ELISA Detection of Morphine in the Horse

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Abstract

A one step enyme-linked immunosorbent assay (ELISA) test for morphine was evaluated as part of a panel of pre- and post-race tests for narcotic analgesics in racing horses. This ELISA test is very sensitive to morphine with an I-50 for morphine of about 400 pg/ml. The test readily detects the presence of morphine or its metabolites in equine blood and urines for 8 or 48 hours longer, respectively, after administration of sub-therapeutic doses. The antibody also cross-reacts with hydromorphone, oxymorphone, nalorphine, levorphanol, and codeine. On initial introduction into post-race urine screning this test flagged 18 of 166 samples positive for opiates. Thirteen of these samples were subsequently confirmed positive for opiates by mass spectrometry.

Introduction

The inability to detect basic drugs is a serious deficiency in an equine race testing system. This is because basic drugs are the medications with the greatest ability to affect the performance of a horse. Basic drugs include the narcotic analgesics, which have been used in racing horses for at least 100 years, and most stimulants, depressants, local anaesthetics, and tranquilizers. Many of these drugs are active in very low concentrations and the doses are commonly in the area of 10 mg horse or less, which makes their detection in post-rac urine challenging. Obviously, therefore, their detectio in pre-race blood is an even more substantial challenge It is a challenge that thin layer chromatography, i its current configuration, is unlikely to meet simpl because the concentration of these drugs in blood ar too low.[1,2]

In an alternative approach we have chosen to utiliz immunoassay as our testing mode. Because of the spee requirement, we have limited ourselves to simple, non radiolabel immunoassays. Among the possible testin modes we have chosen two, PCFIA and one ste ELISA. Work on the utilization of PCFIA in post race testing has been reported previously.[3,4] In thi communication we evaluate a simple one step ELIS/ test for morphine and related narcotic analgesics i racing horses.

Materials And Methods

Horses

Mature Thoroughbred, half Thoroughbred an Standbred horses (400-600kg) were used throughout The animals were kept at pasture and allowed free acces to food and water. The horses were placed in standar box stalls (17 sq M) approximately 12 hours prior t dosing for acclimatization.

Urine and plasma samples from racing horses wer collected by the authorities in charge at the individua racecourses after races and delivered to the drug testin laboratory of the racing jurisdiction.

Dosing and Sampling

Authentic morphine standard[a] was used in *in vitro* studies. For *in vivo* studies, morphine sulfate injection USP[b] was administered by rapid IV injection into the jugular vein. All urine samples were collected by bladder catheterization, and were stored frozen until assayed. Urine samples were diluted and filtered through microcentrifuge filters[c] prior to assay.

All plasma samples were treated with trichloroacetic acid (TCA) to denature and remove excess plasma proteins and endogeous flourescent substances. In this step 300 ul of 4% TCA were added to 300 ul of plasma, vortexed, and allowed to react at room temperature for 20 minutes. The samples were then centrifuged at 8,800 xg for 10 minutes and the supernatant removed. To 50 ul of the supernatant 12 ul of 0.24M sodium carbonate, pH 10 were added, the mixture vortexed, and an aliquot taken for assay.

One Step ELISA Test

The one step ELISA tests were performed as previously described.[4,5] Briefly, anti-morphine antibody was linked[5] to flat bottom wells.[d] Similarly, morphine hemisuccinate was linked to horseradish peroxidase (HRP) to give rise to a covalently linked morphine-HRP complex.[6] All assay reactions were run at room temperature. The assay was started by adding 20 ul of the standard, test, or control samples to each well, along with 100 ul of the morphine-HRP solution. During this step, the presence of free drug or cross-reacting metabolites competitively prevented the antibody from binding to the morphine-HRP conjugate. The degree of antibody-morphine HRP binding was therefore inversely proportional to the amount of drug in the sample. After ten minutes of incubation the fluid was removed from the microtiter wells and the wells washed three times with buffer. Substrate[e] was then added to all wells and their absorbance read at 650 nm in a microwell reader[f] at 0, 5, 10, 15, and 60 minutes after addition of substrate.

