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Note

Determination of the melanotropin-inhibiting factor analogue pareptide in urine by high-performance liquid chromatography

KOON-SEA HUI*, MARIA HUI, KWOK-PING CHENG and ABEL LAJTHA

Center for Neurochemistry, Rockland Research Institute, Ward's Island, NY 10035 (U.S.A.)

and

ISTVAN BOKSAY and MONICA FENCIK

Clinical Research Department, Ayerst Laboratories, 685 Third Avenue, New York, NY 10017 (U.S.A.)

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Melanotropin-inhibiting factor (MIF), Pro-Leu-Gly-NH_2, has recently been shown to greatly potentiate the therapeutic efficacy of L-DOPA in the treatment of parkinsonism [1, 2]. However, MIF is hydrolyzed rapidly in tissues [3, 4]. Pareptide*, an analogue of MIF, was synthesized in the Ayerst Research Labs. This compound mimics the physiological function of MIF, and it has been shown to be more potent and long-lasting because of its resistance to degradation, suggesting that it too may have potential use in the treatment of parkinsonism, and of drug-induced extrapyramidal reactions as well.

MIF derivatized with 5-dibutylaminonaphthalene-2-sulphonyl chloride (Bns-Cl) or dansyl chloride (Dns-Cl) was successfully separated from its metabolites by high-performance liquid chromatography (HPLC) [5]. MIF can be quantitated with the aid of pareptide as an internal standard [3]. Pareptide that was added to human blood plasma was studied by HPLC using 7-chloro-4-nitrobenzyl-2-oxa-1,3-oxadiazole (NBD-Cl) as tagging reagent. However, it gave a high blank and it needed a calibration curve for every experiment [6].

We report here a method that was successfully used for quantitative and qualitative determination of pareptide in urine after oral administration of the drug. The sample is first purified by cation-exchange chromatography, then derivatized with Dns-Cl and studied by HPLC and thin-layer chromatography (TLC). MIF can serve as an internal standard, increasing the speed and

*Pareptide is the commercial name for L-prolyl-N-methyl-D-leucyl-glycinamide.

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the specificity of the method. The method in principle is suitable for following the metabolic rate of administered peptides.

EXPERIMENTAL

Chemicals and reagents

Pareptide was obtained from Ayerst Research Labs. (Montreal, Canada). Dns—Cl was obtained from Pierce (Rockford, IL, U.S.A.). MIF was purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade solvents obtained commercially were used in TLC without further purification. In HPLC, the HPLC-grade acetonitrile was obtained from Waters Assoc. (Milford, MA, U.S.A.). The solvent systems were prepared immediately before use; for HPLC they were filtered (0.45 μm) and degassed before use. TLC polyamide precoated microplates (5 × 5 cm, 25 μm thick) were obtained from Schleicher & Schuell (Keene, NH, U.S.A.). Analytical-grade cation-exchange resin AG 50W-X4, 200—400 mesh, H^+ form, was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Radial Pak C_{18} column (200 × 8 mm) was obtained from Waters Assoc. The size of the spherical packing particle was 10 μm. Other chemicals were of reagent grade and were obtained from available commercial sources.

Chromatographic equipment

The HPLC system consisted of a Waters Model 6000 pump, a Waters U6K septumless injector with a 2-ml injection loop, and a Waters radial compression module RCM 100; chromatographic elution was monitored by a Schoeffel FS 970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.). Detector output was recorded and processed by a Waters data module.

Measurement of pareptide in urine samples

Three normal subjects (DF-1, DF-2, and DF-3) were given 1.5 g of pareptide, the oral dose recently used for the treatment of parkinsonism, in capsules at 6 a.m. Urine samples were collected 0, 2, 4, 6, 8, 12, and 24 h after the administration of pareptide. The samples were kept at −20°C before the assay.

