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An HPLC/RIA method for dynorphin A1-13 and its main metabolites in human blood

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Abstract

A selective HPLC/RIA procedure for the determination of dynorphin A1-13 (Dyn A1-13) and its major metabolites in human blood was developed. In order to block peptidase activity, blood samples were transferred into an aliquot of a blocking solution (5% aqueous $ZnSO_4$ solution–acetonitrile–methanol; 5:3:2, v/v/v). After solid phase extraction, reconstituted aliquots were injected into an isocratic reversed phase HPLC system to separate Dyn A1-13 from its main metabolites (Dyn A2-13, Dyn A1-12 and Dyn A2-12). The isolated and concentrated HPLC-fractions were assayed by RIA using a commercially available antiserum. Intra-day variabilities for quality controls (0.07, 0.25, and 1 ng ml^{-1}) of Dyn A1-13, A2-13, A1-12, A2-12 were between 9 and 41%. Accuracy was between 86 and 132%. Inter-day variability for single quality controls analyzed on five days for Dyn A1-13, A2-13, A1-12, A2-12 was between 4 and 49% for 0.07, 0.25 and 1 ng ml^{-1} samples, respectively. Accuracy was between 72 and 129%. Five different batches of control blood showed blood levels no different from zero. Considering the complexity of the assay, the method is selective, accurate and reproducible with a limit of detection of 0.07 ng ml^{-1} for Dyn A1-13, Dyn A2-13, Dyn A1-12 and 0.21 ng ml^{-1} for Dyn A2-12. The assay was applied to the determination of Dyn A1-13 and its metabolites in blood samples of 2 subjects receiving i.v. infusions of 250 μg or 1000 μg kg^{-1} Dyn A1-13 over 10 min. 1997 Published by Elsevier Science B.V.

Keywords: HPLC; Radioimmunoassay; HPLC/RIA; Dynorphin; Metabolism; Pharmacokinetics

1. Introduction

Dynorphin A1-13 (Dyn A1-13, YGGFLRRIRPKLK) is a potent endogenous opioid peptide [1]. It has been reported to affect pain [2] and to modulate tolerance and addiction. It has been evaluated in clinical studies to attenuate the

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effects of opiate withdrawal [3,4]. The main metabolites of Dyn A1-13 in blood are Dyn A1-12, Dyn A2-13 and Dyn A2-12 [5], all of which show pharmacological activity [6]. The pharmacokinetics of Dyn A1-13 have been reported recently in naive and morphine dependent patients using a RIA technique [7]. However, the technique is unable to distinguish between intact drug and related metabolites. This paper focuses on the design of a selective and sensitive analytical technique which is able to measure Dyn A1-13 and its major metabolites.

Several techniques have been described for the quantitative determination of (opioid) peptides in biological samples. High pressure liquid chromatography (HPLC) with electrochemical detection (ECD) [8,9], fluorescence detection (FD) [10–12]) or off-line radioimmunoassay (RIA) [13,14] has been applied to the analysis of in vitro or in vivo samples. Each of the above mentioned methods has inherent advantages and pitfalls.

A method is presented for the measurement of Dyn A1-13 and its main metabolites (Dyn A2-13, Dyn A1-12 and Dyn A2-12). It includes solid phase extraction (SPE) for sample purification and concentration, an isocratic reversed phase ion-pairing HPLC-system for separation followed by RIA for detection. This assay allows determination of Dyn A1-13 and metabolites in the sub-ng ml^{-1} range.

2. Materials and methods

2.1. Materials

Dynorphin A1-12 (Dyn A1-12) was provided by the sponsor and manufactured by American Peptide Company (Lot # SF 0298 AC). Dynorphin A1-13 (Dyn A1-13) and rabbit anti-Dyn A1-13 antiserum (RAS 8676N) were provided by Peninsula (Belmont, CA). Dyn A1-10 and Dyn A2-13 were obtained from Sigma (St. Louis, MO). Synthesis and amino acid analysis of Dyn A2-12, Dyn A3-12 and Dyn A4-12 were provided by the Protein Chemistry Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida. Peptide purity was usually greater than 70%. All other chemicals were of analytical grade, with exception of pentane sulfonic acid sodium salt (PSA), acetonitrile (ACN) and methanol, which were obtained from Fisher (Pittsburgh, PA) as HPLC grade. Human blood was received from the Blood Bank of Shands Hospital (Gainesville, FL), and stored at 4°C for up to one week. Blood samples from two subjects receiving a 10 min infusion of 250 or 1000 μg kg^{-1} of Dyn A1-13 were obtained from the laboratory of S. Shafer. Further information on this clinical study has been provided elsewhere [7].

