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Radioimmunological Measurement of Beta-Endorphin in Equine Plasma[1] (41670)

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Abstract. Radioimmunoassay procedures were developed and validated for the quantification of beta-endorphin (β-EP)-like immunoreactivity in equine plasma. β-EP could be quantitatively extracted from plasma with silicic acid powder and subsequently assayed, however, valid estimates of this hormone could also be obtained on unextracted plasma. Although beta-lipotropin (β-LPH) cross-reacted in the assay, it was not necessary to correct for β-LPH activity when assaying unextracted plasma because chromatographic analyses showed that 92% of the immunoreactivity in plasma extracts was similar in molecular weight to authentic β-EP (1–31). In addition, electroacupuncture treatment did not alter the relative proportion of immunoreactivity among different molecular weight fractions.

The distribution of peptides derived from the proopiomelanocortin (POMC) precursor in tissue of the pars distalis (PD) and of the pars intermedia (PI) of the equine pituitary gland (1) appears quite similar to that of other species (2). If the secretion of POMC peptides from these lobes of the equine pituitary is also comparable to that in other species, it seems likely that PD tissue should release ACTH, beta-lipotropin (β-LPH), and beta-endorphin (β-EP) into blood plasma. Likewise, PI tissue should release into blood plasma alpha and beta melanocyte stimulating hormone, β-EP, as well as acetylated and proteolytically cleaved forms of β-EP. The specific POMC peptides present in blood plasma have not been determined for normal horses, but Wilson *et al.* (1) reported a chromatographic and immunologic study of POMC peptides from the plasma of one horse with Cushing's disease. Their study revealed substantial concentrations of POMC peptides with the molecular weights of β-EP, gamma-lipotropin, beta-melanocyte stimulating hormone, and ACTH, but nothing in the molecular weight range of β-LPH. The absence of β-LPH immunoreactivity in plasma from this clinically affected horse was unexpected since this horse as well as normal horses contained β-LPH immunoreactivity in the PD of the pituitary (1).

The goal of the present work was to validate a radioimmunoassay (RIA) for measurement of β-EP-like immunoreactivity in plasma of horses before and after electroacupuncture treatment to induce analgesia (3) and to determine if plasma could be assayed without extraction. Because our anti-β-EP serum cross-reacted with β-LPH, it was necessary to determine the relative contributions of β-LPH and β-EP to the total immunoreactivity in plasma from untreated as well as treated horses.

Methods. *Collection and extraction of plasma.* Blood was collected from male and female horses through a Teflon catheter previously inserted into the jugular vein. Each 20 ml sample was mixed immediately with heparin and centrifuged at 4°C. Aliquots of decanted plasma were frozen at −20°C until thawed for extraction or assay. Plasma was combined from several horses to make two pools (H1 and H2) for validation of the assay. Individual horses were sampled before and during electroacupuncture treatment as described elsewhere (3).

Peptides were extracted from 2 ml aliquots of plasma by adsorption to silicic acid powder followed by elution with acid acetone using published procedures (4). Peptide-containing extracts were evaporated to dryness and reconstituted in 0.01 *M* phosphate-buffered saline (PBS) for assay or chromatography.

Gel chromatography. Reconstituted extracts of 15 plasma samples or purified peptides were

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chromatographed on Sephadex G-50 columns (0.9 × 50 cm) maintained at 4°C (gravity flow: 12 ml/hr). Columns were prepared, equilibrated, and eluted with a solution of 0.1% Triton X-100, 0.1% bovine serum albumin (BSA) in 0.01 *M* PBS (pH = 7.4). Sequential 1-ml chromatographic fractions were collected and stored at −20°C until RIA. Duplicate 20-μl aliquots of the reconstituted extract prior to chromatography were also assayed. Chromatographic columns were calibrated using a 1:1 molar mixture of unlabeled β-EP and β-LPH. In order to be identified, peaks of immunoreactivity among chromatographic fractions had to exceed the averaged baseline of immunoreactivity by two standard deviations. This averaged baseline was subtracted from all peaks when calculating the recovery of chromatographed immunoreactivity.

RIA procedures. Human β-EP was radioiodinated with ^{125}I using the chloramine-T method as previously described (4). Antiserum against human β-EP was obtained from V. Höllt (Max Planck Inst. Psychiatry, Munich). This antiserum was reported to recognize human β-EP and β-LPH equally but not to recognize alpha or gamma endorphin or the enkephalins (5). Antiserum was used at a final concentration of 1:240,000 in each RIA tube. Ovine β-EP and β-LPH were used as reference preparations because they closely resemble the equine peptides (6, 7). The double-antibody RIA utilized a buffer diluent of 0.01% BSA, 0.1% gelatin in 0.01 *M* PBS, and employed procedures already described (4) except for a 1-day preincubation of antiserum with standards and unknowns to increase sensitivity. The minimum detectable concentration of β-EP was 31 pg/ml when assaying 200-μl aliquots of plasma.

