.c. dosage. The ratio was very stable, and in both cases changed very little in the slow elimination phase (4-24 h), but concentrations were significantly different in the 10 min-2 h period.

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