Journal of Chromatography, 577 (1992) 239–249
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6322

Measurement of β-endorphin in human plasma by high-performance liquid chromatography with electrochemical detection: validation of a method employing the simultaneous purification and concentration of β-endorphin

Lynda S. Monger* and Cedric J. Olliff

Pharmaceutical Sciences Research Group, Brighton Polytechnic, Moulsecoomb, Brighton, E. Sussex BN2 4GJ (UK)

(First received May 29th, 1991; revised manuscript received February 3rd, 1992)

ABSTRACT

The simultaneous purification and concentration of synthetic human β-endorphin from plasma is described, which when used together with an appropriate isocratic high-performance liquid chromatographic–electrochemical detection (HPLC–ED) system allows the determination of elevated physiological levels of β-endorphin. Purification of plasma was gained by flash-freezing in liquid nitrogen, acidifying with 100 μl of trifluoroacetic acid (10%, v/v) per ml of plasma, thawing at 4°C and centrifuging to remove any precipitate. Solid-phase extraction with silica sorbent was utilised, which allowed further isolation of the analyte, a method of concentration and a procedure whereby β-endorphin could be transferred to the HPLC mobile phase. Silica sorbent demonstrated greater selectivity than C_{18} for synthetic human β-endorphin and, in addition, provided improved recovery of this analyte when utilising elution volumes of 500 μl or less. Proteolytic degradation and heparin-induced high-affinity binding in plasma were shown not to effect the recovery of β-endorphin if blood was rapidly chilled and plasma quickly obtained, frozen and acidified. Validation of this purification/concentration method using [^{125}I]β-endorphin demonstrated a recovery of 85.6% which was not jeopardised when concentrating the sample twenty-fold. This provided an increase in the sensitivity of detection, when used in conjunction with HPLC–ED, from 5 ng/ml to 250 pg/ml.

INTRODUCTION

To date, radioimmunoassay (RIA) is the only method with sufficient sensitivity to measure β-endorphin-like material in physiological fluids. Significant cross-reactivity occurs, resulting from the existence of many structurally related endogenous opioids. In addition, antibodies raised to the C-terminal of β-endorphin recognise β-lipotropin [1].

High-performance liquid chromatography (HPLC) provides a versatile and effective method of separation for β-endorphin and other endogenous opioids, and, if undertaken prior to RIA, enhances its specificity. This combination has been used extensively to determine β-endorphin-like immunoreactivity in cerebrospinal fluid (CSF) [2,3], in tissue extracts [4,5] and in plasma [6]. Whilst these techniques are practicable, the use of an on-line detection system offers obvious advantages in routine automation of analysis. With the advent of new electrochemical detectors, their potential for the analysis of peptides has been demonstrated [7–9]. Further work is, however, required if this method is to be used for the standard analysis of human β-endorphin in biological fluids.

In an attempt to address this problem, this pa-

per describes and validates a method for the simultaneous purification and concentration of human plasma β-endorphin, which, when linked to a compatible isocratic HPLC–electrochemical detection (ED) system, has the potential of increasing the detection limits of this analyte.

EXPERIMENTAL

Materials

Purified β-endorphin (human sequence) was purchased from Sigma (Poole, UK) as was α-endorphin, γ-endorphin, methionine enkephalin and leucine enkephalin. [^{125}I]β-Endorphin was obtained from Amersham International (Amersham, UK) and C_{18} and silica solid-phase Bond Elut extraction cartridges from Anachem (Luton, UK). Acetonitrile (HPLC-S grade) was purchased from Rathburn Chemicals (Walkerburn, UK) and orthophosphoric acid (Aristar) and potassium dihydrogenphosphate (Aristar) from BDH Chemicals (Poole, UK). High-purity water was produced by double distilling, deionising and filtering through a 0.45-μm nylon membrane filter (Gelman Sciences, Northampton, UK).

Equipment and operating conditions

All chromatographic experiments were performed using a SP8750 organiser and SP8770 pump (Spectra-Physics, Hemel Hempstead, UK) with a Rheodyne injection valve, coupled with a single-pen chart recorder. Detection was obtained by a UV–VIS detector (LC871 Pye, Cambridge, UK) at 210 nm or ED by a 5100A Coulochem electrochemical detector, guard cell 5020 and analytical cell 5011 set at +850 mV applied potential (loaned by Severn Analytical, Manchester, UK). Reversed-phase Nucleosil C_{18} 300 Å pore size, 7 μm particle size, analytical (250 mm × 4.9 mm I.D.) and guard (50 mm × 4.9 mm I.D.) columns (Hichrom, Reading, UK) were employed.

An isocratic elution with a mobile phase containing acetonitrile–0.1 *M* potassium dihydrogenphosphate adjusted to pH 2.3 with orthophosphoric acid (32:68, v/v) was degassed with a constant stream of helium (BOC Special Gases, London, UK). A nominal flow-rate of 1.0 ml/min was used. A 50-μl sample was injected manually using the filled loop technique. Sample size varied between 5 ng/ml and 10 μg/ml, but was sufficiently small to avoid column overloading. The detection limit was taken to be the concentration at which the peak was twice the baseline noise [10]. Chromatograms were recorded and analysed with respect to peak height, peak area, retention time, capacity factor (k'), peak asymmetry factor (a_s), effective theoretical plate number (N_{eff}) and effective theoretical plate height (H_{eff}).