2.2. Calibration standards and samples

Dyn A1-13, Dyn A2-13, Dyn A1-12 and Dyn A2-12 calibration standards were prepared in deactivated human blood. Deactivated blood was prepared by mixing blood and an equal aliquot of blocking solution (aqueous $ZnSO_4$ solution (5%, v/v) acetonitrile (ACN), and methanol (5:3:2, v/v/v)) prior to use. Dyn A 1-13 and its major metabolites were stable in deactivated blood over 24 h at room temperature. Preliminary experiments revealed a lower overall recovery for Dyn A1-12. Therefore, standards contained 0, 0.04, 0.1, 0.2, 0.4, 1, 2, 4 and 20 ng ml^{-1} of Dyn A1-13, Dyn A2-13, Dyn A2-12 and 0, 0.12, 0.3, 0.6, 1.2, 3, 6, 12 and 60 ng ml^{-1} blood of Dyn A1-12. Quality control samples (0.07, 0.25 and 1 ng ml^{-1} for Dyn A1-13, Dyn A2-13, Dyn A2-12; 0.21, 0.75 and 3 ng ml^{-1} for Dyn A1-12) were prepared in deactivated human blood and stored at −80°C. On the day of the experiment, standards were thawed and centrifuged at 300 × *g* for 10 min. Supernatant (2 ml) was transferred into a separate vial and diluted with 3 ml of 3% aqueous acetic acid.

2.3. Solid phase extraction

The solid phase extraction was performed with slight modifications as previously described [5] to obtain a more favorable elution profile (reduction of the elution volume from 2.5 to 1 ml). Prior to the sample application, cartridges (Supelclean LC-18 SPE) were activated with 2 ml ACN, followed by 2 ml of 60% ACN in aqueous trifluoroacetic

acid (0.03%, v/v, TFA) and 2 ml of 3% aqueous acetic acid. Standards and samples (diluted with 3% aqueous acetic acid as described above) were applied, the cartridge was then washed with 10% ACN in aqueous TFA and eluted with 1 ml of 60% ACN in aqueous TFA. Using 10% ACN in the washing step reduced the load of blood components on the HPLC system without affecting the recovery when compared to using only TFA solution. The eluent was collected in microcentrifuge tubes, dried in a vacuum centrifuge (RC 10-10, Jouan Inc., Winchester, VA) and reconstituted in 220 μl of 30% ACN in aqueous TFA. The microcentrifuge vials were then centrifuged (15 000 × *g*, 10 min) and 200 μl of clear supernatant were transferred into disposable autoinjector vials and subsequently separated by HPLC.

2.4. Isocratic HPLC

The HPLC system consisted of a pump (CM 4000, LDC, Boca Raton, FL), an auto-injector (ISS-200, Perkin Elmer, Norwalk, CT), an adequate precolumn filled with reversed phase C18 material, a reversed phase column (μ-Bondapack 3.9 × 150 mm C18, Waters, Milford, MA), a variable UV-detector set to 210 nm (SM 4000, LDC, Boca Raton, FL), and a programmable fraction collector (model 203, Gilson, Middleton, WI). This system was preferred over other reversed phase systems as previous detailed studies on the metabolism of DYN A1-13 showed very good separation of related dynorphin metabolites [5]. The mobile phase consisted of a mixture (25:75, v/v) of ACN and aqueous TFA (0.03%,v/v containing 7.5 mM pentane sulfonic acid (PSA)). Precolumn material was replaced before every run. Retention times were verified before every experiment using UV-detection and high concentrations (2 μg ml^{-1}) of Dyn A1-13, Dyn A2-13, Dyn A1-12 and Dyn A2-12.

