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Dynorphin A (1–8) Analog, E-2078, Is Stable in Human and Rhesus Monkey Blood[1]

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ABSTRACT

E-2078 is a dynorphin A (1–8) analog, [N-methyl-Tyr[1], N-methyl-Arg[7]-D-Leu[8]] Dyn A (1–8) ethylamide. Its biochemical stability against enzymatic degradation *in vitro* in human and rhesus monkey blood, and *in vivo* in rhesus monkey blood was studied using matrix-assisted laser desorption/ionization mass spectrometry. *In vitro* studies were carried out in freshly drawn human and rhesus monkey blood, incubated at 37°C for various time periods. *In vivo* studies were conducted by E-2078 i.v. injection to rhesus monkeys, and blood samples were collected at various time points after the injection. It was found that E-2078 was stable against enzymatic degradation *in vitro* in freshly drawn human and rhesus monkey blood. Minor biotransformation products from E-2078, such as E (1–4), E (1–5) and E (3–6), were detected *in vitro* in some human and rhesus monkey blood, but they made up less than 5% of the total starting E-2078 peptide. No biotransformation products were detected in the blood samples from *in vivo* studies. The apparent half-life of elimination of E-2078 *in vivo* from the rhesus monkey blood was determined to be 44.0 min.

Dyn A (1–17) is an endogenous neuropeptide. Studies have indicated that this peptide and its congeners may attenuate the behavioral symptoms of the opioid withdrawal syndrome in morphine-dependent rodents and may be useful as a therapeutic agent for the treatment of opioid dependency in man (Takemori *et al.*, 1992, 1993). It has also been shown that Dyn A peptides have analgesic properties and may have potential application in the management of pain (Hooke and Lee, 1995; Smith and Lee, 1988). However, natural Dyn A peptides are susceptible to enzymatic degradation *in vitro* and *in vivo* in a biological system (Goldstein *et al.*, 1979; Young *et al.*, 1987). Dyn A peptides undergo proteolytic cleavages in a biological matrix to form a variety of biotransformation products (Butelman *et al.*, 1996; Chou *et al.*, 1993, 1994a, 1994b; Yu *et al.*, 1995, 1996a, 1996b).

To stabilize Dyn A peptides against enzymatic degradation, a group of researchers synthesized a variety of Dyn A analogs (Tachibana *et al.*, 1988; Yoshino *et al.*, 1990). These analogs were evaluated and studied for their stability, receptor-binding properties, and biological activities. They finally arrived at [N-methyl-Tyr[1], N-methyl-Arg[7]-D-Leu[8]] Dyn A (1–8) ethylamide, designated as E-2078.

Studies showed that E-2078 was stable against enzymatic degradation (Nakazawa *et al.*, 1990). E-2078 bound to κ-opioid receptor just like Dyn A (1–17) (Yoshino *et al.*, 1990), and exhibited a 2-fold more potent analgesic effect than morphine. This enhanced analgesic action was attributed to its stability against enzymatic degradation (Nakazawa *et al.*, 1990; Yoshino *et al.*, 1990). E-2078 exhibited analgesic properties when used in patients with severe pain after lower abdominal surgery in clinical studies (Fujimoto and Momose, 1995; Tachibana, 1996).

We report studies of E-2078 biotransformation *in vitro* in freshly drawn human and rhesus monkey blood and also *in vivo* in rhesus monkey blood. These studies were conducted using our recently developed techniques of matrix-assisted laser desorption/ionization mass spectrometry, which permits sensitive and specific simultaneous detection of both parent peptide compounds and multiple biotransformation products in a biological matrix.

Materials and Methods

Subjects. Three healthy human male subjects, average age 28.7 yr (range 24–31), served as donors for the blood used in the *in vitro* studies. Five adult rhesus monkeys, *Macaca mulatta*, two females

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ABBREVIATIONS: Dyn A, dynorphin A; 4HCCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; Arg, arginine; Tyr, tyrosine; Leu, leucine; Phe, phenylalanine; Gly, glycine; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry.

and three males, average age 12 yr (range 8–18), mean weight, 8.7 kg (range 6.5–12 kg) were used for the *in vitro* and *in vivo* studies. They were housed singly with free access to water, and were fed approximately 30 Purina (St. Louis, MO) monkey chow biscuits daily and fresh fruit twice per week. The monkeys were housed on a 12:12 light:dark cycle (light on at 7:00). Experiments were carried out between 10:00 and 15:00.