RESULTS AND DISCUSSION

Optimised HPLC–ED system for synthetic human β-endorphin

An optimised isocratic reversed-phase HPLC method linked with UV–VIS and ED for human β-endorphin has been previously described [11,12]; however, its potential to detect plasma concentration of this peptide has as yet not been reported. To investigate the detection limits achievable for β-endorphin, this HPLC system was linked to a coulometric ED system.

The mobile phase comprised acetonitrile–0.1 *M* potassium dihydrogenphosphate adjusted to pH 2.3 with phosphoric acid (32:68, v/v) and was delivered at 1.0 ml/min to C_{18} reversed-phase guard and analytical columns. The ESA Coulochem 5100A dual-channel detector with two porous-graphite in-line working electrodes was used in the screening mode, with the second detector electrode set at a working potential of +0.85 V and the screening potential of the first set at +0.40 V. These settings were based on a current–voltage curve for synthetic β-endorphin, in which C/ng, measured at the second electrode, plateaued between +850 and +950 mV, rising from a base level at an applied potential of +500 mV. The system also incorporated a guard cell detector prior to the injector, set at +800 mV to act as a scrubber for the mobile phase. Chromatographic parameters obtained for synthetic human β-endorphin utilizing these conditions are shown in Table I.

TABLE I

CHROMATOGRAPHIC PARAMETERS OBTAINED FOR β-ENDORPHIN UTILISING ACETONITRILE–0.1 *M* KH_2PO_4 pH 2.3 (32:68, v/v)

Chromatographic parameter	Value
k'	1.11
A_s	1.00
N_{eff}	760.2
H_{eff}	394.6
Detection limit (without prior purification/concentration)	
Injected onto the column	250 pg
Per ml of plasma	5 ng/ml

Purification and concentration of synthetic human plasma β-endorphin

Plasma production

Blood from the antecubital vein was placed in cooled (4°C) polypropylene tubes containing anticoagulant and, when used, protease inhibitor and placed on ice immediately. The lowering of the temperature was a preliminary process utilised to inhibit protease activity. The samples were placed in a refridgerated centrifuge (Sorvall RT 6000B, Du Pont, Bad Nauheim, Germany), pre-cooled to 4°C, for 10 min at 2500 *g*, enabling separation of plasma. The plasma was placed in polycarbonate test tubes.

Solid-phase extraction

Solid-phase extraction of native β-endorphin on C_8 and C_{18} cartridges has been established [13,14]; howver, eluting buffers utilised are those that are compatible with the subsequent method of detection [1,15,16]. Similarly, if this technique is to be utilised prior to HPLC–ED, then the solid-phase eluting buffer must be compatible with the mobile phase above. This compatibility is of primary importance, because β-endorphin has been previously demonstrated to be extremely sensitive to solvent polarity; a slight alteration in its solvent environment prior to HPLC analysis manifests itself as a major change in the chromatographic performance of this peptide [12]. Any solid-phase extraction method used, therefore, must elute β-endorphin from the sorbent with the HPLC mobile phase.

Investigations into the expediency of non-polar supports for the separation of β-endorphin from biological matrices, as opposed to silica, has as yet not been ascertained. Although these sorbents are typically utilized to extract compounds of opposing physical characteristics, because β-endorphin possesses both hydrophobic and hydrophilic regions, silica might be used in an attempt to exploit the polar amino acids existing between residues 6–12 and 24–31. It was proposed, therefore, that the separation of β-endorphin from plasma could be successfully achieved by polar and non-polar mechanisms. A novel solid-phase extraction method was thus developed.

Silica and C_{18} cartridges were prepared for the extraction of β-endorphin by the application of a series of solvents, which were used to solvate the sorbent. Initially, four 1-ml portions of 2% H_3PO_4 in acetonitrile followed by four 1-ml portions of 1% H_3PO_4 in water were applied to the column. The solvent was brought through the column under a negative pressure of 18 kPa, using a Vac-Elut system (Analytichem International, Cambridge, UK). At no time during the extraction were the columns allowed to run dry.

Acidified plasma samples [100 μl of 10% (v/v) trifluoroacetic acid (TFA) per ml of plasma] containing synthetic β-endorphin were applied and drawn through the cartridge under a negligible pressure of 2 kPa. Columns were washed with two 1-ml portions of 1% H_3PO_4 in water (pH 2.3), an identical pH to that of the acidified plasma, ensuring that no changes in the ionisation of β-endorphin occurred. The wash solution was eluted so that the solvent meniscus was in line with the top of the sorbent enabling the accurate determination of sample concentration. Prior to elution of the β-endorphin the cartridge was primed by the addition of a column volume (200 μl) of HPLC mobile phase (acetonitrile–potassium dihydrogenphosphate pH 2.3, 32:68, v/v), to facilitate the removal of the wash solution. Failure to equilibrate the Bond-Elut column in mobile phase altered the subsequent HPLC of β-endorphin, presumably as a consequence of

changes in the polarity of the sample. Finally, a predetermined volume of HPLC mobile phase (0.5–1 ml) was employed as an eluent for β-endorphin.

