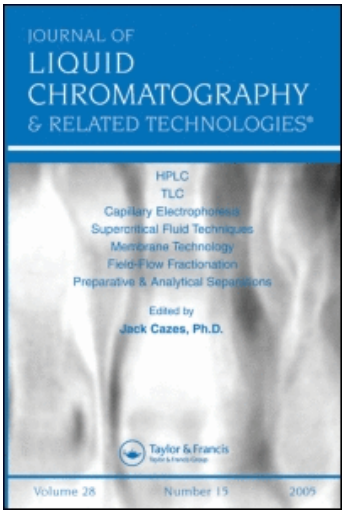

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COMPARISON OF REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND COMPETITIVE PROTEIN BINDING ASSAY IN THE QUANTIFICATION OF CORTISOL IN BOVINE PLASMA

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ABSTRACT

A reversed phase high performance liquid (HPL) chromatographic method was developed for quantification of cortisol in bovine plasma. The reliability of this method was compared to that of a competitive protein binding assay (CPBA). Samples were adjusted to > pH 12 with NaOH, extracted with methylene chloride, evaporated to dryness and reconstituted in methanol/water (55%/45%) for HPLC determination. Satisfactory separation and sensitivity were achieved using an Altex Ultrasphere C-8 column (15 cm x 4.6 mm) with isocratic elution of methanol/water (57%/43%) and UV detection (242 nm). The detection limit of the CPBA (0.16 ng) was lower than for the HPL chromatographic method (0.54 ng); however, precision was significantly better for the HPL chromatographic method than for the CPBA. Values for the CPBA were significantly higher than those obtained using HPL chromatography, probably due to the positive interference of non-specific binding of plasma components in the CPBA. The specificity of HPL chromatography for cortisol was validated by mass spectrometry.

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INTRODUCTION

In a recent study designed to determine the effects of cold stress on cortisol levels in newborn calves (1), the reliability of a competitive protein binding assay (CPBA) was found to be unsatisfactory. Problems associated with both short term and long term precision plus a lack of specificity for cortisol were found to be the major drawbacks with the CPBA. Previously published comparisons of reversed phase high performance liquid (HPL) chromatography versus the CPBA and the CPBA-related radioimmunoassay (RIA) indicated that more satisfactory results might be obtained using HPL chromatography. Several studies have shown that HPL chromatography offers greater precision and specificity than the RIA in the determination of cortisol in human plasma (2-4). Scott and Dixon (5) reported poor correlation between a reversed phase HPL chromatographic technique and a commercially available CPBA kit for measurement of cortisol in human plasma. While these reports indicate that HPL chromatography has certain potential advantages over the CPBA, no thorough statistical analysis comparing these two approaches has been reported.

Aside from two normal phase HPL chromatographic studies on rat adrenals (6) and horse and rabbit sera (7), application of HPL chromatographic methods for quantification of cortisol levels in animals is virtually unexplored. In this report, a reversed phase HPL chromatographic method was developed for quantification of cortisol levels in bovine plasma and its reliability was compared to that of the CPBA.

MATERIALS AND METHODS

Plasma Samples

Heparinized blood samples were obtained by jugular venipuncture of 23 cows and 21 newborn calves. Plasma was collected and frozen at -90°C until analyzed.

Control Sera

Sera from four cows were pooled. One-third of the pooled serum was left untreated and labeled as the normal pool. Decolorizing charcoal (4 g/100 mL) was added to the remaining pooled sera and stirred for 2 hours at room temperature to remove endogenous cortisol. Charcoal was then removed by centrifugation overnight (75,000 X g at 10°C) and by several filtrations with vacuum. The serum was divided in half and cortisol (Sigma Chemicals) in methanol/water (55%/45%) was added to prepare the high pool (100 ng/mL) and the low pool (10 ng/mL) controls.

Instrumentation

A Varian 5000 Series Chromatograph with a Waters U6K injector was programmed to deliver an isocratic mobile phase of 57% methanol in water at 1.5 mL/minute at room temperature. An Altex Ultrasphere C-8 column (15 cm x 4.6 mm with 5 µm particles) and a guard column (7 cm x 9.4 mm, packed with Co:Pell, 40 µm ODS particles [Whatman]) were used for analysis of samples. A Schoeffel SF 770 variable wavelength detector was set at 242 nm, 0.02 AUFS and at a fast time constant setting.