RIA validation. Cross-reaction of ovine β-LPH was tested by assaying amounts of the peptide between 12.5 and 200 pg/tube. RIA inhibition curves (logit vs log) were constructed by assaying between 40 and 320 μl of unextracted plasma and between 40 and 160 μl of extracted and threefold concentrated plasma extracts. To estimate recovery of peptides, we added standard β-EP and β-LPH to plasma pools either prior to silicic acid extraction or just prior to RIA of that plasma.

Results. *RIA validation.* Figure 1 presents the standard curve (logit percentage bound vs log dosage) for ovine β-EP as well as parallel RIA inhibition curves for different volumes of unextracted and extracted plasma. The standard curve for ovine β-LPH (not shown) was parallel to that for β-EP with a cross-reaction of approximately 30% by weight or 90% by moles.

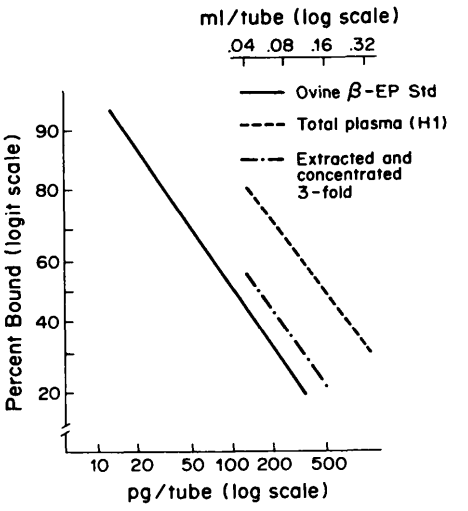

FIG. 1. Logit percentage bound vs log dosage plot of β-EP standard curve and different volumes of plasma pool H1 assayed as total plasma or the same pool after extraction and threefold concentration.

Aliquots of both plasma pools were assayed as total plasma and after silicic acid extraction and reconstitution for assay. Table I presents the estimated concentrations of β-EP like immunoreactivity (IR-β-EP) expressed in terms

TABLE I. COMPARISON OF RADIOIMMUNOASSAY (RIA) OF UNEXTRACTED EQUINE PLASMA WITH SILICIC ACID EXTRACTION, EVAPORATION, AND RECONSTITUTION IN RIA DILUENT

	RIA of unextracted equine plasma	RIA of silicic acid extracts
Estimated IR-β-EP (pg/ml) for each plasma pool:		
H1 pool	710 ± 41 (8)[a]	689 ± 20 (6)
H2 pool	247 ± 11 (14)	386 ± 21 (16)
Within-assay coefficient of variation (%)	9	22

[a] Means are presented ±SEM with the number of observations in parentheses.

TABLE II. ESTIMATED RECOVERY OF PURIFIED PEPTIDES ADDED TO EQUINE PLASMA

	Peptide added to plasma	
	β-EP (1-31)	β-LPH (1-91)
Amount (ng) of peptide added per ml plasma	0.5 to 2.5	2 to 10
Percentage recovery (%R) of peptide added to plasma prior to silicic acid extraction	83% (32)[a]	42% (32)
%R of peptide added to plasma just prior to radioimmunoassay	92% (24)	65% (24)
Estimated efficiency of silicic acid extraction (%R prior to extraction ÷ %R added prior to assay × 100)	90%	65%

[a] Number of estimates in parentheses.

of β-EP equivalents. Although the pools differed greatly in IR-β-EP concentrations, the two methods gave similar estimates. However, variability was less when unextracted plasma was assayed directly.

When standard β-EP was added to plasma prior to extraction, reconstitution, and assay, 83% of the added peptide was recovered in the estimated IR-β-EP (Table II), but only 42% of standard β-LPH added in the same manner was recovered. This relatively low recovery of β-LPH may have resulted from low extraction of β-LPH by silicic acid or from low recovery of added β-LPH in the RIA. To test this latter possibility, standard β-EP and β-LPH were added to total plasma just prior to RIA, and recoveries were 92 and 65%, respectively (Table II). Therefore, part of the low recovery of β-LPH added prior to extraction was due to the RIA. The efficiency of silicic acid extraction for each peptide was also estimated using the two recovery values for each peptide added at different stages. This calculation suggests that silicic acid extracted β-LPH less efficiently than β-EP. In summary, β-LPH recoveries were less than those of β-EP at both extraction and RIA steps.