Particle Concentration Flourescence Immunoassay (PCFIA)

Antibody cross-reaction studies[g] were performed by PCFIA as previously described[3,4,7] on a flouresecence concentration analyzer.[h]

Mass-Spectroscopy

Gas chromatography/mass-spectroscopy (GC/MS) was performed on an instrument[i] equipped with a 12-meter methylsilicone capillary column.[j] The samples were injected directly into the detector in the non-split mode. GC oven temperature was programmed from 70° C to 280° C at 35° C/min. Urine samples were subjected to enzymatic hydrolysis as previously described.[8] Urine samples (50 ml) were incubated (63° C, 3 hr) with β-glucuronidase from *Patella vulgata*[k] (5,000 units/5 ml urine). The samples were made basic (pH 9.5) with ammonium hydroxide and extracted into dichloromethane (DCM)/isopropanol (6:1) (6 ml solvent/5 ml urine). The samples were then back extracted into 2 ml 0.2N sulfuric acid, made basic (pH 9.5) with 2 ml 0.6N ammonium hydroxide, and re-extracted into DCM/isopropanol (6 ml solvent/5 ml urine). The samples were evaporated to dryness, redissolved in methyl acetate, and purified by preparative thin layer chromatography.[l] The solvent system was chloroform/methanol/propionic acid (80:15:5). The plates were scraped and the samples were eluted into 1 ml isopropanol and extracted with 2 ml 0.2N sulfuric acid. The samples were made basic with 2 ml 0.6N ammonium hydroxide (pH 9.5) and extracted into 6 ml DCM. The samples were evaporated to dryness and 30 ul ethylacetate were added to each tube. After thorough mixing, 2 ul of the sample were injected on the GC/MS.

Results

Experimental evaluation of the ELISA test for morphine was performed *in vitro*, *in vivo*, and in the field. The performance of the assay *in vitro* was characterized with respect to the time course and sensitivity to added morphine. Figure 1 shows that in the absence of added morphine the reaction runs rapidly to completion, with an apparent absorbance value of about 2.0 being attained between 10 and 15 minutes after starting the reaction. The addition of increasing concentrations of morphine acts to inhibit the reaction, with virtually complete inhibition occurring after addition of 10 ng/ml of morphine.

The sensitivity of the anti-morphine antibody was also tested *in vitro*. Figure 2 shows that half-maximal inhibition occurs at about 400 pg/ml of morphine for a one-step ELISA test. With the PCFIA technique previously described, using the same anti-morphine antibody and a morphine β-phycoerythrin complex to

construct the assay, the I-50 occurs at about 1 ng/ml.

To be maximally effective a test should not simply detect morphine, but also related medications. The anti-morphine anti-body was therefore tested *in vitro* for cross-reactivity with other medications by PCFIA over a range of drug concentrations from 0.1 to 10 ng/ml. As Table I shows, the antibody cross-reacted with morphine, oxymorphone, hydromorphone, levorphanol, nalorphine, and codeine, and in fact appears more reactive with hydromorphone than with morphine. Figure 3 represents the structural forms of the family of morphine congeners which cross-react with the morphine antibody.

In a series of *in vivo* studies, the ability of the ELISA test to detect morphine or its metabolites in equine plasma and urine was investigated. Figure 4 shows the results of an ELISA assay of plasma levels of morphine after IV administration of a 50 mg/horse dose. The ELISA reaction was initially inhibited about 90%, which is sufficient to be readily visible to the naked eye. The inhibition appeared within minutes after administration of the drug, and remained at a high level for the eight hours over which this inhibition was followed.

In an *in vivo* cross-reactivity study, the ability of the anti-morphine antibody to detect morphine and its analogs in equine plasma by PCFIA was investigated for a range of dosing levels and post-dose times. Table II shows that doses of morphine as low as 1 mg/horse, or hydromorphone as low as 2 mg/horse and oxymorphone as low as 3 mg/horse are easily detectable by PCFIA using this antibody. The inhibition appeared within 30 minutes or less of dosing, and was still present in all horses tested at 2 or more hours after drug administration. In each case the inhibition was greater than 50% and as such is sufficient to allow easy identification of samples from dosed horses.

The one-step ELISA test readily detected morphine or it metabolites in equine urine. Figure 5 shows that the ELISA reaction is essentially completely inhibited in urine samples for the first 24 hours after IV administration of a 50 mg/horse dose of morphine to three horses. By 48 hours after dosing the ability of these urine samples to inhibit the ELISA reaction was declining, however. At 72 hours after dosing the ELISA readings had returned to control levels in one horse, and were only about 50% inhibited in the other two.

In other *in vivo* experiments on the ability of this test to detect morphine congeners in equine urine, urine samples provided by the Quality Assurance Program of the National Association of State Racing Commissioners were tested for opiates. All samples were drawn at 2 hours post-dose, though the dose levels and administration routes varied with the drug administered. Table III shows that the test readily indicated the presence of an opiate in the urines of horses dosed with codeine, hydromorphone, levorphanol, and oxymorphone.