Extraction Procedure

Extraction procedures were carried out on all plasma samples, control sera and distilled water analyzed by the HPL chromatographic method and the CPBA. For the HPL chromatographic analysis, 2.5 mL of sample was added to 5 mL of water containing 500 ng of dexamethasone as an internal standard. Five cortisol standards, each in 55% methanol and containing 6.25, 12.5, 25, 50 or 100 ng/100 µL cortisol and 200 ng/100 µL dexamethasone, were added to water or serum and used to prepare the standard curve for the HPL chromatographic method. For the CPBA, 0.5 mL of sample was added to 1 mL of water containing 5000 cpm/100 µL [1,2-^{3}H(N)] corticosterone (specific activity 58.9 µCi/mmole, New England Nuclear).

All mixtures were adjusted to pH $\geq$ 12 with 1N NaOH (approximately 200 µL/mL sample). Glass-distilled methylene chloride (5

mL for CPBA samples, 13 mL for HPL chromatographic samples) was then added to extract the cortisol in the samples. All mixtures were hand-shaken 50 times and then centrifuged for 15 minutes at 5000 X g at room temperature. The top aqueous layer was removed and the remaining sample was washed with water and recentrifuged. The bottom layer containing methylene chloride and cortisol was pipetted into 12 x 75 mm tubes and evaporated under N_2 gas in a 45°C water bath. Samples used for the CPBA were reconstituted with 500 µL phosphate buffered saline solution (0.5M, pH 7) and 250 µL were added with scintillation fluid for a test of recovery. Samples used for the HPL chromatographic assay were reconstituted with 250 µL of 55% methanol.

Determination of Cortisol

For the CPBA, extracted samples (12.5 - 50 µL) were diluted to make 500 µL. Five hundred µL of 2% corticosteroid binding globulin from dog plasma, labeled with 40,000 cpm/mL [1,2-^{3}H(N)] corticosterone, was added to each tube. Tubes were incubated in a 45°C waterbath for 10 minutes then in an ice waterbath for 10 minutes. Florisil, 40 mg, was added to remove unbound corticosteroids and the tubes were rapidly shaken exactly 1 minute. Following a 30 minute incubation in ice water, 0.5 mL from each tube was pipetted into a vial with scintillation fluid (Omnifluor, New England Nuclear). Cortisol values were determined from the standard curve and corrected for extraction losses (8).

Before analysis by HPL chromatography, samples were sonicated for 10 minutes and then aspirated into 100 µL micropipets that were stoppered with a Dade Miniseal (American Scientific Products). These were centrifuged for 5 minutes in an Autocrit centrifuge (Clay Adams). The clarified samples were then ejected into tubes (9). All samples were run in duplicate. An injection volume of 100 µL (equivalent to 1 mL of plasma) was used. Cortisol and dexamethasone peaks were identified by their respective retention times (3.8 cm and 5.5 cm). There was no evidence of contamination of the cortisol and dexamethasone peaks with other

components. Peak heights were measured to compute concentrations of cortisol. Linear regression analysis of the standard curve (cortisol/dexamethasone ratio versus ng cortisol) permitted calculation of cortisol values in unknown samples from their cortisol/dexamethasone ratio.

RESULTS

The recovery of ^{3}H-corticosterone from the control sera showed the extraction procedure to have an efficiency of 64.9 ± 7.0% (M±S.D., N=30). Because recovery could range from 46.8% to 80.0%, internal standards (^{3}H-corticosterone in the CPBA; dexamethasone in the HPL chromatographic method) were added to the samples before extraction.

In the HPL chromatographic method, the internal standard (dexamethasone) extracted 4.8% more efficiently than cortisol so that use of a nonextracted standard curve artificially lowered the cortisol values. This necessitated the extraction of the standard curve with every analysis to obtain the greatest accuracy. An unpaired t-test revealed no significant difference ($P<0.01$) in cortisol/ dexamethasone extraction from either water or serum matrices.

Results of cortisol concentrations obtained by HPL chromatography and the CPBA of plasma from cows and calves are summarized (Tables I and II). Paired t-test results indicated that the mean values of cortisol estimated from analysis of cow and calf samples by the CPBA were significantly greater ($P<0.01$) than those values calculated by HPL chromatography. The mean difference in cortisol concentrations between the two assays was 3.46 ng/ml for cow plasma and 14.11 ng/ml for calf samples. Linear regression analysis also indicated that differences existed between the two methods (slope = 0.805, y intercept = 2.59).