An identical process could be utilised for the extraction of synthetic β-endorphin from plasma using C_{18} and silica sorbent. It is apparent, therefore, that the mechanism of extraction must be more complex than typical normal-phase separation. A possible explanation is that a polar–polar interaction between analyte and sorbent occurs, whilst elution is achieved hydrophobically by the mobile phase. Thus, β-endorphin has a greater hydrophobic affinity than hydrophilic under elution conditions.

The observation that α-endorphin, γ-endorphin, methionine enkephalin and leucine enkephalin can be successfully extracted by C_{18} stationary phase, whilst α-endorphin and γ-endorphin, but not methionine and leucine enkephalin, can be extracted on silica seems to support this explanation (Table II). All the above peptides contain identical N-terminal amino acids, and thus the hydrophobic part of the molecule, whilst the enkephalins, having a shorter chain length, lack the mid region polar amino acids contained in all the endorphins. Thus, it might be expected that, if a polar–polar interaction is occurring that neither of the enkephalins would be successfully extracted on silica sorbent under these conditions, as was found experimentally.

A comparison of C_{18} and silica stationary phases was undertaken to determine the existence of any inherent advantages of either sorbent. Because the total recovery of the peptide is of primary importance, recoveries of β-endorphin from C_{18} and silica columns were monitored with respect to elution volumes. Results are shown graphically in Fig. 1. Synthetic β-endorphin was applied to the cartridge in acidified water and processed according to the procedure outlined above. Elution with 900, 500, 400, 300 and 200 μl of mobile phase demonstrated a similar profile for both types of sorbent, but recoveries were lower for the C_{18} sorbent. Statistical significance between recoveries gained by varying the elution volumes for silica as opposed to C_{18} were assessed by means of the Mann Whitney *U*-test [17]. Although no significant difference was found between silica and C_{18} at an elution volume of 900 μl, a significant increase in the recovery at 500 μl ($p < 0.05$), 400 μl ($p < 0.0122$), 300 μl ($p < 0.0122$) and 200 μl ($p < 0.0122$) was demonstrated using silica columns. Therefore, whereas no distinguishable benefits exist when eluting with a volume of 900 μl, a potential advantage is provided by silica sorbents at elution volumes of 500 μl and less. Obviously, smaller elution volumes provide the opportunity to concentrate a sample.

TABLE II

RECOVERIES OF α-ENDORPHIN, γ-ENDORPHIN, METHIONINE ENKEPHALIN AND LEUCINE ENKEPHALIN AFTER EXTRACTION ON SILICA AND C_{18} BOND-ELUT SORBENT

Compound	Recovery (%)	
	C_{18}	Silica
α-Endorphin	91.7	92.3
γ-Endorphin	94.3	95.3
Methionine enkephalin	93.1	7.6
Leucine enkephalin	96.2	5.9

Protein precipitation: the comparison of three acids

After plasma preparation, removal of high-molecular-mass proteins is essential to minimise

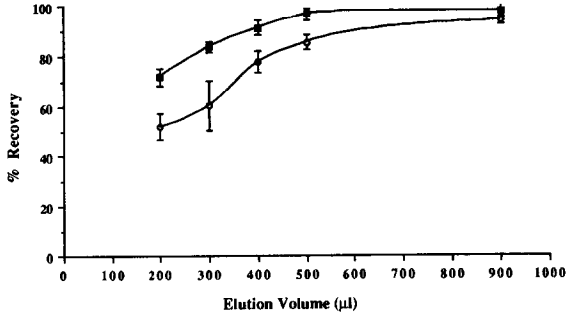

Fig. 1. Comparison of the recovery of synthetic β-endorphin gained when extracted on C_{18} (○) and silica (■) Bond-Elut columns and eluted with varying volumes of acetonitrile–0.1 *M* KH_2PO_4, pH 2.3 (32:68, v/v).

interference in subsequent Bond-Elut separation. The use of acid not only enables the deproteinisation of plasma but, by substantially lowering the pH, reduces the susceptibility of β-endorphin to proteolytic degradation. A variety of acids have been utilised within protocols for the purification of β-endorphin and include 1 *M* HCl [15,16,18], 0.5% TFA [14] and 0.1 *M* acetic acid [19,20]. The acid chosen must, however, provide maximal precipitation of undesired plasma proteins without affecting β-endorphin and be compatible with the HPLC and detection of this molecule.

