ENKEPHALIN CONTAINING PEPTIDES IN HUMAN BLOOD

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Contract N00014-79-C-0796

1981

The opioid pentapeptides met-enkephalin and leu-enkephalin[1] are widely distributed both within the central nervous system, the pituitary, and other tissues[2-5] including the adrenal gland[6]. Following the initial observation that the met-enkephalin sequence was contained within the structure of β-lipotropin (and hence β-endorphin), it has recently become apparent that the enkephalin sequences are also to be found within a variety of other peptides[7-14]. It now seems unlikely that met-enkephalin is derived from a β-endorphin-like precursor[15-18]; other enkephalin-containing sequences remain as candidates for a precursor role. Of these other sequences, two groups of peptides may be described: dynorphin is localized in terminal structures within the neural lobe of the pituitary, while the family of enkephalin-containing sequences described by Udenfriend and colleagues is localized within the chromaffin cells of the adrenal medulla. While in both these groups of peptides the localization is suggestive of a hormonal role, two possibilities must be considered. The enkephalin-containing peptides may serve as precursors to enkephalins, which are known to circulate in human plasma[19], or they may themselves have a hormonal function and be released into the circulation. In order to investigate such a role in humans, we have examined blood from nonpathological volunteers, using both radioreceptor assay and radioimmunoassay techniques. We report the existence in human blood of two forms of dynorphin immunoreactive material and a separate group of multiple molecular weight forms of enkephalin-containing compounds with apparently similar structure to those previously characterized in the bovine adrenal medulla[7-9]. The large amount of these enkephalin-containing compounds in human blood relative to the reported levels of met-enkephalin[19] raises the possibility of a hormonal role for the larger peptides.

The investigation of dynorphin in human blood was based upon immunoreactivity. The procedure for the generation of the dynorphin antibodies in rabbits, and subsequent characterization of the antisera used in this study is described elsewhere[20]. The antiserum recognizes the first nine residues of dynorphin and does not cross-react with enkephalins or β-endorphin. No suitable antisera were available to measure in blood the variety of enkephalin-containing sequences found in the adrenal medulla, and so the procedure used by other laboratories, of tryptic digestion followed by radioreceptor assay, was adopted (see legend to Fig. 1 for details). This procedure will measure those enkephalin sequences where there is a basic residue adjacent to the N-terminal tyrosine of the enkephalin sequence. Digestion with trypsin will then expose this N-terminal tyrosine, and the peptide will compete in the radioreceptor assay. All opioid peptides so far identified contain an N-terminal enkephalin sequence.

Three volunteers (male, 25-30 years old) donated blood on at least two occasions each. The blood was extracted with acid acetone followed by gel filtration on Sephadex G75 in 1 N acetic acid. An aliquot of each fraction was used for dynorphin radioimmunoassay. Two peaks of dynorphin immunoreactivity were identified(Figure 1A), one co-eluting with authentic dynorphin 1-13 and one much larger peak which co-elutes with cytochrome C (molecular weight 12,400). Material eluting in the salt volume with enkephalin is also apparently immunoreactive; enkephalin is not immunoreactive in this assay, and the nature of this material is unknown.

Further aliquots of the same fractions were used for radioreceptor assay, and in Figure 1B it can be seen that two major peaks (α and β) of opiate radioreceptor activity can be detected in human blood without prior trypsinization. Following digestion of the aliquots of the G75 fractions with trypsin the

amount of activity at these two peaks is increased approximately 3-fold (Figure 1C). In addition, multiple peaks of opioid receptor activity are unmasked from higher molecular weight material in the blood. These results suggest that the larger enkephalin containing sequences do not have measurable amounts of enkephalin at the free N-terminus, but do have enkephalin sequences with a lysine or argine residue next to the N-terminal tyrosine of enkephalin. Comparison in Figure 1, of the elution pattern of dynorphin immunoreactivity with receptor active material shows that the major peaks of activity in the receptor assay cannot be accounted for by dynorphin immunoreactive material. It is not possible from this analysis to know whether the enkephalin sequences exist singly or in multiples in each of the larger sequences; analogy with material from the bovine adrenal medulla suggests multiple enkephalins within the larger sequences[9]. In order to compare the human blood peptides with those previously characterized from bovine adrenals, we prepared chromaffin granules from the adrenal medulla as described by Smith and Winkler[21], extracted peptides and separated on the same Sephadex G75 column as the human blood. The result of radioreceptor assay of an aliquot of each fraction is shown in Figure 1D. This result is essentially in agreement with that reported by Lewis et al.[22]. The large amount of small molecular weight material which displaces in the radioreceptor assay is preceded by a peak which co-elutes with our peak α from human blood (Fig. 1B); this appears to be similar to peak IV of Lewis et al.[22]. The human blood materials appear to differ from the bovine chromaffin granule peptides in at least two respects. Firstly, the very large amounts of small molecular weight activity seen in chromaffin granule extracts (which include enkephalins and single enkephalin sequences with C terminal extensions as described in refs. 7 and 12) are not apparent in our assays of human blood extracts (Fig. 1B). Secondly, the larger molecular weight material