TABLE I
Concentrations of Cortisol in Cow Plasma as Determined by High Performance Liquid Chromatography (HPLC) and the Competitive Protein Binding Assay (CPBA)

Batch No.	Samples/Batch	Mean Cortisol Concentration (ng/mL) HPLC	CPBA
1	5	20.24	22.76
2	4	21.57	23.30
3	6	9.83	12.04
4	3	12.88	22.09
5	5	6.78	10.63
Overall Mean		13.87	17.33
Standard Deviation		± 8.78	± 9.15

TABLE II
Concentrations of Cortisol in Calf Plasma as Determined by High Performance Liquid Chromatography (HPLC) and the Competitive Protein Binding Assay (CPBA)

Batch No.	Samples/Batch	Mean Cortisol Concentration (ng/ml) HPLC	CPBA
1	4	85.74	81.64
2	5	85.83	93.15
3	5	70.75	102.38
4	5	104.11	120.84
5	2	76.38	93.50
Overall Mean		85.69	99.78
Standard Deviation		± 21.69	± 29.79

The corrected recovery of cortisol (i.e., after adjustment by the recovery of the internal standards) by the two methods varied no more than ±5% from ideal and was not influenced by the quantity of cortisol present (Table III). The mean recovery of cortisol added to serum was 96% for HPL chromatography and 105% for the CPBA.

The precision was generally greater for HPL chromatography than for the CPBA (Table IV). The average day to day precision for the three control sera in six tests was 16.6% for the CPBA and 8.6% for HPL chromatography. Within batch precision averaged 7.4% for the CPBA and 4.4% for HPL chromatography.

The degree of specificity appeared to differ between the two methods. Analysis by mass spectrometry of the cortisol peak from several pooled plasma samples from the HPL chromatographic eluent verified the identity and purity of the cortisol peak. The retention times for cortisol and dexamethasone were 3.8 minutes and 5.5 minutes, respectively (Fig. 1). Several peaks other than cortisol and dexamethasone occurred in the typical plasma sample chromatogram (Fig. 2, peaks 1, 2, 3); however, the appearance of one or more of these peaks on a sample chromatogram did not always correlate with an increased cortisol value for a sample in the CPBA. One peak, possibly corticosterone, was seen occasionally as a shoulder off the dexamethasone peak but did not alter cortisol quantification (Fig. 3). The apparent nonspecificity of the CPBA was demonstrated in another experiment in which serum cortisol was analyzed by the CPBA before and after separation by HPL chromatography. A paired t-test indicated that the amount (%) of cortisol before separation by HPL chromatography was greater ($P<0.01$) than that obtained after treatment by HPL chromatography (Table V). This suggests that the CPBA was quantifying other substances besides cortisol. The values from the CPBA before HPL chromatographic separation averaged 25.5% more than those measured by the CPBA after HPL chromatographic separation.

TABLE III
Comparison of Corrected Recovery Between High Performance Liquid Chromatography (HPLC) and the Competitive Protein Binding Assay (CPBA)

Extracted Sample	Amount of Cortisol Added (ng)	Cortisol Recovered (ng)		% Recovery		Average % Recovery	
		HPLC	CPBA	HPLC	CPBA	HPLC	CPBA
Cortisol in	50	47.75	50.60	95.5	101.2		
Serum Matrix	100	95.70	110.30	96.4	110.3	96.0	105.0
	200	192.80	204.00	96.4	102.0		

TABLE IV
Within Batch and Day to Day Precision of High Performance Liquid Chromatography (HPLC) and the Competitive Protein Binding Assay (CPBA)

Extracted Samples	Coefficient of Variation (%)			
	Within Batch Precision		Day to Day Precision	
	HPLC	CPBA	HPLC	CPBA
High Pool	2.8	17.0	2.9	24.6
Normal Pool	4.1	3.3	4.3	20.0
Low Pool	4.7	6.0	18.5	5.2
Calves	3.5	6.6	--	--
Cows	6.7	4.0	--	--
Mean	4.4	7.4	8.6	16.6