Three acids at varying concentrations were compared for their suitability. Plasma samples containing undetectable levels of β-endorphin were prepared and then acidified using 10% (v/v) TFA (50 μl/ml of plasma), 10% (v/v) TFA (100 μl/ml of plasma), 1 *M* HCl (100 μl/ml of plasma), and 1 *M* and 1.5 *M* H_3PO_4 (100 μl/ml of plasma). Samples were vortex-mixed and centrifuged (20 000 *g* at 4°C for 10 min) to pellet the precipitated proteins. The supernatant was further processed using silica and C_{18} solid-phase extraction and analysed by HPLC–UV to establish the extent of purification. All acids provided better purification on the silica Bond-Elut columns than on the C_{18}. Distinct benefits of employing one acid in preference to another could be discerned when using the silica columns. The rank order of purification was H_3PO_4 < HCl < TFA, with 10% (v/v) TFA (100 μl/ml of plasma) being the most effective. Purification with all acids was enhanced when plasma was flash-frozen in liquid nitrogen prior to acidification and thawed at 4°C. Flash-freezing and 10% (v/v) TFA (100 μl/ml of plasma) with extraction on silica provided the most efficient purification.

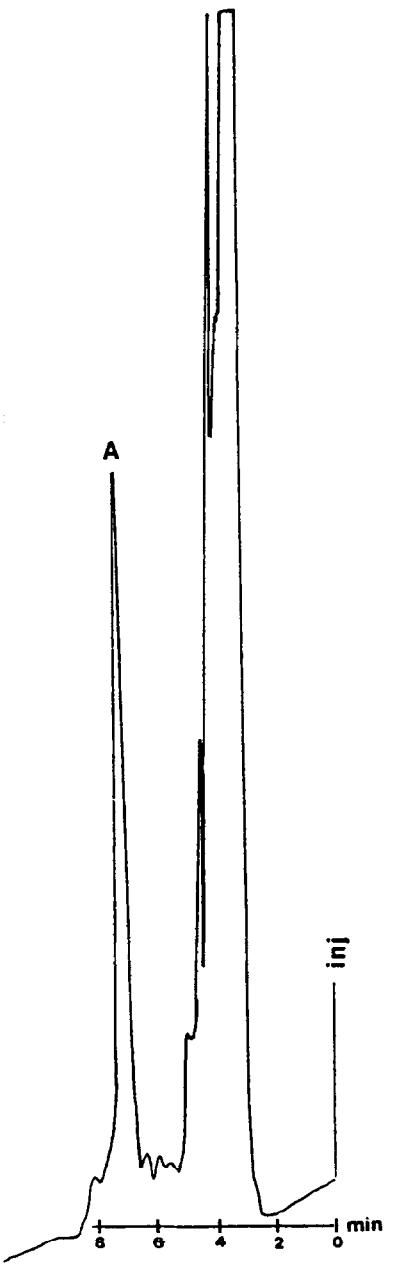

Fig. 2. Chromatogram of synthetic β-endorphin (peak A) after purification utilising 10% (v/v) TFA (100 μl/ml), flash-freezing and silica solid-phase extraction.

Protein precipitation: is β-endorphin affected?

Although it was demonstrated that flash-freezing and acidification [10% (v/v) TFA, 100 μl/ml of plasma] provided superior purification of plasma, it was not known if the β-endorphin would be precipitated under these conditions. To determine the effects of deproteinisation on β-endorphin, plasma was added to a known concentration of freeze-dried synthetic β-endorphin, vortex-mixed, flash-frozen and acidified with 10% (v/v) TFA (50 and 100 μl/ml of plasma), 1 *M* HCl (100 μl/ml of plasma) or 1.5 *M* H_3PO_4 (100 μl/ml of plasma) and thawed at 4°C. Samples were extracted on silica and analysed by HPLC–UV. None of the acids precipitated β-endorphin and,

as already reported, 10% (v/v) TFA (100 μl/ml of plasma) gave more adequate purification of plasma, attaining baseline separation of β-endorphin (Fig. 2).

The purification process

The following procedure was the method of purification utilised.

1. Take blood from the antecubital vein and place in pre-cooled polypropylene test tubes containing EDTA (1 mg/ml of blood), vortex-mix and immediately place on ice.
2. Centrifuge the blood at 4°C for 10 min at 2500 *g*.
3. Pipettte-off the plasma placing 1-ml aliquots in polycarbonate centrifuge tubes and flash-freeze in liquid nitrogen.
4. Add 100 μl of TFA (10%, v/v) per ml plasma and thaw at 4°C.
5. Centrifuge the acidified plasma at 20 000 *g* for 10 min at 4°C and place on ice while solvating the Bond-Elut columns.
6 Solvate silica Bond-Elut columns with 4 × 1 ml of 2% H_3PO_4 in acetonitrile followed by 4 × 1 ml of 1% H_3PO_4 in water, applying a negative pressure of 18 kPa.
7. Add the acidified plasma, changing the pressure to 2 kPa.
8. Wash the columns with 2 × 1 ml of 1% H_3PO_4 in water, allow the wash solution to elute only so far as the top of the sorbent. Further elution should be carried out in an identical way.
9. Elute the column void volume by adding 200 μl of mobile phase (acetonitrile–KH_2PO_4 adjusted to pH 2.3 with H_3PO_4, 32:68).
10. Elute the β-endorphin from the column with a predetermined volume (0.5–1.0 ml) of mobile phase.
11. Analyse by HPLC.