from human blood which is active on tryptic digestion (Fig. 1C) does not assume such a large percentage of the total trypsinized activity in the chromaffin granule extraction (data not shown).

In human blood extracts, the smaller active peaks α and β (Fig. 1) have activity prior to tryptic digestion, suggesting the existence of a free N-terminal enkephalin sequence, and increased activity after tryptic digestion, suggesting further cryptic sequences with basic residues at the N-terminus. Material from peak α was pooled from the G75 column and separated on C18 ODS reverse-phase high-pressure liquid chromatography (HPLC). Recovery on this column was 80-100 percent. Radioreceptor assay was used after tryptic digestion of the HPLC fractions. While four minor peaks are apparent, the native enkephalin-containing material elutes in a single peak under the conditions we used for reverse phase separations (Fig. 2). This suggests that the free N-terminal enkephalin and the embedded enkephalin sequences are contained within the same peptide. There is on average a 4-fold increase in opioid receptor activity following tryptic digestion, which with the apparent molecular weight of peak α (2000-2500) suggests that the sequence contains a maximum of three embedded enkephalin sequences in addition to one at the free N-terminus. The peak from bovine chromaffin granules which co-eluted with peak α off the G75 (Figure 1D) was separated in the same way on reverse phase HPLC. This also yielded a single dominant peak of receptor active material with a different retention time from the human blood peptide (Fig. 2).

Digestion of pooled human blood material from peak α with trypsin, followed by gel chromatography on Sephadex G50 in 50 percent acetic acid, reveals several receptor active fragments, including peaks the size of six residues, five residues and smaller (Fig. 3). These products probably correspond to enkephalin with a basic residue, enkephalin and additional

smaller as yet unidentifed material. Radioimmunoassay of fractions from this G50 chromatography with antibodies against met-enkephalin[23] show that both the five and the six residue sequences are immunoreactive (Fig. 3). This antibody has 20 percent cross reactivity with met-enkephalin (arg^6). From Figure 3 we can see that the major products of tryptic digestion were not met-enkephalin but immunoreactivity co-eluting with met-enkephalin (arg^6).

A recent report describes a similar enkephalin-containing peptide in a human adrenal medullary tumor[24]. This peptide has a similar molecular weight to peak α described here from human blood and generates mainly met-enkephalin (arg^6) and met-enkephalin (lys^6) on tryptic digestion. However, these authors describe two forms of this molecular weight peptide, separable by reverse phase HPLC. Our observation of a single active peak in a different reverse phase separation could mean that only one of the two forms are present in the blood, or that our separation does not distinguish between these two forms.

It seems most likely that these circulating peptides have their origin in the chromaffin cells[7,19,25,26]. Since these opiate-related materials appear to be stored in the same granules as catecholamines[25,26], their release may be under similar control. By contrast present knowledge of the localization of dynorphin suggests that it is released from the neurohypophysis. In this report we have shown that both these groups of peptides circulate in human blood. The circulating levels of the larger peptides are many times greater (in enkephalin equivalents) than the amounts of enkephalin (reported to be 0.028-0.28 pmoles/ml of plasma by Clement-Jones et al.[19]). This probably does not represent the ratio of material released into the blood, since the different compounds may have very different half lives. However, the high levels of the larger enkephalin-containing forms means that the possibility

must be considered that they have a function as hormones independent of a role as enkephalin precursors. It is not known whether the physiological role of these peptides in the blood is related to their actual or potential opiate activity or to some other unknown hormonal function.