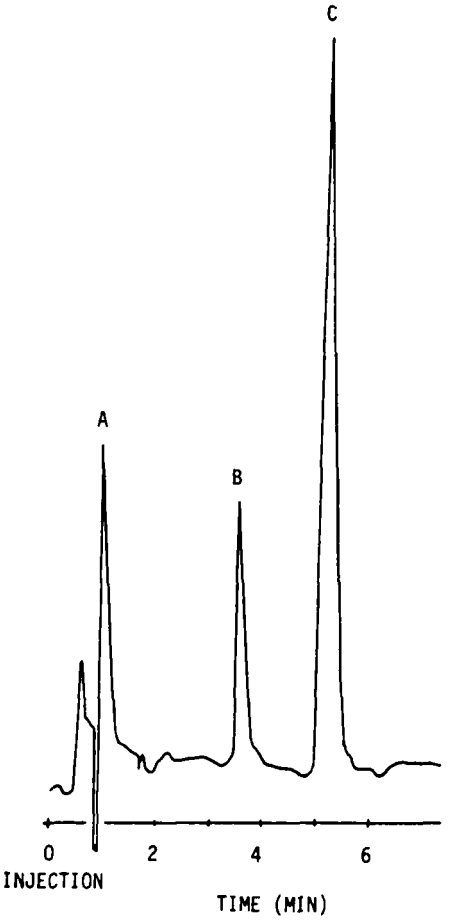

Figure 1 Chromatogram of extracted cortisol standard. A = solvent front, B = cortisol (50 ng), C = dexamethasone (200 ng). Column, Altex Ultrasphere C-8 (5 μm); mobile phase, 57% MeOH:43% H_2O; flow rate, 1.5 ml/min; detector, Schoeffel 770 set at 242 nm, 0.02 AUFS.

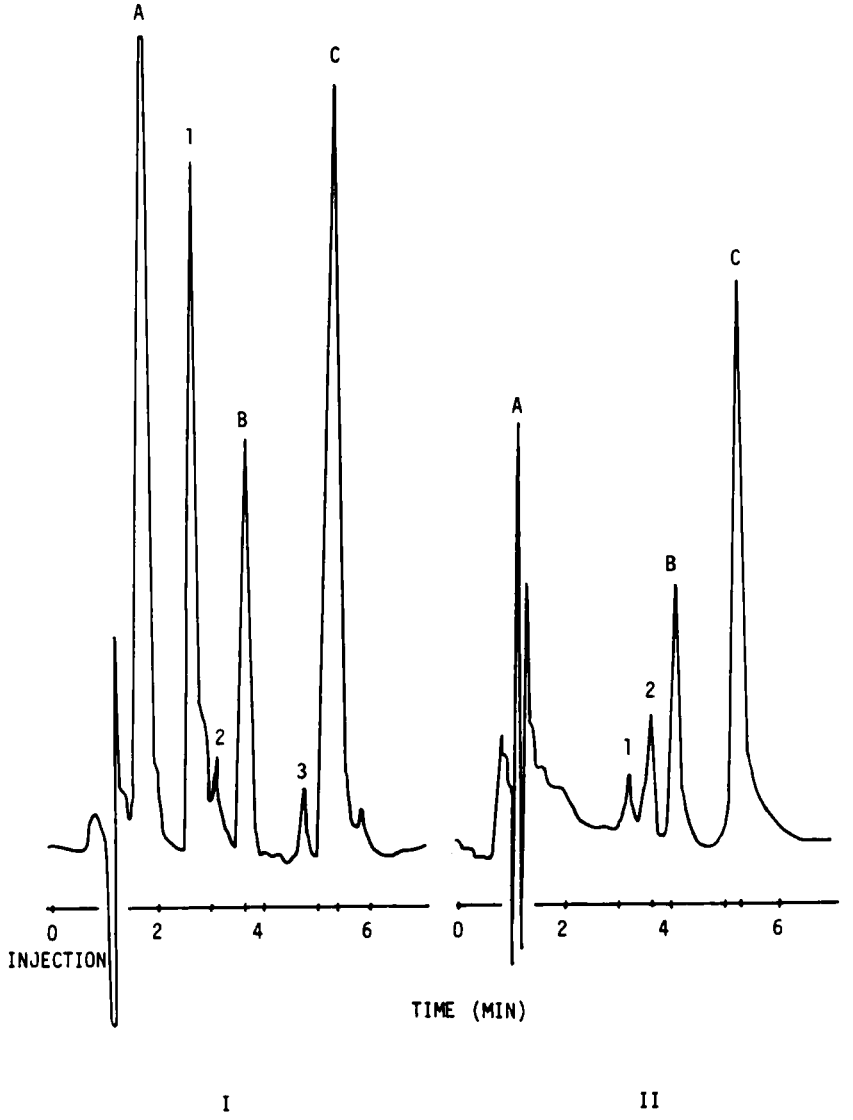

Figure 2 Chromatograms of a calf plasma (I) and the normal pool sera (II). A = solvent front, B = cortisol, C = dexamethasone, 1,2,3 = commonly occurring peaks. Conditions as in Figure 1.