An extensive washing cycle with ACN and aqueous TFA (0.03%, v/v, containing 7.5 mM PSA (70:30, v/v)) was performed over 60 min. During this washing cycle, DMSO (200 μl) was injected every 15 min. The system was then switched to the analytical mobile phase for an additional 15 min washing and equilibration. Processed blood samples (200 μl) were then injected. During a run, every fifth injection consisted of ACN to remove lipophilic built-up that could affect the retention time of dynorphin fragments. Under these conditions, retention times were constant over 10 h. The HPLC-fractions containing the relevant dynorphin fragment were collected. These fractions were 4–6 min (Dyn A2-13), 6.8–8.8 min (Dyn A2-12), 9.3–12.3 min (Dyn A1-13) and 16.8–20.8 min (Dyn A1-12) post injection. HPLC fractions containing separated dynorphin fragments were dried in a vacuum centrifuge (RC 10-10, Jouan, Winchester, VA), reconstituted in 200 μl of blocking solution, and 50 μl aliquots were then assayed in duplicate by RIA.

2.5. Radioimmunoassay

2.5.1. Buffers and solutions

Incubation buffer consisted of a mixture of 0.5 g bovine serum albumin, 1.25 ml solution of 10% Triton X 100 and 250 ml of 0.1 M phosphate buffer (pH 7.4 containing 0.1% sodium azide).

To prepare rabbit anti-Dyn A1-13 dilutions, 50 μl distilled water was added to freeze dried serum. Aliquots of a dilution of 1:100 representing antiserum stock solution 2 was stored at −60°C in microcentrifuge tubes. The portion of the stock solution 2 in use was stored at −20°C. The working solution (1:650 000) was prepared when needed by diluting stock solution 2 (75 μl) in 9 ml of assay buffer. The working solution was prepared daily, mixed and stored on ice.

[^{125}I]Dyn A1-13 (10 μCi, Peninsula, CA) was dissolved in 1 ml incubation buffer and stored in 0.2 ml aliquots at −20°C. On the day of assay, these aliquots were diluted with incubation buffer to obtain 4–6000 CPM/100 μl.

20 ml Phosphate buffered saline pH 7.4 was mixed with 1 g polyethylene glycol 6000, 42 μl normal rabbit serum (NRS; Pel-Freeze) and 80 μl goat antiserum against rabbit IgG (GARGG; Pel-Freeze) in order to prepare the anti-rabbit buffer solution.

2.5.2. RIA incubation

All RIA incubations were performed in duplicate at 4°C. 50 μl Reconstituted sample, 450 μl

incubation buffer, 100 μl antibody working solution, and 100 μl tracer (4-6000 CPM) were added to 1.5 ml microcentrifuge tubes (Marsh, Rochester, NY). After 24 h incubation 250 μl of anti-rabbit buffer solution was added and incubated for 3 h. After centrifuging for 10 min in a microcentrifuge (15 000 × *g*) the supernatant was aspirated and the precipitate remaining in the vial counted in a γ counter. Total radioligand binding was determined in the absence of competitor and non-specific binding in the presence of excess (200 ng ml^{-1}) dynorphin fragment.

To test the selectivity of the antibody towards various dynorphin fragments calibration curves were prepared in blood–blocking solution supernatant and assayed directly by RIA (no SPE and HPLC-separation). Based on the IC_{50} value of these calibration curves (see Section 2.6), the cross-reactivity (CR, in %) was obtained as $CR = (IC_{50DF}/IC_{50A1\text{-}13}) \times 100$. Whereas IC_{50DF} represents the IC_{50} value of the investigated dynorphin fragment and $IC_{50A1\text{-}13}$ the IC_{50} value for Dyn A1-13 respectively.

2.6. Data analysis

Using the non-linear curve fitting program MINSQ (Micromath, Salt Lake City, UT), displacement curves (measured in counts per minutes, PM versus competitor concentration, *C*) were fitted to the logistic function:

$$CPM = TB - \frac{TB \cdot C^N}{C^N + IC_{50}^N} + NSB$$

Estimates of NSB (non-specific binding, in CPM), the total specific binding TB (CPM in the absence of competitor – nonspecific binding NSB), *N* (Hill slope factor) and IC_{50} (concentration to decrease specific tracer binding by 50%, expressed in ng per assay tube) were used to transform CPM of the unknowns (quality controls and clinical samples) into the corresponding concentrations.