A 50-μl volume of 6 *N* hydrochloric acid was added to a 0.5-ml urine sample. The sample was then loaded on a small AG 50W-X4 cation-exchange column (5.5 × 0.5 cm), the resin of which was prewashed and swollen with 0.1 *N* hydrochloric acid. After loading of the sample, the column was washed with 1 ml of 0.1 *N* hydrochloric acid followed by 5 ml of water; it was then eluted with 1 *N* ammonia. When the eluate became alkaline, 3 ml of it was collected.

MIF (1.08 μmol) was mixed with 100 μl of the eluate. The mixture was dried by a stream of nitrogen at 60°C. The residue was mixed with 40 μl of water and 10 μl of 0.5 *M* sodium bicarbonate. A 20-μl volume of Dns-Cl in acetone (1 mg/ml) was added to the sample, and the dansylation was carried out at 60°C for 30 min. After the dansylation, 200 μl of acetonitrile was added to the sample, which was mixed well and centrifuged at 8000 *g*; portions of the supernatant were used for TLC and HPLC at ambient temperature.

A 1-μl volume of the dansylated mixture was applied to the TLC plate with the aid of a Hamilton microsyringe. The plate was first developed with solvent system 1: formic acid—water (3:97). When the solvent front reached the top of

the plate, it was removed and dried by a stream of warm air. The plate was then turned 90° and developed with solvent system 2: benzene—acetic acid (9:1). After the development in the second direction, the plate was air dried and examined under UV light (365 nm).

Solvent system acetonitrile—0.01 *M* sodium sulfate buffer pH 7.0 (45:55) was pumped isocratically at a flow-rate of 3 ml/min, resulting in a pressure of 215—430 kg/cm^2. Volumes of 10—20 μl of the samples were used for the study. The elution was detected by fluorescence with the excitation set at 350 nm and the emission at 470 nm, which are the maximal wavelengths for excitation and emission of Dns-MIF and its related peptides [5]. The other settings of the fluoromonitor were: sensitivity, 4.0; full-scale expansion fluorometric response range, 0.02 μA; and time constant, 6.0 sec.

Degradation of MIF and pareptide by tissue

A 45-nmol amount of MIF or pareptide was incubated with tissue homogenate in 150 μl of 0.05 *M* Tris buffer, pH 7.5, at 37°C for 1 h. The reaction was stopped with 7.5 μl of 20% trichloroacetic acid. The sample was centrifuged, and the supernatant was dansylated and studied by HPLC as previously described [3].

RESULTS AND DISCUSSION

Specificity of the method

MIF and its possible metabolites were derivatized with Dns, ethansyl, propansyl, Bns, and monoisopropansyl chloride [5]. NBD has been used as a derivatizing reagent for the determination of pareptide in plasma; however, fluorescence with Dns-pareptide is about five times greater than with the NBD-pareptide derivative [7]. Dns-MIF and its Dns metabolites could be detected at the lowest levels. Dansylation was not used before because the relative instability and high background of plasma derivatized with Dns offsets the higher sensitivity of the determination with this reagent [6]. Since pareptide is more hydrophobic than the natural MIF, its retention time in the reversed-phase column was longer. The retention time for MIF was 6.7 min, and for pareptide it was 9 min (Fig. 1B).

Passing the urine sample through the ion-exchange column purified and stabilized it. Pareptide could be fully (100%) recovered from the column. MIF was eluted from the ion-exchange column earlier than pareptide, and thus any MIF (a natural peptide in the body) was separated from the pareptide. Since pareptide could be fully recovered, MIF added after the ion-exchange column could serve as an internal standard.