Chemicals. [N-methyl-Tyr^1, N-methyl-Arg^7-D-Leu^8] Dyn A (1–8) ethylamide (E-2078) was synthesized and kindly supplied by Eisai Co. Ltd. (Ibaraki, Japan). Dyn A (1–8) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Angiotensin III and diphenhydramine HCl were purchased from Sigma Chemical Co. (St. Louis, MO). Ketamine HCl was obtained from Fort Dodge (Fort Dodge, IA). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ). Saline (0.9% NaCl) and dextrose were from Abbott Laboratories (North Chicago, IL). 4HCCA was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). High-performance liquid chromatography grade acetonitrile was purchased from Burdick & Jackson (Muskegon, MI) and TFA from Fisher Scientific (Fair Lawn, NJ).

Dyn A (1–8) and its analog, E-2078, *in vitro* biotransformation in human and rhesus monkey blood. Blood was drawn from subjects into vacutainer tubes preconditioned with EDTA (Becton Dickinson, Rutherford, NJ). In the E-2078 processing experiments, 14.0 ml of freshly drawn blood was placed in a Falcon polypropylene tube, 50 ml (Becton Dickinson, Lincoln Park, NJ). After removal of 1.0 ml blood as control, E-2078 in amount of 7.67 mg, which was dissolved in 500 μl of saline, was immediately added to the blood (*i.e.*, 0.59 mg/ml). This E-2078-containing blood was incubated in a water bath at 37°C (Bench Scale Equipment Co., Inc., Dayton, OH). Blood samples were taken from the incubating tube at the following time-points after the addition of E-2078: 0, 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480 min and 24 hr. One ml of blood was used for each time point. In the similar Dyn A (1–8) processing experiments, 8 and 11 ml of rhesus monkey and human blood, respectively, were used. Dyn A (1–8), 4.13 mg for monkey blood and 5.90 mg for human blood, was used (0.59 mg/ml). Blood samples, 1 ml, were taken at the following time-points: rhesus monkey blood, 0, 1, 5, 10, 15, 30 min; human blood, 0, 1, 5, 10, 15, 30, 45, 60 and 90 min.

Immediately after removal of the blood from the incubating tube, plasma was separated from the blood by centrifugation in a Sorvall RC-5B refrigerated (0–5°C) superspeed centrifuge (Sorvall Instruments, Du Pont Company, Newtown, CT), at 3400 $\times$ *g* for 5 min. A total of 200 μl of plasma containing E-2078 and proteolytic products was added to 1.8 ml of 1.0% TFA aqueous solution. The samples were frozen at -70°C until analysis.

Before analysis, the samples were thawed at room temperature. To 2.0 ml of sample plasma solution was added 100 μl of 630 μM (0.029 mg/ml) Dyn A (1–8) in 0.05% aqueous TFA as the internal standard for the E-2078 processing studies. For the Dyn A (1–8) processing studies, 100 μl of 500 μM (0.022 mg/ml) angiotensin III in 0.05% aqueous TFA as the internal standard was added to 2.0 ml of sample plasma solution. The solution containing plasma, the peptide, the internal standard and TFA, was centrifuge-filtered with Centricon-SR3 concentrators (Amicon Inc., Beverly, MA), with molecular weight cut-off of 3000 Da. A total of 20 μl of the filtrate was mixed with 10 μl of acetonitrile for analysis by mass spectrometry.