Chromatography. The calibration of one chromatographic column is shown in Fig. 2 (lower left panel). β-LPH (1–91) with a molecular weight (MW) of about 10K Da eluted between fractions 23 and 29 whereas β-EP (1–

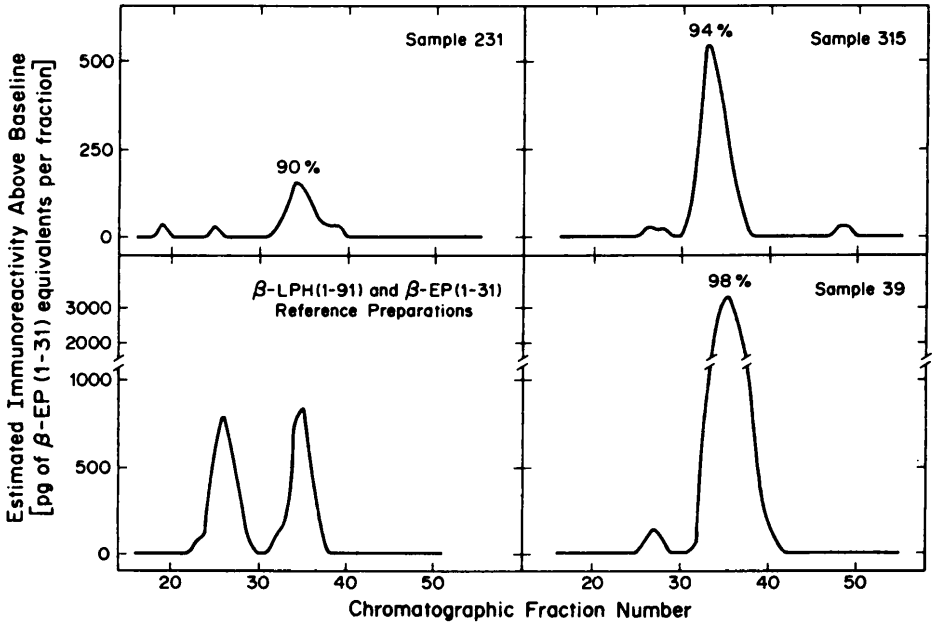

FIG. 2. Chromatographic analyses of reference preparations (lower left panel) and plasma extracts of selected samples (other panels). The percentage of recovered immunoreactivity found in the molecular weight range of authentic β-EP (1–31) is given in each panel over the presumed β-EP peak.

31) with a MW of 3.4K Da eluted between fractions 32 and 37.

When extracts of plasma samples were chromatographed and the fractions assayed for IR-β-EP, a large peak corresponding in MW to β-EP was always observed (Fig. 2). Other small peaks were occasionally observed in the MW range of β-LPH (9K–11K Da) or in the void volume (>12K Da) of the column as shown in Fig. 2. In a few cases a peak of immunoreactivity was found in fractions subsequent to β-EP (i.e., <2.5K Da) as shown for sample 315 in Fig. 2.

Table III presents the chromatographic results for all 15 samples some of which were collected during electroacupuncture treatment. The various chromatographic peaks contained 77.5 ± 5.2% of the IR-β-EP which had been applied to the column. Immunoreactivity was detected in the void volume (>12K Da) in 11 of 15 samples and averaged 5.3% of the total recovered IR-β-EP. β-LPH-like immunoreactivity was detected in only 6 of 15 samples and averaged 2.4% of the recovered IR-β-EP. With the exception of one unusual sample (No. 337), the small MW fraction contained IR-β-EP in 7 of 14 samples and averaged 5.8%. Sample No. 337 was unique because of its high proportion (73%) of IR-β-EP in the small MW fraction.