2.7. Assay validation

In order to obtain intra-day estimates, eight replicates of frozen quality control samples (0.07, 0.25 and 1 ng ml^{-1} for Dyn A1-13, Dyn A2-13 and Dyn A2-12; 0.21, 0.75 and 3 ng ml^{-1} for Dyn A1-12) were analyzed on a given day. Quality controls were designed to be close to the IC_{50} value (e.g. 0.25 ng ml^{-1} for Dyn A1-13), at the lower and upper end of the calibration curve (for Dyn A1-13, 0.07 and 1 ng ml^{-1}, respectively). To obtain inter-day estimates, calibration curve sample sets and quality controls were assayed on five days.

Inter- and intra-day precision was determined from the S.D. of their observed concentrations. Inter- and intra-day accuracy was determined by comparing calculated mean obtained from two measurements of aliquots of the same sample concentration with the nominal concentration at each concentration level. The comparison is shown in percent in Table 2.

3. Results and discussion

The schematic for the HPLC/RIA assay for Dyn A1-13 and its major metabolites (Dyn A1-12, Dyn A2-13, Dyn A2-12) is shown in Fig. 1. The instability of dynorphins in blood [5] made it necessary to apply a blocking solution consisting of a solution of aqueous $ZnSO_4$, ACN and methanol to ensure deactivation of peptidases. Under these conditions the peptides were stable over a period of 24 h at room temperature as demonstrated by direct HPLC analysis of deactivated blood samples containing high concentrations of dynorphin A1-13 or metabolites (10 μg ml^{-1}).

Initial experiments tested the selectivity of the commercial antibody towards various dynorphin fragments (Table 1). According to these results, the antibody showed significant cross-reactivity to all dynorphin fragments larger than Dyn A3-10 (Table 1). In general, the specificity of an HPLC-RIA is determined by the cross-reactivity of the antibody and the chromatographic resolution (Table 1). The developed isocratic HPLC system separated Dyn A1-13 and its main in vitro metabolites (Fig. 2). The rank order of elution was in agreement with general principles of reversed phase HPLC as less charged species (DYN A1-12, and DYN A2-12) showed increased reten-