E-2078 *in vivo* studies in rhesus monkeys. Monkeys received injections with diphenhydramine (1.2 mg/kg, intramuscularly) as a pretreatment to limit the possible consequences of histamine release after administration of relatively large amounts of E-2078. Thirty min later, they were anesthetized with ketamine (10 mg/kg, i.m.). The back of the lower leg area was carefully shaved to avoid any adventitious bleeding. An indwelling catheter (Angiocath, 22 gauge, 1 inch long, Becton Dickinson, Sandy, UT) was acutely placed in a superficial vein of each leg, secured and flushed with heparinized saline (20 U/ml), and connected to a multisample injection port. One milliliter of blood sample was obtained as control and placed in a 2-ml vacutainer preconditioned with EDTA on ice. All blood sampling was followed by port and catheter flushing with heparinized saline. The animal was then placed on a heating pad (37°C) on a surgery table.

During the sampling period, the animal received a dextrose/saline i.v. infusion (approximately 30 ml/kg/hr) from the catheter that had previously been used for the E-2078 injection. Supplemental ketamine (approximately 5 mg/kg) was administered at hourly intervals to maintain the animal in an anesthetized state throughout the sampling period.

The required amount of E-2078 was dissolved in saline (10 mg/kg in 20 mg/ml solution), and injected in one of the leg catheters (injection time was approximately 15 sec). At the end of injection, a timer was started, and the injection catheter was flushed with heparinized saline. Blood sampling was performed at the following time points from the contralateral catheter: 0, 5, 15, 30, 60, 90, 120 and 180 min. Samples were centrifuged (3400 $\times$ *g* at 0–5°C for 5 min). A 0.2-ml aliquot of plasma was placed in a cryovial containing 1.8 ml of 1% TFA and kept at -40°C until the time of analysis.

In vivo blood samples were treated following the same procedures described earlier for *in vitro* blood samples with the exception that 40 μl of 80 μM (1.54 μg/ml) of Dyn A (1–8) was used as the internal standard for 2.0 ml of plasma sample solution.

Mass spectrometry. The samples were analyzed with a custom matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-MS) constructed at the Rockefeller University (Beavis and Chait, 1989, 1990). The instrument incorporates an HY-400 Nd-YAG laser (Lumonics Inc., Kanata, Ontario, Canada) operated at a wavelength of 355 nm, a pulse duration of ca. 10 ns, and a pulse energy density of ca. 15 mJ/cm^2; a 30 KV ion acceleration potential; and a 2 m long linear time-of-flight mass analyzer. Ion detection and signal amplification are accomplished with a hybrid microchannel plate detector-discrete dynode electron multiplier assembly (detector potential being 3.6 KV).

For mass spectrometric measurement, the samples with acetonitrile [2:1 (v/v)] were mixed with matrix solution. A saturated matrix solution was prepared by dissolving excess amount (5 mg/ml) of α-cyano-4-hydroxycinnamic acid in acetonitrile and 0.1% aqueous TFA [1:2 (v/v)]. Plasma containing E-2078 biotransformation products was mixed with matrix solution in a ratio of 1:2 (v/v). A 0.5-μl aliquot of the resulting solution was applied to the mass spectrometer sample probe tip, and allowed to evaporate to dryness in the air. The sample probe was inserted into the mass spectrometer vacuum system where solvents in the sample were completely removed. After 5 min, a working pressure of approximately 10^{-7} torr was achieved. To obtain adequate statistics, *i.e.*, to achieve a signal-to-noise ratio of the peak of interest of more than 10:1, the results from 100 laser shots were added to produce each mass spectrum. In this study, one spectrum was obtained for each sample from each subject.

A standard curve was constructed using E-2078 standard solutions. The standard solutions were made by serial dilution of E-2078 in 0.05% TFA aqueous solution. A constant amount of internal standard, Dyn A (1–8) (1 μl of 136 μg/ml, or 0.136 μg), was added to 100 μl of standard solutions. The E-2078 standard solutions containing Dyn A (1–8) were each mixed with acetonitrile (2:1, v/v), and the resulting solutions were separately mixed 4HCCA matrix solution (1:2, v/v). Half-microliter aliquots of the resulting solutions were applied to the mass spectrometer sample probe tip for MALDI MS analysis. Mass spectrometric measurements were carried out in triplicate for each aliquot of the standard solutions. The ratio of the signal intensity from E-2078 to that from the internal standard, and the S.E. of the triplicate measurements were calculated.