In 14 of 15 samples, β-EP like immunoreactivity constituted a very large proportion (92%) of the recovered IR-β-EP. This proportion did not appear to vary with the magnitude of the IR-β-EP estimate on unextracted plasma (correlation coefficient = 0.31, $P > .10$). These results suggest that plasma from normal horses contains very little β-LPH to interfere

TABLE III. CHROMATOGRAPHIC FRACTIONATION OF β-EP IMMUNOREACTIVITY IN SILICIC ACID EXTRACTS OF EQUINE PLASMA

Sample number	Total plasma IR-β-EP estimate[a] (pg/ml)	Activity recovered in fractions[b] (% total put on column)	Percentage of recovered activity in each molecular weight range (% total recovered)			
			<2.5K	2.8–3.8K[c]	9–11K[d]	>12K
231[e]	158	62.8		89.5	4.5	6.0
232	1000	76.2	4.2	91.2		4.6
233	600	69.7		97.0		3.0
341[e]	200	66.5		95.5		4.5
342	500	83.0	1.7	86.6	1.5	10.2
343	340	90.0	4.0	94.8		1.2
336[e]	250	49.7	7.2	92.8		
337	550	128.8	73.3[f]	26.7[f]		
338	400	55.2	4.2	88.1		7.7
313[e]	600	69.7	16.0	75.9		8.1
314	1000	74.4		94.3		5.7
315	700	58.6	3.0	93.6	3.4	
39[e]	1000	83.4		97.7	2.3	
40	2600	103.3		97.7	1.0	1.3
41	1500	91.3		92.7	1.6	5.7
Overall						
Number		15	7	14	6	11
Mean		77.5	5.8	92.0	2.4	5.3
SEM		5.2	1.8	1.5	0.5	0.8

[a] Estimate obtained from duplicate aliquots of unextracted plasma. Average coefficient of variation between duplicates was 13%.

[b] Total recovered immunoreactivity above baseline from those peaks which exceeded the baseline by two standard deviations.

[c] Molecular weight range including β-EP.

[d] Molecular weight range including β-LPH.

[e] Each sample with this footnote was collected prior to electroacupuncture treatment. The next two sequentially numbered samples were collected after 30 and 70 min of treatment.

[f] Values excluded from the overall calculations below.

with the RIA estimate of β-EP in unextracted plasma. Furthermore, electroacupuncture treatment which increased plasma IR-β-EP concentrations in some horses to very high levels (i.e., four samples of 1000 pg/ml or higher) did not alter the preponderance of β-EP-sized material in the plasma IR-β-EP estimate.

Discussion. The present RIA for β-EP had significant cross-reaction with β-LPH as do many published assays for β-EP. If equine plasma were to contain significant quantities of β-LPH, it would be necessary to interpret our estimates of IR-β-EP in total plasma as reflecting some contribution from β-LPH. Fortunately, plasma from normal horses as well as those receiving electroacupuncture treatment does not appear to contain very much immunoreactivity in MW ranges other than that of β-EP (1–31). This result is consistent with chromatographic analysis of plasma from one horse with Cushing's disease (1). The relative absence of β-LPH in equine plasma contrasts with the abundance of β-LPH like immunoreactivity in the pars distalis of the equine pituitary (1). Perhaps β-LPH in pituitary tissue serves almost exclusively as a precursor for other peptides such as β-EP and gamma-lipotropin (6, 7) and is only occasionally secreted into blood. Electroacupuncture treatment as in the present study and Cushing's disease (1) apparently do not alter this role of pituitary β-LPH as a precursor.

Silicic acid powder extracted β-EP from equine plasma with high efficiency, and this β-EP could be eluted from silicic acid with acid acetone, dried, and reconstituted for RIA in agreement with Wilson *et al.* (1). However, we also showed that untreated plasma could be assayed directly and that the β-EP estimates were similar to those from extracts of the same plasma. However, our estimates of β-EP in unextracted plasma from normal horses were substantially higher than those reported (1) for normal horses using RIA of silicic acid extracts of plasma. Reasons for this difference are not clear. Nevertheless, the ability to perform our RIA on unextracted equine plasma represents a distinct advantage when large numbers of samples are to be assayed. Other workers have validated direct RIA of unextracted plasma from rats (8) and humans (9).

One plasma sample (No. 337) collected during treatment contained an exceptionally large proportion of immunoreactivity of small MW. Although the β-EP in this sample may have been degraded more than in others, all samples were treated alike. It should be noted that samples No. 336 (before treatment) and No. 338 (during treatment) from the same horse showed only small proportions of the small MW IR-β-EP. Further work will be necessary to clarify the significance of sample No. 337.

Plasma samples (Nos. 39, 40, and 41) from one horse in the present study contained very high concentrations of IR-β-EP (1.0 to 2.6 ng/ml). These concentrations are similar to the levels reported by Orth *et al.* (10) for one horse suffering a mild case of Cushing's disease. Unfortunately, our horse was not available for clinical testing when its high plasma concentration of IR-β-EP was discovered. Because of its possible clinical syndrome, data from this horse were excluded from our investigation of the relationship between electroacupuncture, cutaneous analgesia, and plasma IR-β-EP which is published elsewhere (11).

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