The ELISA test for morphine proved to be particularly effective in field trials. The results of analysis of a day's complement of post-race urine samples from a track are shown in Figure 6. The samples fell into two groups. Most of the samples gave ELISA readings which were greater than a morphine standard. However, two samples did not. One of these was a test urine sample from a morphine-dosed horse and the other was a track sample. The track sample behaved like a typical drug-containing sample in that little or no color developed over the test period. Mass spectral analysis of this sample in fact shows the presence of oxymorphone.

Table IV summarizes the results obtained on preliminary introduction of this test into post-race urine analysis. The ELISA test was applied to nine days of post-race samples from tracks in two southwestern racing jurisdictions in October 1987. From a total of 166 samples 18 were flagged positive for opiates. Thirteen were subsequently confirmed positive for either oxymorphone or hydromorphone by mass spectral analysis. For some of these samples insufficient urine was available for complete confirmation analysis. Consequently, the confirmation rate of 72% is likely an underestimate of the true number of opiate positives in this population of post-race urines.

Discussion

The ELISA test for morphine reported here is both rapid and sensitive. Since this test detects opiates in

urine without the necessity of a hydrolysis step, samples likely to contain opiates can be identified within thirty minutes of receipt of the samples in the laboratory. These samples can then be immediately subjected to further appropriate analysis for opiates at a substantial saving in time and effort over the old procedures.

As well as being rapid, this test is also very sensitive to morphine. As shown in Figure 2, the test is able to detect morphine with a sensitivity of about 400 pg/ml, which is considerably more sensitive than thin layer chromotographic technology. While this improved sensitivity is not critical in post-race testing, where the concentrations of drug in the system tend to be higher, these differences are critical in pre-race testing, where the concentrations of drug in the blood are low. For morphine itself, sensitivity is clearly more than adequate, since it appears that about 50 mg of morphine have to be administered to an adult horse to produce a pharmacological effect. However, if a more potent narcotic analgesic is being used whose effective dose will be less than that of morphine, and which may not react with this anti-morphine antibody as readily as morphine, then the sensitivity of the test becomes very important.

This test will also detect narcotic analgesics other than morphine. As shown in Tables I and II, PCFIA will detect doses of morphine of as little as 1 mg/horse, and doses of hydromorphone and oxymorphone of approximately similar size. Similarly, there are suggestions that the test may also be able to detect nalorphine and codeine administration. These broad spectrum characteristics of the test are very useful, since none of these drugs has ever been detected in a pre-race test.

This test is effective in post-race urine testing. Because of the concentrating power of the kidney and the fact that most of these drugs are excreted as glucuronide conjugates, these drugs are much easier to detect in urine than in blood. As shown in Figure 5 morphine was detectable in post-dose urine for at least 48 hours and likely longer. This sensitivity raises the possibility that in practice these drugs will detect a relatively wide range of morphine congeners and contribute to control of these agents.

This possibility is further supported by the data of Table III, which shows that the test is capable of detecting administration of codeine, oxymorphone, hydromorphone, and levorphanol. Given this range of sensitivity, this simple one step ELISA assay is likely to be very effective as a post-race test for illegal medication in racing horses.

This likelihood has been borne out in our initial experience with this assay in the field, since this test detected use of oxymorphone and hydromorphone in racehorses in two Southwestern racing jurisdictions. On its initial introduction into routine testing this test flagged 18 of 166 post-race urines as possible opiate positives, and 13 of these were confirmed positive for opiates by mass spectral analysis. The test appears to be particularly effective and has contributed substantially to equine medication control in racing horses.

In summary, therefore, we have developed a simple one step ELISA for morphine that readily detects morphine in blood or urine after its administration at therapeutic and sub-therapeutic doses. The test also detects administration of small doses of codeine, oxymorphone, hydromorphone, and levorphanol in blood or urine after their intravenous administration to horses. Utilization of this test in pre-race and post-race testing programs would appear to offer swift and sensitive testing for morphine and a significant number of its congeners at very reasonable cost.

References

1. Tobin, T., Maylin, G.A. Henion, J., .Woodward, C., Ray, R., Johnston, J., Blake, J.W.: An evaluation of pre- and post-race testing and blood and urine testing in horses. *J. Equine Med. Surg.*, 3, (1979): 85-90.

2. Tobin, T.: *Drugs and the Performance Horse.* Charles C. Thomas, Springfield, IL. (1981): 363-365.

3. McDonald, J., Gall R., Wiedenbach, P., Bass, V.D., DeLeon, B., Brockus, C., Stobert, D., Wie, S., Prange, C.A., Yang, J.-M., Tai, C.L., Weckman, T.J., Woods, W.E., Tai, H.-H., Blake, J.W., Tobin, T.: Immunoassay detection of drugs in horses. I. Particle concentration fluoroimmunoassay detection of fentanyl and its congeners. *Res. Comm. Chem. Pathol. Pharmacol.*, 57, (1987): 389-407.