In the Dyn A (1–8) *in vitro* biotransformation studies, the use of angiotensin III as the internal standard was to show the appearance and disappearance of Dyn A (1–8) and the processing products.

***In vivo* pharmacokinetic analysis.** The pharmacokinetics of the disappearance of E-2078 *in vivo* in rhesus monkey blood was analyzed with SAAM II (version 1.0.2, SAAM Institute, University of Washington, Seattle, WA), a program for kinetic analysis. The ki-

netic curves were plotted with both the experimental data and calculated data obtained with SAAM II using KaleidaGraph (Version 3.0.5, Abelbeck Software, Reading, PA) with nonlinear curve-fitting routine. The half-life of E-2078 was calculated by plotting the logarithm of the relative signal intensity *vs.* the time after injection.

Results

Dyn A (1–8), on *in vitro* incubation at 37°C in freshly drawn human and rhesus monkey blood, was rapidly processed to form two biotransformation products: Dyn A (2–8) and Dyn A (1–6) (fig. 1). Dyn A (1–8) and the biotransformation products were cleared from human blood at 90 min incubation, and cleared from the rhesus monkey blood at 30 min incubation. E-2078 was also incubated in freshly drawn human and rhesus monkey blood at 37°C for up to 24 hr. In stark contrast to the results observed with Dyn A (1–8), no major biotransformation products were detected. As shown in figure 2, at 24 hr incubation time point, intact E-2078 was still the major species present in the blood. Minor biotransformation products such as E (1–4), E (1–5) and E (3–6) were detected in blood of some human and rhesus monkey subjects at certain incubation time points (data not shown). Mass spectrometric analysis of the control blood for *in vitro* Dyn A (1–8) and E-2078 processing studies in human and rhesus monkey blood recorded a flat baseline, besides a few very small background peaks.

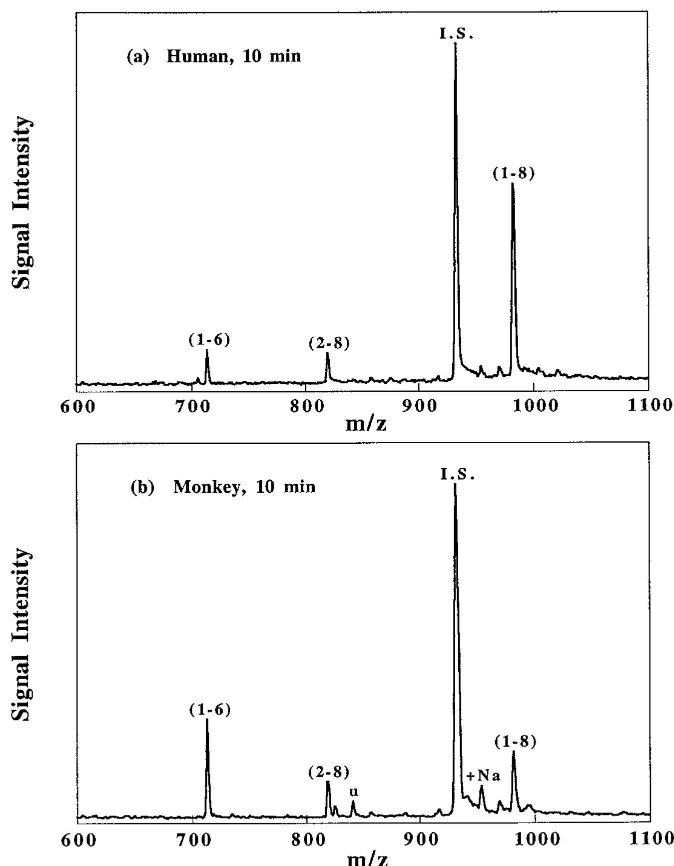

Fig. 1. Matrix-assisted laser desorption/ionization mass spectra of dynorphin A (1–8) (0.59 mg/ml) *in vitro* biotransformation, incubation at 37°C for 10 min, in freshly drawn blood of (a) human (b) rhesus monkey subjects. The numbers refer to dynorphin A fragments; I.S., the internal standard, angiotensin III (0.022 mg/ml); u, unidentified peaks; +Na, signal corresponding to sodium adduct species.