Dansylated MIF and pareptide were resolved from each other by reversed phase HPLC using solvent systems containing acetonitrile in phosphate buffer. With excitation at 360 nm and emission at 487 nm the fluorescence of the two compounds was equal. However, when we used the optimal excitation and emission wavelengths (350 nm and 470 nm, respectively) [5] the sensitivity of the detection was increased tenfold (minimal detectable amount was 20 pmol) and pareptide fluorescence was two times that of MIF. The amount of pareptide or MIF derivatized with Dns-Cl had a linear relationship to the

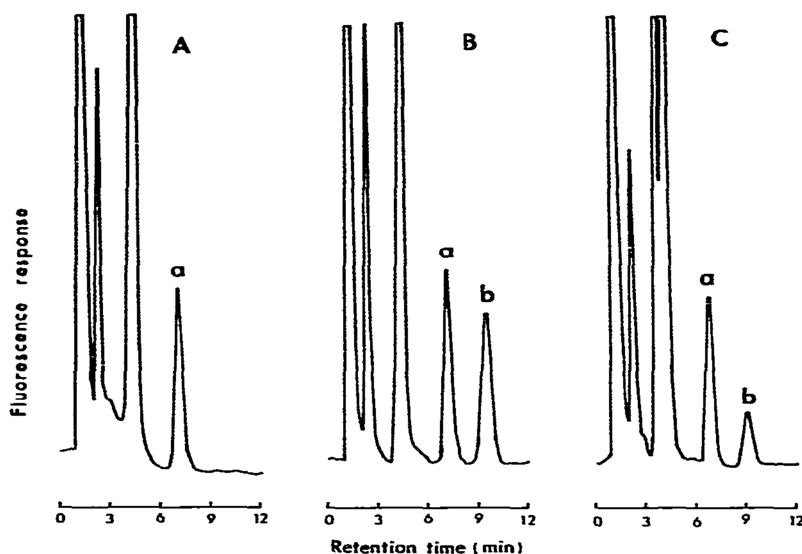

Fig. 1. HPLC chromatograms of urine samples after oral administration of pareptide. A 0.5-ml volume of urine was acidified and purified by cation-exchange chromatography. The isolated pareptide fraction was mixed with internal standard (1.08 μmol MIF), dansylated, and analyzed by HPLC. For details, see Experimental section. (A) 0 time; (B) authentic pareptide (0.54 μmol); (C) 2 h after oral administration of pareptide. Peaks: a = MIF, b = pareptide.

peak heights (or areas) in HPLC [3]. The peak heights or areas of the equivalent amounts of MIF and pareptide were in a straight line, with a ratio of 0.5 within the range of 100 pmol—20 nmol. The experimental error of the method was within 5%. The use of fluorescence had another advantage: there was some absorption at the pareptide region of the HPLC when UV_{254} was used for monitoring. When both MIF and pareptide were derivatized with Dns-Cl, their concentration and the height of their corresponding separated peaks were in linear relationship. MIF was a good internal standard for pareptide, and vice versa, in HPLC.

Two-dimensional TLC on polyamide layers has proved to be a fast and high-resolution qualitative method not only for Dns-amino acids [8] but also for dansylated small peptides. MIF has been separated from its metabolites by such a method [5]. Since pareptide is an analogue and more hydrophobic than MIF, its R_F is greater than that of MIF in each of the two solvent systems we used. With solvent system 2, the R_F values are 0.71 and 0.84, respectively. Dns-MIF and Dns-pareptide are resolved from each other clearly. Even though the compounds are separated at one run, two-dimensional separation isolated them from other dansylated compounds from the urine that interfere in the observation.

At zero time, we did not find any peak in the region of 9 min (pareptide peak) in the chromatogram (Fig. 1A). This proves that after the ion-exchange chromatography and dansylation compounds in the urine did not interfere in our assay by HPLC, which was monitored with fluorescence with excitation at 350 nm and emission at 470 nm. In TLC, there was no fluorescence zone around the Dns-pareptide region.

When authentic pareptide was added to the urine (0 time), we found a symmetrical peak (retention time, 9 min) in the HPLC chromatogram and a confined zone on the TLC plate with an R_F of 0.61 for solvent system 1, and 0.84 for solvent system 2. Pareptide was found by HPLC in the urine collected 2—4 h after oral administration of 1.5 g of pareptide, and the findings were confirmed by TLC.