The use of heparin as opposed to EDTA as an anticoagulant

EDTA [21–24] and heparin [19,20] have been used routinely as anticoagulants in the purification of human plasma β-endorphin. It has been reported that heparin, unlike EDTA, has the propensity to induce high-affinity binding sites for β-endorphin within plasma and this reaction is time- and temperature-dependent [25,26]. Consequently, the validity of using heparin in the presence of β-endorphin has been questioned [15]. Furthermore, it has been demonstrated that the presence of heparin results in a loss of β-endorphin-like material such that no analyte was contained in the sample [16]. The use of heparin may thus promote erroneous results due to artificially low levels of free β-endorphin being measured. Although 150 I.U./ml heparin has been utilized to induce binding of [^{125}I]β-endorphin [26], 75 I.U./ml is the concentration routinely used to prevent coagulation. Thus, an investigation to establish the effects of heparin on the final recovery of β-endorphin after its purification from plasma was undertaken, utilising 150 and 75 I.U./ml heparin and EDTA (1 mg/ml of blood).

Aliquots (1 ml) of plasma including heparin (150 and 75 I.U./ml) and EDTA were added to a known concentration of freeze-dried, synthetic β-endorphin, vortex-mixed and kept at 4°C and, alternatively, in a water bath at 37°C for 3 h. Samples were purified by the process of flash-freezing, acidifying and thawing at 4°C, and separated using silica Bond-Elut columns as described previously. Samples were analysed using HPLC–UV. No difference in the concentration of recovered β-endorphin was noted for any of the samples kept at either 4 or 37°C. It might be concluded, therefore, either that heparin at both 150 and 75 I.U./ml failed to induce high-affinity binding sites within the plasma or the purification process disrupted the binding of β-endorphin to plasma proteins. The latter suggestion is plausible, as it is possibile that acidification of the plasma induces dissociation of β-endorphin from the binding sites.

The use of the protease inhibitor aprotinin

Numerous studies measuring β-endorphin have employed protease inhibitors to ensure minimal loss due to proteolytic degradation. The majority utilise aprotinin [18–20,24,27]; however, bacitracin [22] and N-ethylmaleimide [23] have also been used. These studies provide no evidence

of the inhibitory efficiency or the potential benefits of using protease inhibitors and do not discuss the susceptibility of β-endorphin to various proteases and the mode of action of the inhibitor.

It has been shown that β-endorphin is resistant to exopeptidase activity in the form of aminopeptidases [28–32] and carboxypeptidases [29,32], but the shortening of the molecule's chain length increases its susceptibility to this type of degradation. The unusual stability of β-endorphin is due possibly to folding of the peptide chain [32] that renders the NH_2-terminal tyrosine and COOH-terminal sequence highly resistant to attack. The central section, however, is accessible to cleavage by endopeptidases [28,29].

Although endopeptidases are present in tissue and brain extracts [28], in serum only aminopeptidase activity is present in significant amounts [33]. When [^{125}I]β-endorphin in human serum was incubated for more than 180 min, only a very slow release of label was noted [33]. It might be supposed, therefore, that β-endorphin samples, if purified and analysed at low temperatures and within a short time period, will not suffer unacceptable levels of degradation. The activity of a protease inhibitor is perhaps, therefore, dependent on the biological matrix containing β-endorphin and the experimental procedure utilised. An alternative investigation, however, found ^{125}I-labelled β-endorphin degradation in serum to be most effectively inhibited by the endopeptidase inhibitors 2-mercaptoethanol (a thiol agent) and phenylmethylsulphonyl fluoride (PMSF) (a serine agent) [31]. This evidence suggests endopeptidase activity and thus contradicts the assumption that endopeptidases are present only in insignificant amounts in human plasma [33]. It was, therefore, decided to investigate the effect of using aprotinin, an alternative, extensively used endopeptidase inhibitor on the recovery of β-endorphin under varying conditions.

Blood was added to either freeze-dried aliquots of aprotinin (500 k.I.U./ml of whole blood) with a known concentration (2.5 μg) of synthetic β-endorphin or control samples containing only synthetic β-endorphin. Plasma was obtained, kept at varying temperatures for set time periods and purified according to the procedure previously outlined. Temperatures were chosen to reflect conditions that might be encountered during the purification processes. Samples were analysed by HPLC–UV to determine any differences in the recovery of β-endorphin in the presence and absence of aprotinin. Results are displayed as the mean peak area (±S.D.) in Table III. Statistical significance has been assessed by the Mann Whitney *U*-test [17].

No significant difference in the recovery of synthetic β-endorphin was demonstrated between samples that contained aprotinin to control samples at any temperature. This observation supports that of others [16], suggesting that aprotinin is either ineffective or unnecessary if blood is rapidly chilled, and plasma is quickly separated from cells, frozen and acidified. Additionally, it has been demonstrated that aprotinin, rather than halting proteolytic degradation of β-endorphin in plasma, inhibits changes in the molecular