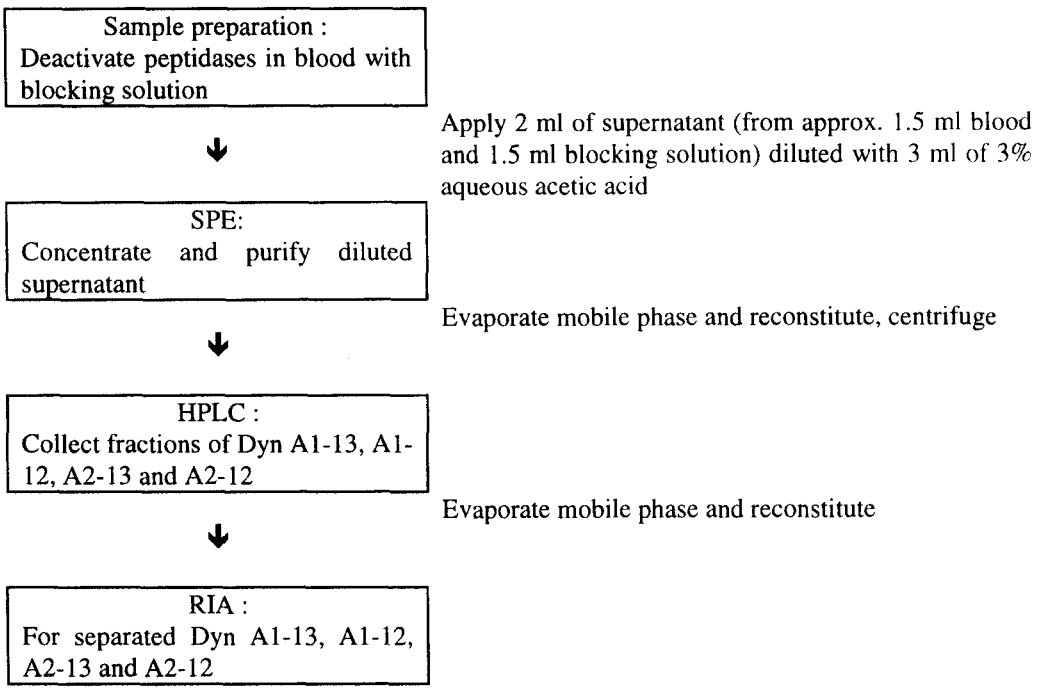

Fig. 1. Scheme of the HPLC-RIA assay.

tion over derivatives with highly charged Lys residues in position 13 (DYN A1-13, DYN A2-13). These results also suggests that this residue is not complexed by the ion pair reagent PSA, as under these conditions a reversed elution order should have been observed. Typical retention times were 4.85 min for Dyn A2-13, 7.85 min for Dyn A2-12, 10.8 min for Dyn A1-13 and 18 min for Dyn A1-12. Therefore, time windows of 3.8–5.8 min (Dyn A2-13), 6.8–8.8 min (Dyn A2-12), 9.3–12.3 min (Dyn A1-13) and 16.8–20.8 min (Dyn A1-12) post injection were collected. We were, however, unable to separate Dyn A2-12 from Dyn A3-12 (Table 1). Based on in vitro data, the amounts of Dyn A3-12 were assumed to be negligible [5]. Dyn A4-12 coeluted with Dyn A2-13 but does not interfere due to its low immunoreactivity. The assay of five different batches of blood obtained from the blood bank and the negative control of the assayed patient showed no interference from endogenous dynorphins or any other blood components. This is in agreement with the very low physiological levels of 20–40 fmol ml^{-1} total dynorphin immunoreactivity reported in plasma [15].

Typical calibration curves of Dyn A1-13, Dyn A1-12, Dyn A2-13 and Dyn A2-12 observed under assay conditions (i.e. after SPE and HPLC, see Section 2) are shown in Fig. 3. The calculated binding parameters (TB, NSB, N, IC_{50}) and their standard deviation for the calibration curves obtained during the inter-day evaluation (one calibration curve assayed on a given day for 5 days) are listed in Table 2. Non-specific binding (about 30% of 1400 CPM of total) was relatively high, quite in agreement with the stickiness of the peptide. Attempts to minimize the non-specific binding by changing incubation tubes (polypropylene, glass tubes, polystyrene tubes) did not improve the results. In addition, changing the buffer composition (modifying detergents and albumin concentration) was not successful. The results suggested that the antiserum displayed the behavior of a homogeneous population of high affinity

Table 1
Possible sources of interference in the HPLC-RIA

Dynorphin fragment	Cross-reactivity (%)	HPLC retention time (min)	In vitro plasma formation (%)[a]
Dyn A1-13	100	10.8	100
Dyn A1-12	90 (60–122)	18.0	80
Dyn A2-13	46	4.85[b]	15
Dyn A2-12	115–116	7.85	75
Dyn A3-12	110	8.35	15
Dyn A4-12	0.4–1.8	4.50[b]	40
Dyn A1-10	44	7.95	15
Dyn A2-10	—	3.50[b]	20
Dyn A1-8	<17	7.25	>5
Dyn A4-8	0	Not detected[b]	30

[a] Percent Dyn A1-13 transformed in specific metabolite in blood (data taken from [5]). Cross-reactivity is based on molar IC_{50} values (0.5 ng ml^{-1} for Dyn A1-12) The HPLC retention time is based on two runs with a difference <5%. Only fragments generated in larger amounts in plasma in vitro [5] were tested.
[b] Partial or total matrix interference.

binding sites (N close to unity) for all four assayed dynorphin fragments.