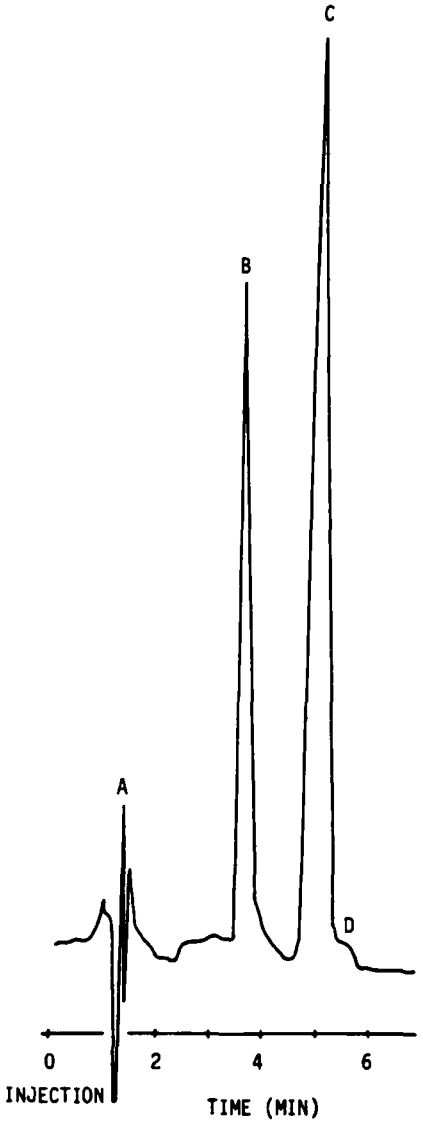

Figure 3 Chromatogram of cortisol standard with corticosterone. A = solvent front, B = cortisol, C = dexamethasone, D = corticosterone. Conditions as in Figure 1.

TABLE V
Measurements of Plasma Cortisol by the Competitive Protein Binding Assay (CPBA) Before and After Chromatographic Separation by High Performance Liquid Chromatography (HPLC)

	CPBA Cortisol Value Before HPLC (ng/ml)	CPBA Cortisol Value After HPLC* (ng/ml)	$\frac{\text{Before HPLC}}{\text{After HPLC}} \times 100$
Range (n=26)	1.33 - 78.78	0.00 - 80.35	83 - 202%
Mean	24.95	21.39	125.5±31.5%

* Values are corrected for recovery loss by HPLC.

The sensitivity of both methods was more than adequate for quantifying all levels of cortisol in bovine plasma. From the fiducial limits (10) calculated for 21 plasma samples with concentrations of cortisol $\leq$ 5.5 ng/mL, the HPL chromatographic method had a detection limit of 0.54 ng (P=0.01). When 1 ng of cortisol was injected into the HPL chromatographic apparatus, peaks were obtained with heights averaging 5.3 times the peak-to-peak baseline noise. With detection limits set at three times the baseline noise, this would be equivalent to 0.56 ng which agrees favorably with the sensitivity value obtained by fiducial limits.

The water controls for the CPBA (11) were used to determine sensitivity. From the fiducial limits (P=0.01), the greatest sensitivity was 0.16 ng while the 99% confidence limits for the same samples indicated 0.19 ng as the smallest amount of cortisol that the method could distinguish from zero.

DISCUSSION

Results of the present study showed differences in reliability between the HPL chromatographic and the competitive protein

binding assay methods for quantification of cortisol in bovine plasma. The differences in reliability between the two methods were more apparent as cortisol levels increased. Aside from the difference in instrumentation used for each approach, other factors that could have accounted for these differences were examined. For example, special effort was taken to insure that the cortisol standards for HPL chromatography and the CPBA were quantifiably identical even though it was necessary to prepare them in different solutions and concentrations. The use of internal standards in each procedure eliminated other possible sources of error in extraction, pipetting and injection. Thus, the differences between the values obtained by the two methods could be explained by one or more of the aspects of reliability.

Results from the present study showed that the corrected recovery of both methods was good. Extracts of cortisol standard in serum were underestimated by HPL chromatography (96%) and overestimated by the CPBA (105%). This difference of 9% did not account for the entire disparity between the two techniques, however.