TABLE III

EFFECT OF APROTININ ON THE RECOVERY OF SYNTHETIC β-ENDORPHIN KEPT AT VARYING TEMPERATURES

	Recovery (mean ± S.D., n = 6) (peak area mm^2)				
	Samples immediately purified, and acidified plasma thawed at temperatures of			Samples kept at set temperatures for 1.5 h prior to purification	
	4°C	24°C	37°C	4°C	24°C
Aprotinin	139 ± 16	129 ± 15	125 ± 5	131 ± 14	128 ± 23
No Aprotinin	128 ± 7	130 ± 6	128 ± 10	129 ± 14	116 ± 11

volume of the molecule [34]. β-Endorphin in acidified plasma eluted from a Sephadex G-50 column in a position corresponding to a smaller molecular size than β-endorphin in neutral conditions; this effect was prevented by the addition of aprotinin [34]. Any measured loss of β-endorphin may therefore be attributable to alteration in the environmental conditions of the molecule inducing conformational change. Because an identical pH is utilized throughout the purification process and subsequent HPLC analysis outlined within this paper, it might be expected that β-endorphin will maintain its molecular volume, negating any potential loss in measurement.

Recovery of synthetic β-endorphin after purification

Strategies to optimise the purification of plasma β-endorphin have been outlined, but paramount to the validity of such a process is the quantification of the analyte's recovery. Although the recovery of synthetic β-endorphin from silica extraction columns is high when applied in water, the total recovery from human plasma, utilizing the purification process described, has not yet been ascertained. To assess this recovery, ^{125}I-labelled β-endorphin was employed. Concentrations of β-endorphin were representative of those routinely used during method development and of physiological levels. In addition, the use of [^{125}I]β-endorphin enabled monitoring of loss during discrete stages of the purification process and also allowed accurate determination of the extent of non-specific binding.

Plasma (1.25 ml) was placed in polypropylene test tubes containing a predetermined amount of freeze-dried [^{125}I]β-endorphin. Samples of physiological concentration contained 0.1 μCi of [^{125}I]β-endorphin, which, with a specific activity of 2075 μCi/nmol, provided a concentration of 128 pg/ml. It was necessary to add 5 μg of unlabelled β-endorphin to 0.5 μCi of [^{125}I]β-endorphin in the higher-concentration samples so that the final concentration of 4 μg/ml could be obtained. Spiked samples were mixed thoroughly, allowed to equilibrate for 10 min at 4°C, before two 100-μl aliquots were taken to use as controls, and 1 ml was transferred to a Pegasus polycarbonate centrifuge tube and purified as stated previously. During the purification, samples of the following were collected: the initial 1 ml of wash solution eluted from the Bond-Elut column (W1), the second 1 ml of wash solution eluted from the Bond-Elut column (W2), the 200 μl sorbent void volume eluted with 200 μl of mobile phase (VV), 1 ml of mobile phase passed through the column after the initial elution (E2), the Bond-Elut column (B-E), the contents of the Pegasus centrifuge tube, *i.e.* the pelleted protein that had been precipitated earlier, plus 1 ml of mobile phase used to rinse the tube (P-T).

These samples were analysed for their [^{125}I]β-endorphin content, enabling the identification of the potential amount and site of loss of β-endorphin during the purification process.

All samples were dispensed into scintillation vials and standardised so that each contained 1 ml of mobile phase, 1 ml of water and 19 ml of scintillant (Cocktail T) irrespective of the volume or proportion of solvent initially in the sample. To ensure the complete transfer of sample, test tubes were rinsed with the above. Total radioactivity in these samples was measured by liquid scintillation counting using a Rack Beta 1209 Primo liquid scintillation counter (LKB Wallac, Milton Keynes, UK). Data were corrected for background activity and quenching and expressed in disintegrations per minute (dpm). Recoveries of [^{125}I]β-endorphin after purification from plasma are shown as a percentage of the control values and displayed in Table IV.

High recoveries of [^{125}I]β-endorphin of 89.9 ± 2.1 and 85.6 ± 2.1% were recorded for concentrations of 4 μg/ml, and 128 pg/ml, respectively. Values compare favourably with those reported by others [16] of 85–97%, and are greater than the 31% recovery gained by Wiedemann and Teschemacher [15] who also used solid-phase extraction to purify β-endorphin.

Table V demonstrates that only minimal losses of [^{125}I]β-endorphin were experienced as a consequence of washing the column, eluting the void volume or retention on the column after the first

TABLE IV

RECOVERY OF [^{125}I]β-ENDORPHIN ATTAINED AFTER ITS PURIFICATION FROM HUMAN PLASMA

Control (dpm)	Purified (dpm)	Recovery (%)
4 μg		
683 719	610 218	89.2
620 980	548 167	88.3
647 294	597 550	92.3
Mean		89.9
S.D.		2.1
128 pg		
76 439	65 636	85.9
71 823	61 166	85.2
74 129	65 421	88.2
69 681	58 027	83.3
Mean		85.6
S.D.		2.1

elution with mobile phase as measured by the [^{125}I]β-endorphin content of the second elution. Loss due to non-specific binding of [^{125}I]β-endorphin to the Bond-Elut column was also negligible. The contents of the Pegasus centrifuge tube, however, contained a mean of 4.0% of the total [^{125}I]β-endorphin in the orginal sample, with a wide range of between 1.4 and 9.0%. This loss was more irregular and higher than the other samples taken and can perhaps be explained by the entrapment of [^{125}I]β-endorphin with other high-molecular-mass proteins during ultracentrifugation.