ACKNOWLEDGEMENTS

This work was supported by an ONR Selected Research Opportunity Award and NIMH Program-Project Grant, MH 23861.

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FIGURE LEGENDS

Figure 1. Sephadex G75 separation of extracts. For A, B, and C, blood from a male volunteer was collected (23 ml) into a heparinized tube containing aprotinin (Sigma, 1.4 trypsin inhibitory units per 10 ml), and centrifuged at 6000 x g for 15 min. Plasma (12 ml) was extracted into 5 volumes of acid acetone (75 percent acetone, 25 percent 0.2 N HCl, 0.1 percent thiodiglycol), and centrifuged at 30,000 x g for 20 min. The supernatant was dried under nitrogen to 3-4 ml, insoluble material was removed by centrifugation and the supernatant was applied to a Sephadex G75 column (1.6 x 96 cm) which was eluted with 1 M acetic acid with 0.01 percent thiodiglycol (flow rate 12 ml/h; fraction volume 1.5 ml). The molecular weight markers indicated are: a, dextran blue (void volume); b, cytochrome C (12,400); c, human β-endorphin (3,300); dynorphin 1-13 (1,700); met-enkephalin (574).

A. Dynorphin assay was with 0.25 ml of each fraction, dried down and assayed as described in detail elsewhere[17]. The antiserum was used at a dilution of 1:3000, in phosphate buffer (pH 6.0) containing 0.1 percent gelatin and 0.1 percent Triton X-100, using about 12,000 cpm of ^{125}-dynorphin. The sensitivity of the assay was 5 pg dynorphin.

B. Radioreceptor assay was with 0.25 ml of each fraction, dried and taken into 100 µl of (50 mM Tris pH 7.7). The membrane preparation was from whole rat brain, homogenized (Brinkman Polytron) in 30 ml of 50 mM Tris pH 7.7, centrifuged at 30,000 x g for 20 min. followed by resuspension of the pellet in Tris buffer and incubation at 0°C for 1 h. The membrane pellet was washed twice in buffer and finally

suspended in 30 ml Tris buffer per brain. Incubation of 100 µl of each sample was with 300 µl membrane preparation, 50 µl bacitracin (0.5 mg/ml) and 50 µl [^{3}H]naloxone (New England Nuclear; 30,000 cpm at a final concentrtion of 1.0 nM) at 30°C for 15 min. Leu-enkephalin standards were used. Separation of bound from free was on Whatman GF/B filters which were counted in 10 ml of Cytosint (West Chem Products, California). Results are expressed as leu-enkephalin equivalent per ml of original blood.

C. 0.125 ml of each fraction was dried and trypsinized in 100 µl Tris (50 mM, pH 8.4) containing 10 µg TPCK trypsin (Worthington) overnight at 37°C. The reaction was stopped by the addition of 10 µg ovomucoid trypsin inhibitor (Worthington) and the radioreceptor assay was performed as above. Leu-enkephalin standards were used with the trypsinization procedure to construct the standard curve.

D. Chromaffin granules were prepared by the method of Smith and Winkler[18] from 50 g of bovine adrenal medulla. Tissue was cooled within 15 min of death of animals, and the preparation was started within 2 h. Extraction of chromaffin granules was into 200 ml of 75 percent acetone, 25 percent 0.2 N HCl with thiodiglycol (0.1 percent), phenylmethylsulphonylflouride (200 µg/ml) and pentachlorophenol (20 µg/ml) with a Brinkman Polytron. Half of the total extract was applied to the G75 column and eluted as described.

Figure 2. A 1.2 ml pooled sample from peak α (Fig. 1) was dried and applied to an Altex C18 ODS reverse phase column (5 µ, 4-6 mm I.D., 22 cm length) eluted with 50 mM ammonium acetate (pH 4) with an acetonitrile gradient at 1.5 ml/min. The gradient was 5-20 percent

(5 min), 20–50 percent (25 min), 50–100 percent (20 min). Fractions of 1.5 ml were dried and assayed by radioreceptor assay after tryptic digestion as described in Fig. 1. A sample from the chromaffin granule peak which co-eluted with peak α on the Sephadex G75 column was similarly separated with the same gradient on the same column. The standards indicated are: a, α-neo-endorphin; b, dynorphin 1–13; c, human β-lipotrophin; d, human β-endorphin.