Peptide absorption and excretion

Pareptide appeared in the urine 2 h after oral administration (Table I) and was present in the urine 12—24 h after its administration. The highest concentration of pareptide in the urine was found 2—4 h after drug administration. In 24 h the total amount of pareptide excreted by urine was 13 mg, which is 0.9% of the amount given orally. In a separate study, using NBD-Cl as a derivatizing agent, the highest peptide concentration (360 ng/ml) was found in blood 2 h after 1.5 g of pareptide was administered orally; this is compatible with our other findings.

The poor absorption of pareptide from the gastrointestinal tract after oral administration was not due to its rapid degradation. When we compared the degradation of MIF with that of pareptide in vitro, we found that virtually no pareptide was split by rat brain, liver, intestinal mucosa, spleen, lung, or plasma (Table II). Pareptide was resistant to hydrolysis even after 19 h of incubation. Under conditions where all of the added MIF was metabolized, pareptide remained intact [4].

Peptides are transported less efficiently than amino acids into the mucosal tissue by carriers different from those that transport amino acids [9]. A number of proline peptides less sensitive to peptidases can pass intact through sacs of everted rat intestine [10]. The absorption of several, perhaps most, of the unmetabolized peptides is very limited. Since the carriers for peptides are specific, and pareptide is an N-methylated D-leucine compound, it is likely that most of the administered drug was not taken up. Pepstatin, a potent cathepsin D inhibitor and a peptide analogue, is not metabolized and is vir-

TABLE I

PAREPTIDE IN URINE AFTER ORAL ADMINISTRATION

Urine samples were collected at the indicated time intervals after oral administration of 1.5 g of pareptide. A, B, and C represent three normal subjects.

Time (h)	Urine collected (ml)			Total amount excreted (mg)		
	A	B	C	A	B	C
0	174	165	146	0	0	0
0— 2	58	69	40	1.3	0.7	0.9
2— 4	150	67	74	5.7	2.8	2.7
4— 6	102	87	93	3.5	1.4	1.8
6— 8	151	83	204	2.9	2.7	1.8
8—12	401	176	374	2.4	3.9	1.7
12—24	266	589	401	0	1.6	1.0
Total	1302	1231	1332	15.8	13.1	9.9

TABLE II

THE DEGRADATION OF MIF AND PAREPTIDE IN RAT TISSUES

An amount of 4.5 nmol of MIF or pareptide was incubated with 0.63 mg tissue in 150 ml of 0.05 *M* Tris buffer, at 37°C for 1 h. The reaction was terminated with 7.5 µl of 20% trichloroacetic acid. The sample was centrifuged, and the supernatant was dansylated and assayed by HPLC. Results for MIF are given; under these experimental conditions there was no detectable metabolism of pareptide by any of the tissue tested. The specific activity for plasma is expressed as nmol MIF split per ml plasma per hour.

Tissue	MIF metabolized (nmol) per mg tissue per hour
Brain	19
Liver	21
Intestinal mucosa	19.5
Spleen	2.8
Lung	1.2
Plasma	53.1

tually unabsorbed on oral administration, but it is excreted in feces [11]. In its absorption in the gastrointestinal tract, pareptide is similar to pepstatin. The fate of pareptide in the body could be studied more quantitatively with the aid of radioactive labelling, which is not yet available. Even though pareptide is poorly absorbed after oral administration, it is still effective as a drug for the treatment of parkinsonism in various laboratory models (Ayerst Research Labs., unpublished observations). Neuropeptides are potent drugs, only a few molecules of which are needed at the target cell to trigger a series of reactions. Furthermore, the affinity between the drug and the receptor plays a critical role in the drug effect. The affinity of MIF and pareptide to a receptor remains to be shown.

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