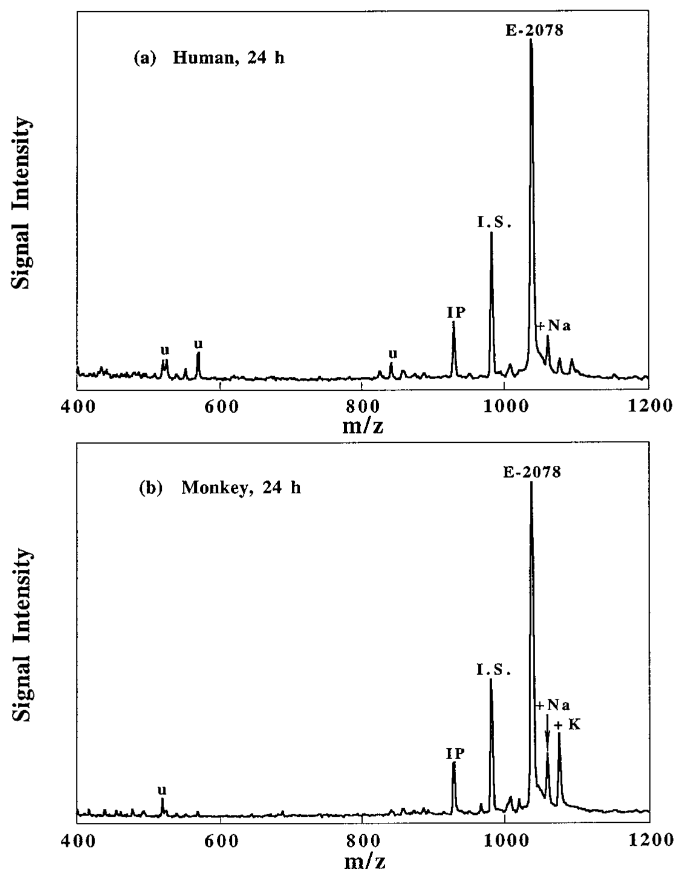

Fig. 2. Matrix-assisted laser desorption/ionization mass spectra of E-2078 (0.59 mg/ml) *in vitro* biotransformation, incubation at 37°C for 24 hr, in freshly drawn blood of (a) human (b) rhesus monkey subjects. IP refer to impurity detected in the starting peptide; I.S., the internal standard, dynorphin A (1–8) (0.029 mg/ml); u, unidentified peaks; +Na and +K, signals corresponding to sodium and potassium adduct species, respectively.

In vivo studies of E-2078 after i.v. injection to rhesus monkey indicated that E-2078 was stable against enzymatic cleavage in blood. No biotransformation products were detected in any of the samples collected at the various time points up to 180 min after the injection.

With the use of an internal standard, Dyn A (1–8), quantitative analysis of the peptide in the samples could be carried out by using the relative signal intensity of the signal from E-2078 against that from the internal standard. As shown in the calibration curve (fig. 3) within the concentration range between 22.22 pg and 555.55 pg of E-2078 applied to the sample probe tip, the relative signal intensity is linearly correlated to the amount of peptide applied.

The pharmacokinetics of disappearance of E-2078 *in vivo* in rhesus monkey blood was analyzed by using Dyn A (1–8) as the internal standard. The pharmacokinetic curves were plotted (fig. 4). The plots constructed based on experimental data and data obtained from kinetic calculation coincided with each other (fig. 4a). Assessment of the goodness-of-fit (Foster, 1995) indicated that the fit for the monoexponential model