Precision of the HPL chromatographic method was excellent over most concentration ranges of cortisol. The low day to day precision of HPL chromatography at levels of 10 ng of cortisol resulted, in part, from the increased error of measurement for small peaks of cortisol. More importantly, a problem with the linearity of the standard curve below 10 ng caused an underestimation of cortisol by HPL chromatography, especially if either of the low standard curve points, 6.25 or 12.5 ng, were missing. A least squares parabola has been suggested (12) to increase the linearity of the standard curve and decrease the imprecision at low cortisol concentrations. However, at 10 ng the standard deviation was ±1.82 ng (18.5%) which represents a variation that is of little consequence in most studies on cortisol levels.

The day to day precision for the CPBA was good for the low levels of cortisol (5.2%), especially when compared to the values obtained for the high and normal levels of cortisol (24% and 20%,

respectively). In contrast, a preliminary study of six CPBAs revealed day to day precisions of 20% for each of the three control sera. Also, 1 ng of unextracted cortisol standard had a day to day precision of 14.1%. The unaccountably high precision of the CPBA in the present study at low levels of cortisol could not be explained. The discrepancy in the precision values obtained did not seem to result from error in the standard curve as all three control sera were within the acceptable limits of the standard curve. Mechanical error in pipetting and delivery of florisil accounted for less than 1.4% of the error observed and could not be considered as an important factor in altering precision. Errors due to other factors such as extraction losses, sample amounts and efficiency of scintillation counts also failed to explain the large range of day to day precision for the CPBA.

As previously noted, there appeared to be differences in specificity between the CPBA and HPL chromatography and this may account for most of the discrepancies in cortisol values obtained by the two methods. Prednisolone, an analog of cortisol used in corticosteroid therapy, was the only interfering substance found in the reversed phase HPL chromatographic method used in the present study. Others (5,13) have also reported interference caused by prednisolone. Lack of specificity of the CPBA was indicated by analysis of the same samples before and after chromatographic purification. Corticosterone, cortisone, 11-deoxycortisol and other steroids can cause erroneously high values for cortisol in the CPBA unless both extraction and chromatographic separation of these components are done before analysis for cortisol (8,14-17). Besides steroids, unknown nonspecific competitors that do not fluctuate predictably with endogenous cortisol may account for overestimation of cortisol by the CPBA (18-20).

Extraneous peaks, that could have hindered cortisol quantification by HPL chromatography and distorted the serum profile on the sample chromatogram were detected by use of a water control extracted along with the samples and control sera. Undistilled

methylene chloride was found to produce large UV absorbing peaks and ultrapure water stored in a Nalgene container was also a source of extra peaks. In another instance, two sets of couplet peaks, previously invisible at 254 nm wavelength, appeared in the chromatogram at a wavelength of 242 nm. These couplet peaks resulted from substances extracted (by 55% methanol used to dilute the samples) from the sealant used to stopper the sample-containing micropipets before centrifugation. Use of another sealant eliminated these peaks. The extracted water control also revealed the presence of a peak co-eluting with cortisol. The source of this peak was determined as some unknown substance(s) extracted by methylene chloride from the polyethylene lid liners of the extraction tube caps. Teflon lid liners eliminated this problem.

Lack of sensitivity was not a problem with either method although the CPBA is three to five times more sensitive than the HPL chromatographic method. Sensitivity of the HPL chromatographic method was improved by using a wavelength of 242 nm (i.e., λ maximum of cortisol) which produced a 30% increase in the signal-to-noise ratio than that seen at 254 nm. The peak height ratio at either wavelength remained constant, however (21,22).

CONCLUSION

Results from the present study indicated that the HPL chromatographic method was more reliable than was the CPBA for the quantification of cortisol in bovine plasma. Reliability of the HPL chromatographic method has been shown by subsequent analysis of cortisol in over 200 bovine plasma samples. Moreover, the specificity of the HPL chromatographic method avoids the problem of overestimation of cortisol in plasma. The HPL chromatographic method also has greater overall precision than the CPBA. Sensitivity is the only aspect of reliability in which the CPBA is superior to that of the HPL chromatographic method. However, the HPL chromatographic method used in the present study is more than

sufficiently sensitive to quantify most naturally occurring cortisol levels and is more sensitive than other HPL chromatographic methods reported (13,22). The HPL chromatographic method is also more serviceable than the CPBA. For example, HPL chromatography has the potential for multi-component analysis so that other plasma corticosteroids such as cortisone, aldosterone and corticosterone can be quantified. In addition, individual peaks of interest can be collected from the HPL chromatographic eluent and further tested.

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