To estimate the recovery in the HPLC-RIA procedure, IC_{50} values obtained in the HPLC-RIA were compared to those obtained from a direct RIA. Considering the 10-fold concentration during solid-phase extraction, the overall recovery based on average IC_{50} values after SPE and HPLC-RIA for Dyn A1-13 was 35, 6 for Dyn A1-12, 29 for Dyn A2-13 and 16% for Dyn A2-12. Similar low recoveries have been observed for high molecular β-endorphin derivatives [16]. The rather low overall recovery is not related to the solid phase extraction procedure alone as the recovery for this step was shown for Dyn A1-12 to be $84 \pm 18\%$, similar to results for endorphins [17]. A varying and low recovery has been described by others for similar HPLC/RIA assays [16]. It has been speculated that the low recovery in HPLC procedures is related to an irreversible adsorption to the packing material [16]. This low recovery is responsible for the pronounced inter- and intra-day variability observed in the assay. Attempts to increase the recovery by changing the mobile phase composition were not successful. The overall recovery of Dyn A1-12 showed the highest variability (IC_{50}-values, Table 2). One possible explanation could be the chromatographic retention behavior of Dyn A1-12. It elutes latest and possesses the broadest chromatographic peak. The lower recovery of this peak could be in part due to incomplete collection in the collected retention time window.

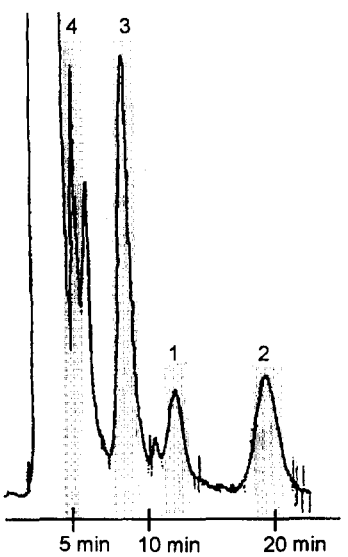

Fig. 2. Chromatogram showing the resolution of Dyn A1-13 (1), Dyn A1-12 (2), Dyn A2-12 (3) and Dyn A2-13 (4) on a reversed phase C18 column with a mobile phase of 24% ACN in aqueous TFA (0.03% v/v) containing 7.5 mM PSA. UV monitoring was performed at 210 nm.

Intra-day variability for quality control samples of Dyn A1-13 was 17, 41 and 28% at 0.07, 0.25 and 1 ng ml^{-1}, respectively (for others see Table