Figure 3. From peak α in Fig. 1, 1.2 ml samples of pooled eluate were dried and digested overnight in 200 µl of 50 mM Tris pH 8.4 containing 100 µg trypsin (at 37°C). This was then applied to a Sephadex G50 column (1.1 cm x 110 cm, in 50 percent acetic acid) and eluted into 1.5 ml fractions (12 ml/min), of which 500 µl was dried down for radioreceptor assay (see legend to Fig. 1) and 250 µl for met-enkephalin immunoassay (see ref. 17). Blue dextran eluted in fractions 31–33, I^{125} human-β-endorphin in fractions 39–42, and the salt in fractions 80–83. The marker peptides shown were: a, met-enkephalin (arg^6); b, met-enkephalin.

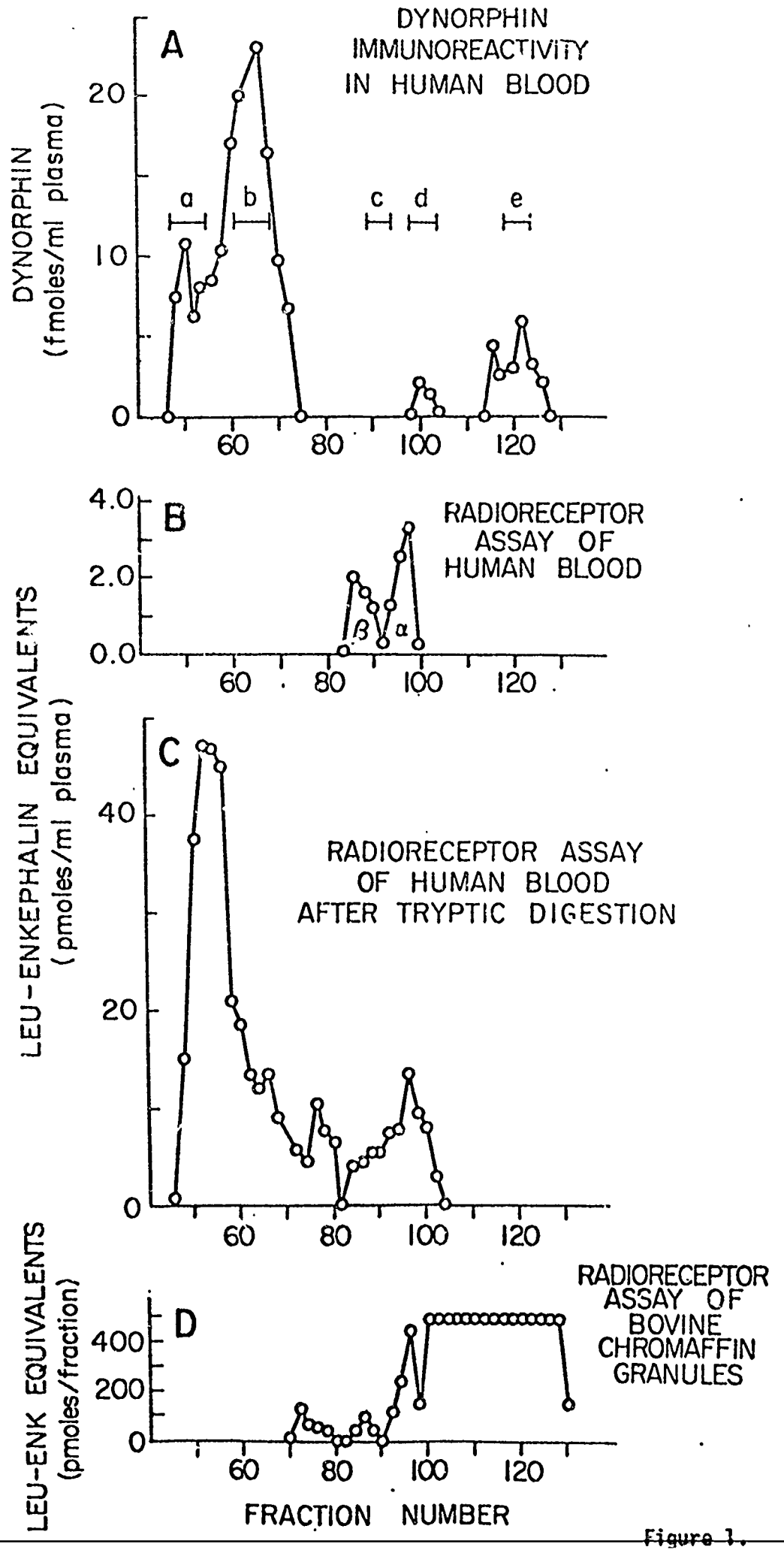
A
DYNORPHIN IMMUNOREACTIVITY IN HUMAN BLOOD
DYNORPHIN (fmoles/ml plasma)
20
10
0
a
b
c
d
e
60
80
100
120
B
RADIORECEPTOR ASSAY OF HUMAN BLOOD
4.0
2.0
0.0
β
α
60
80
100
120
C
RADIORECEPTOR ASSAY OF HUMAN BLOOD AFTER TRYPTIC DIGESTION
LEU-ENKEPHALIN EQUIVALENTS (pmoles/ml plasma)
40
20
0
60
80
100
120
D
RADIORECEPTOR ASSAY OF BOVINE CHROMAFFIN GRANULES
LEU-ENK EQUIVALENTS (pmoles/fraction)
400
200
0
60
80
100
120
FRACTION NUMBER