$$y(t) = 6.41 * \exp(-0.311 * t) + 1.46 * \exp(-0.0103 * t)$$

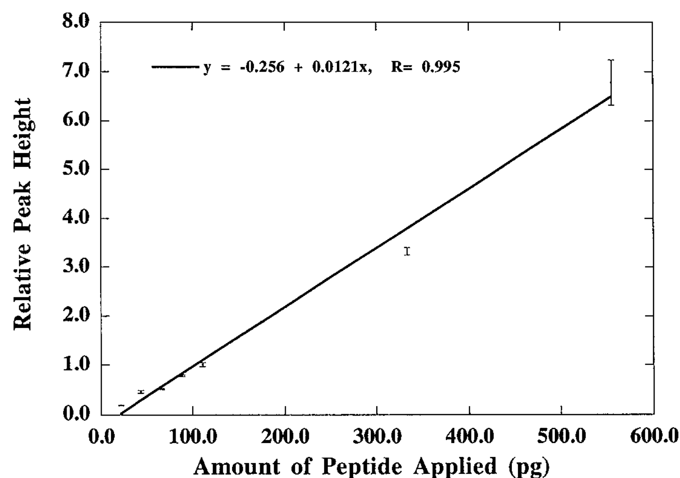

Fig. 3. Linear curve fit for the calibration of the mass spectrometric system with various amount of E-2078 in 0.05% TFA. Error bars represent the S.E.M. of triplicate measurements for each standard solution. Relative peak height is the ratio of peak height of E-2078 to that of the internal standard, dynorphin A (1–8).

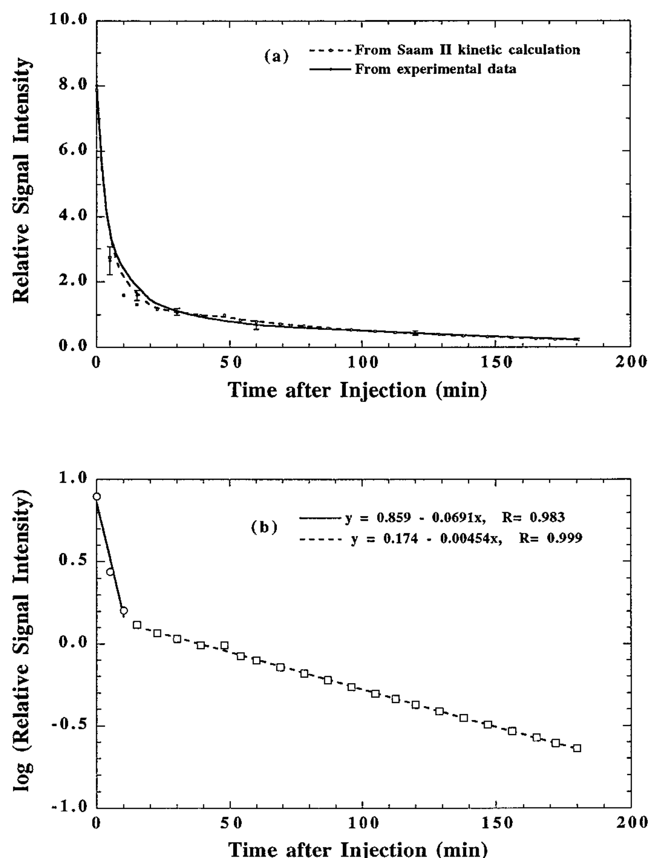

Fig. 4. a, Pharmacokinetics of disappearance of E-2078 *in vivo* from rhesus monkey blood. The measurements were an average of data obtained from five rhesus monkey subjects. Error bars represent the S.E.M. for each time point. b, Replot of the pharmacokinetics: logarithm of relative signal intensity from Saam II kinetic calculation *vs.* the time after E-2078 intravenous injection to rhesus monkeys.

was acceptable with a 5% significance level. By replotting logarithm of the relative signal intensity *vs.* the time after E-2078 injection, it can be seen in figure 4b that the pharmacokinetics exhibited a two-compartment model process, one compartment representing the blood, the other compartment representing the tissues (Wartak, 1983). The first linear section, from 0 to 10 min, represented a rapid distribution of the peptide out of the vascular system into various tissues after injection. The second linear section, from 15 to 180 min, represented the elimination of E-2078 from both the blood and the tissues, once the distribution had been completed and the plasma and tissue concentration were in equilibrium. The calculated apparent half-life was $t_{1/2}(\beta) = 44.0$ min; thus it would take approximately 4.4 hr (around six elimination half-lives) for E-2078 to be eliminated from the rhesus monkey (Wartak, 1983).