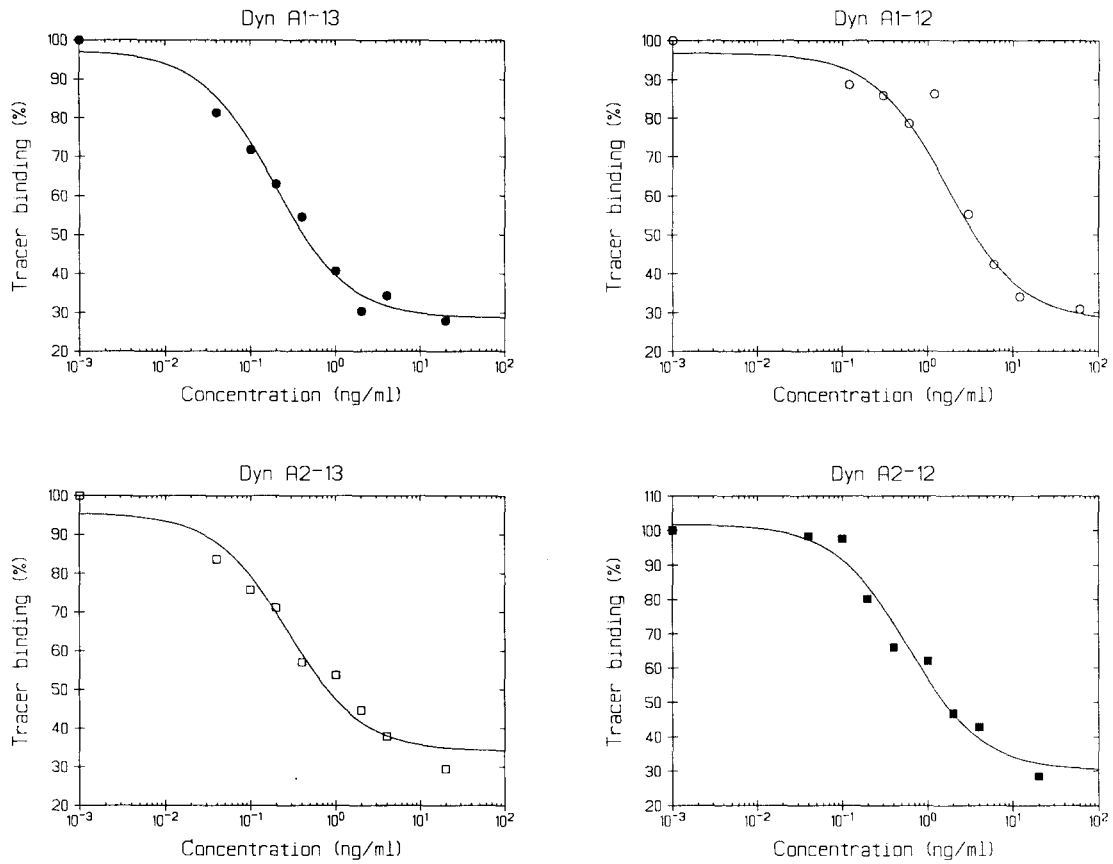

Fig. 3. Typical calibration curves for Dyn A1-13 (●), Dyn A1-12 (○), Dyn A2-13 (□) and Dyn A2-12 (■) obtained under established assay conditions (see Section 2). The tracer binding (as percent of the experimental total binding) is plotted against Dyn A1-13, Dyn A2-13, Dyn A1-12 or Dyn A2-12 concentrations (pg ml^{-1} in plasma). The IC_{50} values were Dyn A1-13 (0.189 pg ml^{-1}), Dyn A1-12 (1.64 pg ml^{-1}), Dyn A2-13 (0.237 pg ml^{-1}) or Dyn A2-12 (0.493 pg ml^{-1}).

2). Accuracy was between 109 and 123% (Table 2). Inter-day variability for single quality controls analyzed on five days (Table 2, for Dyn A1-13) was 49, 48 and 47% for 0.04, 0.25 and 1 ng ml^{-1} samples, respectively. Accuracy for Dyn A1-13 was between 92 and 129% (Table 2). Intra-day accuracy and precision was comparable among all four dynorphin fragments. Based on the intra-day comparison data, the assay shows a limit of quantification of 0.07 ng ml^{-1} for Dyn A1-13, Dyn A2-13 and of 0.21 ng ml^{-1} for Dyn A2-12 (accuracy between 70–130%, variability less than 40%. The assay is significantly more variable than a direct RIA. This was to be expected considering the complexity of the assay. However, important information on the metabolism of dynorphin in vivo can be extracted from such experiments.

Application of the assay to the determination of Dyn A1-13, Dyn A2-13, Dyn A1-12 and Dyn A2-12 in human plasma after constant rate infusion of either 250 or 1000 μg kg^{-1} Dyn A1-13 of Dyn A1-13 over a period of 10 min to human subjects are shown in Fig. 4a and b for 2 subjects. All the dynorphins gradually reached steady state and were eliminated rapidly after the end of the infusion. Half-lives of the α-phases were similar to results obtained by the direct RIA levels of Dyn A1-12 in the subjects are higher than levels of