Figure 1.

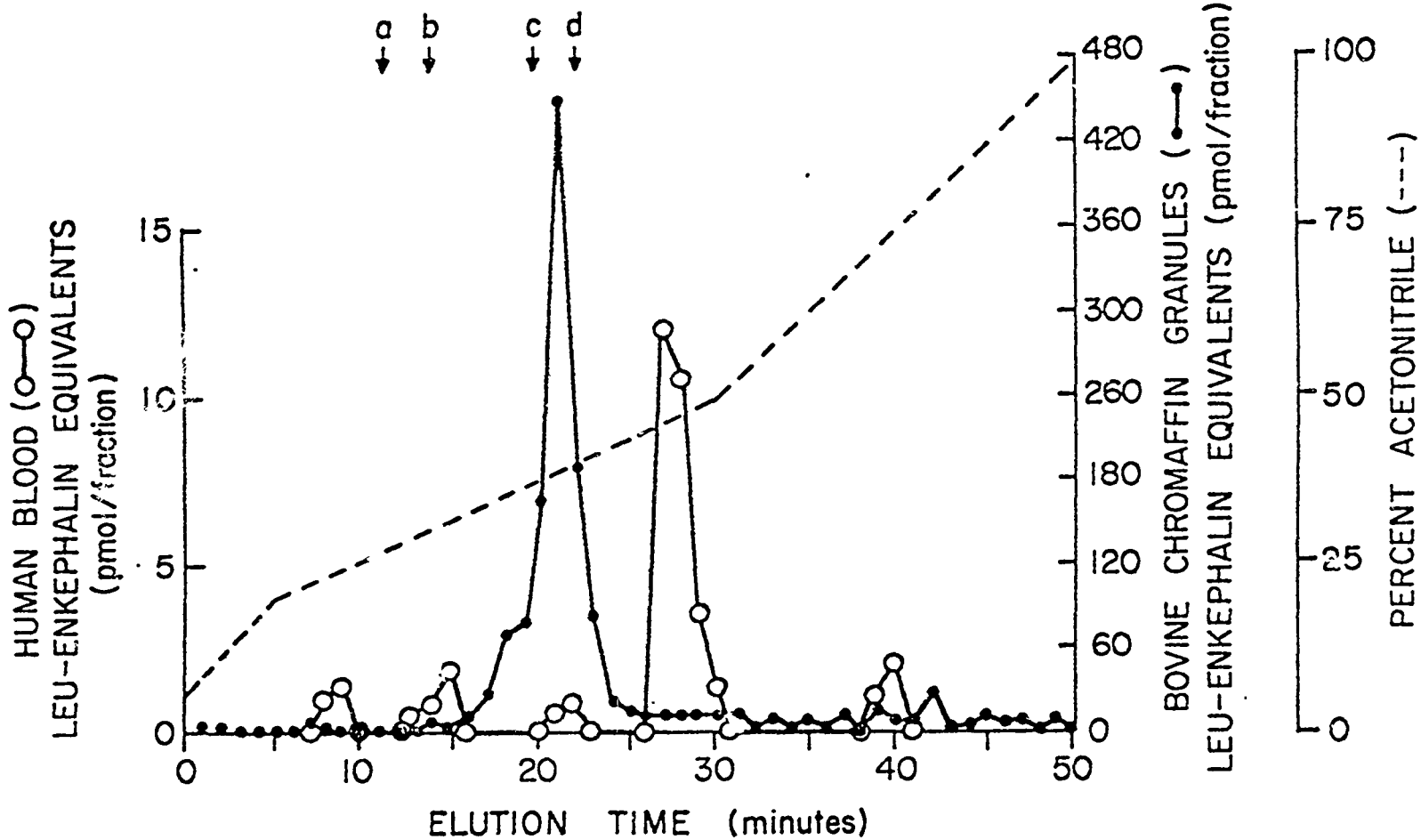
a
b
c
d
HUMAN BLOOD (o—o)
LEU-ENKEPHALIN EQUIVALENTS
(pmol/fraction)
15
10
5
0
480
420
360
300
260
180
120
60
0
BOVINE CHROMAFFIN GRANULES (•—•)
LEU-ENKEPHALIN EQUIVALENTS (pmol/fraction)
100
75
50
25
0
PERCENT ACETONITRILE (---)
0
10
20
30
40
50
ELUTION TIME (minutes)