Discussion

Matrix-assisted laser desorption/ionization mass spectrometry is an analytical technique developed in recent years. It permits sensitive, simultaneous as well as specific detection of the presence of multiple components in a sample. This technique can tolerate complex sample conditions such as biological matrices, requiring minimal sample pretreatment. In our laboratories, this technique has been successfully applied to several studies of biotransformation of neuropeptides including Dyn A peptides in a variety of biological matrices, such as blood, brain tissues and cerebrospinal fluid (Butelman *et al.*, 1996; Chou *et al.*, 1993, 1994a, 1994b; Yu *et al.*, 1995, 1996a, 1996b, 1996c).

In this study, biotransformation of Dyn A (1–8) in human and rhesus monkey blood was found to exhibit the same patterns as that of the endogenous opioid peptide Dyn A (1–17) (Yu *et al.*, 1995, 1996a, 1996b). Two major biotransformation pathways could be identified. Cleavage of N-terminal, tyrosine, to form Dyn A (2–8), and the cleavage between Arg(6) and Arg(7) linkage to form Dyn A (1–6). No cleavage from the C-terminal was detected. Biotransformation of Dyn A (1–8) in rhesus monkey blood occurred at a faster rate than in human blood, as seen in figure 1. This result corroborates our previous findings in studies of Dyn A (1–17) (Yu *et al.*, 1995, 1996a, 1996b).

The modified Dyn A (1–8), E-2078, did not exhibit significant biotransformation either *in vitro* in freshly drawn human and rhesus monkey blood, or *in vivo* in rhesus monkey blood. Modification with a methyl group at Tyr^1, N-methyl-Tyr^1, effectively protected the cleavage at the N-terminal Tyr^1 position. No biotransformation products from this processing were detected. Peptide linkage of Arg(6)-Arg(7) is usually the other site of cleavage of Dyn A peptides in a biological matrix. Modification with a methyl group at the N-Arg^7 position effectively blocked this biotransformation.

Minor biotransformation products of E-2078, E(1–4), E(1–5) and E(3–6), were detected *in vitro* in human and rhesus monkey blood. Based on the signal abundance of all the peptides, these biotransformation products made up less than 5% of the total starting E-2078 peptide. These biotransformation products were not constantly detectable at all time points. No biotransformation products were detected *in vivo* in rhesus monkey blood, possibly because the formation of processing products were in very small amount that they were absorbed onto the tissues or blood cells or rapidly eliminated from the system.

Peptide biotransformation may be tissue specific. Reported stability studies on E-2078 in comparison with Dyn A (1–17)

have shown that no E-2078 degradation product was detected after incubation with mouse serum (10%) for 2 hr, whereas Dyn A (1–17) was degraded completely in that time period (Nakazawa *et al.*, 1990). However, E-2078 was biotransformed in mice brain homogenate (1%) with a half-life about 4 hr; Dyn A (1–17) biotransformation half-life was about 0.5 hr (Nakazawa *et al.*, 1990). The cleavage sites of E-2078 in mouse brain homogenates were identified to be Gly(3)-Phe(4), Phe(4)-Leu(5), and Leu(5)-Arg(6) by high-performance liquid chromatography (Tachibana, 1996). In this study, no biotransformation products from Gly(3)-Phe(4) cleavage were detected.

In summary, Dyn A (1–8) analog, E-2078, was very stable *in vitro* in freshly drawn human and rhesus monkey blood and *in vivo* in rhesus monkey blood. Minor processing products from E-2078, such as E(1–4), E(1–5) and E(3–6), were detected *in vitro* in some human and rhesus monkey blood. The apparent half-life of elimination of E-2078 *in vivo* from the rhesus monkey blood was about 44.0 min. Based on these studies, E-2078 is a suitable candidate for further neurobiological and pharmacological studies, also for possible development as a therapeutic agent for the management of pain, opioid addiction and cocaine addiction (Kreek, 1996; Spangler, 1993, 1996).

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