The discrepancies between the loss of [^{125}I]β-endorphin calculated by the sum of W1 to P-T (Table V) and loss indicated by the total recovery of [^{125}I]β-endorphin (Table IV) can probably be accounted for through some β-endorphin failing to bind to the Bond-Elut column when the sample was loaded.

Concentration effect as measured by [^{125}I]β-endorphin

Because the Coulochem electrochemical detector is unable to measure physiological levels of β-endorphin, concentration of the sample is required prior to HPLC analysis. The feasibility of

TABLE V

IDENTIFICATION OF β-ENDORPHIN LOSS INCURRED AT DISTINCT STAGES OF THE PURIFICATION PROCESS

W1 = initial ml of wash solution eluted from the Bond-Elut column; W2 = second ml of wash solution eluted from the Bond-Elut column; VV = 200 μl sorbent void volume eluted with 200 μl of HPLC mobile phase; E2 = 1 ml of mobile phase passed through the Bond-Elut column after the initial elution; B-E = the Bond-Elut column; P-T = contents of the Pegasus centrifuge tube, *i.e.* the pelleted precipitant plus 1 ml of HPLC mobile phase used to rinse the tube.

Stage	*n*	Percentage of control value (mean ± S.D.)
W1	12	1.7 ± 0.99
W2	12	1.20 ± 0.40
VV	9	0.16 ± 0.10
E2	12	0.99 ± 0.15
B-E	12	0.13 ± 0.07
P-T	12	4.05 ± 2.77
Sum of mean loss		8.23 ± 4.48

using solid-phase extraction as a concentration step during sample purification was investigated. Plasma spiked with [^{125}I]β-endorphin was employed and recoveries assessed.

Plasma, 5.25 ml (sample A) and 10.25 ml (sample B), was placed in polypropylene test tubes containing 0.5 and 1.0 μCi of freeze-dried [^{125}I]β-endorphin, respectively. Samples were mixed thoroughly, allowed to equilibrate for 10 min at 4°C, before two 100-μl aliquots were taken to use as controls, and 5 ml (A) and 10 ml (B) of the remaining plasma were transferred into polycarbonate centrifuge tubes, sample A containing 0.095 μCi/ml of plasma (= 152 pg/ml) and sample B similarly containing 0.097 μCi/ml of plasma (= 157 pg/ml). All samples were purified as previously described, the total volume of plasma being added to the column as one aliquot and eluted in 0.5 ml of mobile phase. This process provided a concentration effect for sample A of ten-fold, and sample B of twenty-fold.

Recoveries of [^{125}I]β-endorphin after purification and concentration were established by the measurement of total radioactivity in the sample.

TABLE VI

RECOVERY OF [^{125}I]β-ENDORPHIN AFTER UTILISATION OF THE PURIFICATION/CONCENTRATION METHOD

Control (dpm)	Purified/concentrated (dpm)	Recovery (%)
Sample A (5–0.5 ml)		
707 997	632 970	89
597 609	539 778	90
583 746	476 613	82
413 185	362 602	88
Mean		87.25
S.D.		3.6
Sample B (10–0.5 ml)		
1 087 860	10 799 110	99
1 126 250	1 081 443	96
1 242 329	1 177 272	95
654 270	529 359	81
Mean		92.75
S.D.		8.0

All samples were prepared for liquid scintillation. The recoveries of [^{125}I]β-endorphin for A and B are shown in Table VI. It is evident that no further loss of [^{125}I]β-endorphin is sustained as a consequence of concentrating the sample. Recoveries for A and B are comparable with those obtained for purification alone. Thus, it is evident that successful concentration of β-endorphin samples is possible when employing the purification/concentration process outlined.

CONCLUSIONS

Because synthetic β-endorphin has been shown to be extremely sensitive to alterations in mobile phase constituents and, moreover, that the chromatographic behaviour of the peptide changes when injected onto the column in any solvent other than the mobile phase [12], it was essential that the extraction method utilised be compatible with the solvents used in the subsequent HPLC analysis.

Investigations were undertaken to ascertain the most effective preliminary purification of plasma samples. It was found that flash-freezing in liquid nitrogen, acidification with 100 μl of TFA (10%, v/v) per ml of plasma, thawing at 4°C and centrifugation to remove any precipitate provided adequate deproteinisation of plasma. This process did not cause any loss of β-endorphin and, furthermore, seemed to protect the sample from proteolytic degradation and heparin-induced high-affinity binding of β-endorphin in plasma.

Silica solid-phase extraction as opposed to C_{18} was found to provide more effective isolation and concentration of β-endorphin, the recovery of [^{125}I]β-endorphin of 89.9% not being jeopardised when concentrating the sample twenty-fold. An insight into the possible mechanism of separation of β-endorphin by silica sorbents was provided by the isolation of related, shorter-chain peptides. A polar–polar interaction between analyte and sorbent was identified with elution probably being gained hydrophobically.