Figure 2.

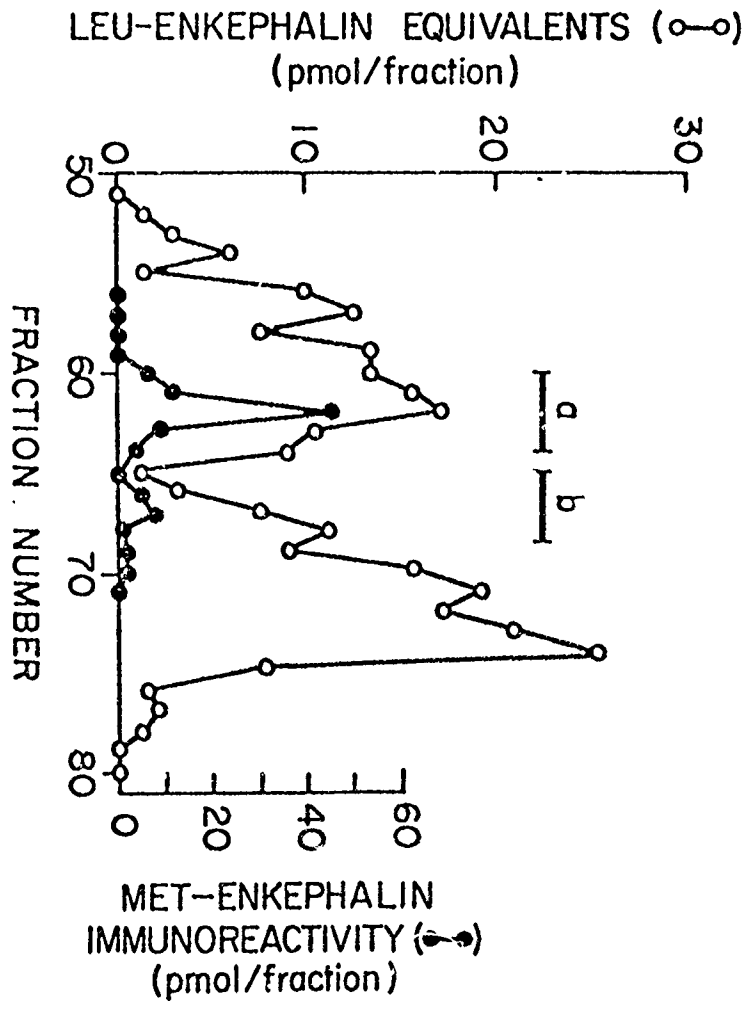
LEU-ENKEPHALIN EQUIVALENTS (o—o)
(pmol/fraction)
0
10
20
30
50
60
70
80
FRACTION NUMBER
a
b
0
20
40
60
MET-ENKEPHALIN
IMMUNOREACTIVITY (•—•)
(pmol/fraction)

Figure 3.