Table 2
Assay validation of the HPLC-RIA

	n	Dyn A1-13	Dyn A2-13	Dyn A1-12*	Dyn A2-12
Parameter	Inter-day reproducibility based on calibration set data (average ± S.D.)				
IC_{50} (ng ml^{1})	7	0.12 ± 0.04	0.16 ± 0.05	0.49 ± 0.32	0.18 ± 0.11
r^2	7	0.995 ± 0.00	0.992 ± 0.004	0.99 ± 0.01	0.994 ± 0.002
TB (CPM)	7	1055 + 135	1043 ± 181	1063 ± 89	1088 ± 173
NSB (CPM)	7	413 ± 67	451 ± 101	459 ± 51	438 ± 70
N	7	1.09 ± 0.52	1.13 ± 0.34	1.04 ± 0.28	1.07 ± 0.35
Concentration (ng ml^{1})	Inter-day accuracy of quality controls (in % of theoretical concentration)				
0.07 (0.21)[a]	5	129	113	114	112
0.25 (0.75)[a]	5	92	89	88	84
1 (3)[a]	5	107	84	88	72
Concentration (ng ml^{-1})	Inter-day precision of quality controls (S.D. of above percentage)				
0.07 (0.21)[a]	5	49	15	32	49
0.25 (0.75)[a]	5	48	17	36	21
1 (3)[a]	5	47	4	47	21
Concentration (ng ml^{-1})	Intra-day accuracy of quality controls (in % of theoretical concentration)				
0.07 (0.21)[a]	8	123	120	95	114
0.25 (0.75)[a]	8	114	111	132	103
1 (3)[a]	8	109	86	99	91
Concentration (ng ml^{-1})	Intra-day precision of quality controls (S.D. of above percentage)				
0.07 (0.21)[a]	8	17	15	23	32
2.25 (0.75)[a]	8	41	12	28	32
1 (3)[a]	8	28	9	35	30

[a] Concentrations of Dyn A1-12 3-fold higher in calibration curve and quality controls.

Dyn A2-13, in agreement with the in vitro data [5] which suggests the main metabolic pathway going through Dyn A1-12. In contrast to the in vitro experiments, we were unable to differentiate between the half-lives of the dynorphin metabolites. For the low dose, $t_{1/2}$ of the α-phase was between 1.2 and 1.5 min, while the high dose patient's $t_{1/2}$ ranged from 0.6–1.1 min. The estimates for some metabolites were shorter than those found in in vitro experiments in human blood at 37°C. This indicates that, at least for the two subjects investigated, blood vessels and other organs are involved in the clearance of Dyn A1-13 and its metabolites in vivo. In addition, we found a rather deep compartment with terminal half-lives of about 30 min for all measured metabolites after the high dose treatment. This deep compartment was also identified in a direct RIA assay for the same patient. Further studies need to identify the physiological reason for this phenomena.

In conclusion, the described assay allows the sensitive and specific determination of Dyn A1-13, Dyn A2-13, Dyn A1-12 and Dyn A2-12 in sub-ng ml^{-1} range. This assay should be suitable for the detailed pharmacokinetic analysis of Dyn A1-13 and its major metabolites (Dyn A1-12, Dyn A2-13, Dyn A2-12) in vivo.

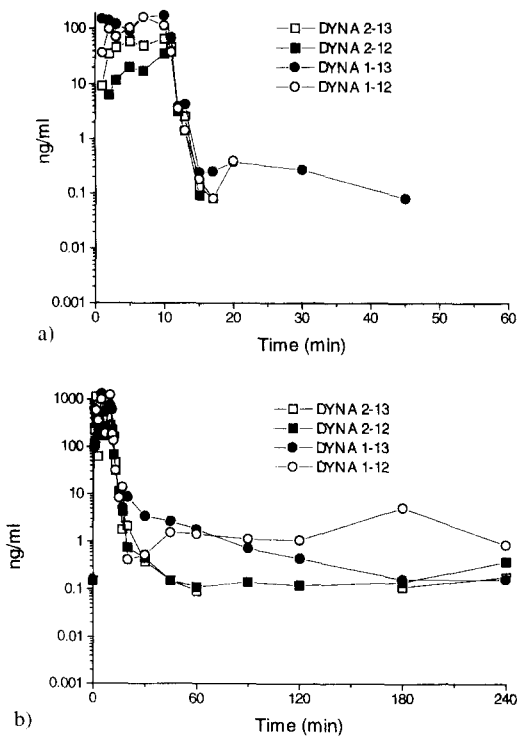

Fig. 4. Blood concentration-time curves for Dyn A1-13 (●), Dyn A1-12 (○), Dyn A2-13 (□) and Dyn A2-12 (■) after 250 μg (a) and 1000 μg (b) Dyn A1-13 kg^{-1} was infused at a constant rate over 10 min to an individual subject. The plasma concentrations of dynorphin A1-13 and metabolites could only be followed in the low dose patient over 20–45 min as concentrations were below the limit of detection at later time points.

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