The process of plasma purification/concentration outlined using synthetic human β-endorphin provides a practical method by which the sensitivity of β-endorphin can be dramatically increased, and, when used in conjunction with the HPLC–ED system, increases the measurable range of β-endorphin from 5 ng/ml to 250 pg/ml. Therefore, the measurement of β-endorphin in human plasma by HPLC–ED is possible if the purification/concentration method presented here is utilised prior to the analysis.

REFERENCES

1 R. Venn, S. Capper, J. Morley and J. Miles, in E. Reid, B. Scales and I. Wilson (Editors), *Bioactive Analytes Including CNS Drugs, Peptides and Enantiomers*, Plenum Press, New York, 1986, pp. 55–63.
2 R. Venn, *J. Chromatogr.*, 423 (1987) 93.
3 F. Bach, R. Ekman and F. Jensen, *Reg. Peptides*, 16 (1986) 189.
4 M. Dennis, C. Lazure, N. Seidah and M. Chretrien, *J. Chromatogr.*, 266 (1983) 163.
5 D. Smyth, personal communication, 1986.
6 K. Hermann, R. Lang, T. Unger, C. Bayer and D. Ganten, *J. Chromatogr.*, 312 (1984) 273.
7 L. Fleming and N. Reynolds, *J. Chromatogr.*, 375 (1986) 65.
8 G. Bennett, J. Johnson and C. Marsden, in E. Reid, B. Scales and I. Wilson (Editors), *Bioactive Analytes Including CNS Drugs, Peptides and Enantiomers*, Plenum Press, New York, 1986, pp. 37–43.

9 S. Mousa and D. Couri, *J. Chromatogr.*, 267 (1983) 191.
10 C. Simpson, *Practical High Performance Liquid Chromatography*, Heyden & Son, London, 1976, pp. 269–289.
11 L. Monger and C. Olliff, *J. Pharm. Pharmacol.*, 42 (1988) 111P.
12 L. Monger and C. Olliff, *J. Chromatogr.*, 595 (1992) 125.
13 D. Gay and R. Lahti, in W. Hancock (Editor), *CRC Handbook of HPLC Separation of Amino Acids, Peptides and Proteins*, Vol. 2, CRC Press, Boca Raton, FL, 1984, pp. 423–428.
14 S. Giovanninetti, *Extraction of β-Endorphin from Plasma. Application Data Exchange, M504* Analytichem International, 1986.
15 K. Wiedemann and H. Teschemacher, *Pharm. Res.*, 3 (1986) 142.
16 A. Cahill, J. Matthews and H. Akil, *J. Clin. Endocrinol. Metab.*, 56 (1983) 992.
17 A. Wardlaw, *Practical Statistics for Experimental Biologists*, Wiley, Chichester, 1987.
18 L. McLoughlin, P. Lowery and A. Ratter, *Clin. Endocrinol.*, 12 (1980) 287.
19 T. Laatikkainen, K. Salminen, U-H. Stenman and J. Leppaluoto, *Clin. Chem.*, 31 (1985) 134.
20 U.-H. Stenman, T. Laatikkainen, K. Salminen and M. Lusa, *J. Chromatogr.*, 297 (1984) 399.
21 P. Farrell, W. Gates, M. Maksud and W. Morgan, *J. Applied Physiol.*, 52 (1982) 1245.
22 P. Farrell, W. Gates, W. Morgan and C. Pert, in H. Knuttgen, J. Vogel and J. Poortmann (Editors), *Biochemistry of Exercise*, Human Kinetics Publishers, Champaign, IL, 1983, p. 673.
23 R. Steinbrook, D. Carr, S. Datta, J. Naulty, C. Lee and J. Fisher, *Anesth. Analg.*, 61 (1982) 893.
24 K. Nakoa, Y. Nakai, S. Oki, S. Matsubara, T. Konishi, H. Nishitani and H. Imura, *Clin. Endocrinol. Metab.*, 50 (1980) 230.
25 A. Hildbrand and H. Teschemacher, *Naunyn-Schmiedebergs Arch. Pharmacol.*, 325 (1984) R75.
26 A. Hildbrand, L. Schweigerer and H. Teschemacher, *J. Biol. Chem.*, 263 (1988) 2436.
27 I. Raisanen, H. Peatero, K. Salinen and T. Laatikkainen, *Obstet. Gynecol.*, 64 (1984) 783.
28 B. Austen and D. Smyth, *Biochem. Biophys. Commun. Res.*, 76 (1977) 477.
29 B. Austen and D. Smyth, *Biochem. Biophys. Commun. Res.*, 77 (1977) 87.
30 C. Li and D. Chung, *Int. J. Protein Res.*, 26 (1985) 113.
31 J. Silberring, N. Majeed and W. Lason, *J. Pharm. Pharmacol.*, 40 (1988) 17.
32 D. Smyth, B. Austen, M. Geisow and C. Snell, in I. MacIntyre and A. Szelke (Editors), *Molecular Endocrinology*, Elsevier, Amsterdam, 1977, pp. 327–336.
33 J. Burbach, J. Loeber, E. Verhoef, E. de Kloet and D. de Weid, *Biochem. Biophys.*, 86 (1979) 1297.
34 J. Bolton, J. Livesay and M. Hearn, *J. Liq. Chromatogr.*, 7 (1984) 1089.