4. Yang, J.-
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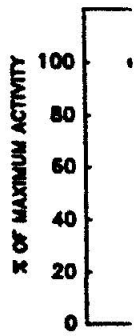
Figure 2. *I-systems.*



4. Yang, J.-M., Tai, C.I., Weckman, T.J., Tai, H.-H., Blake, J.W., Tobin, T., McDonald, J., Gall, R., Wiedenbach, P., Bass, V.D., DeLeon, B., Brockus, C., Stobert, D., Wie, S., Prange, C.A.: Immunoassay detection of drugs in racing horses. II. Detection of carfentanil in equine blood and urine by RIA, PCFIA and ELISA. *Res. Comm. Subs. Abuse*, 8(2), (1987): 59-75.

5. Voller, A., Bidwell, D.W., Bartlerr, A.: The enzyme linked immunosorbent assay (ELISA). *Bull. Wld. Hlth. Org.*, 53, (1976): 55-56.

6. Wie, S., Hammock, B.D.: The use of enzyme linked immunosorbent assay (ELISA) for the determination of Triton-X nonionic detergents. *Anal. Biochem.*, 125, (1982): 168-176.

7. Jolley, M.E., Wang, C.-H., Ekenberg, S.J., Zuelke, M.S., Kelso, D.M.: Particle concentration fluorescence immunoassay (PCFIA): A new, rapid immunoassay technique with high sensitivity. *J. Immunol. Meth.*, 68, (1984): 21-35.

8. Combie, J., Blake, J.W., Nugent, T.E., Tobin, T.: Morphine glucuronide hydrolysis: Superiority of -glucuronidase from *Patella vulgata*. *Clin. Chem.*, 28, (1972): 83-86.

Figure 1. *Time course of ELISA reaction in the presence of increasing concentrations of morphine.*

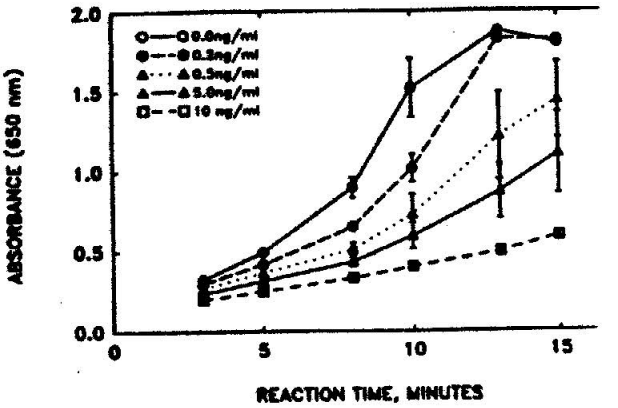


Figure 2. *I-50 for morphine in one-step ELISA and PCFIA systems.*

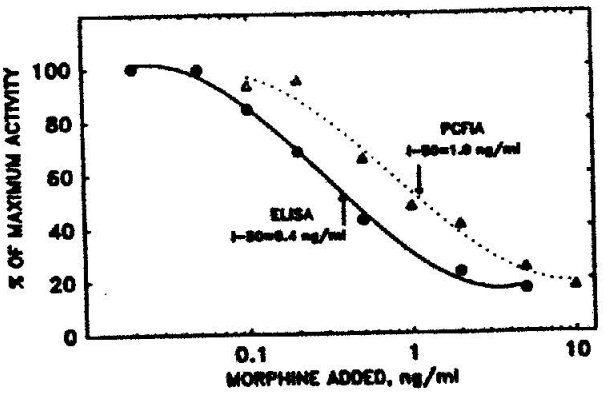


Figure 3. *Morphine and morphine congeners reacting with morphine ELISA.*

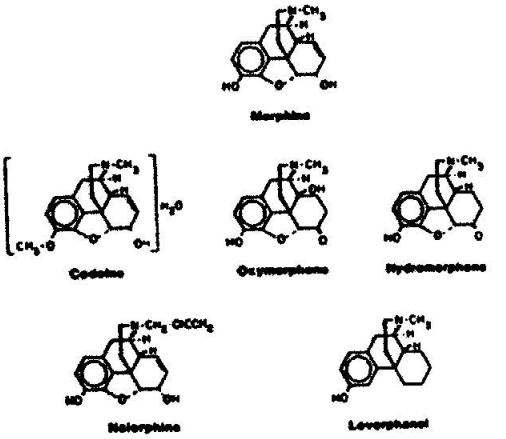


Figure 4. *ELISA inhibitory activity in equine plasma after 50 mg/horse IV administration of morphine.*

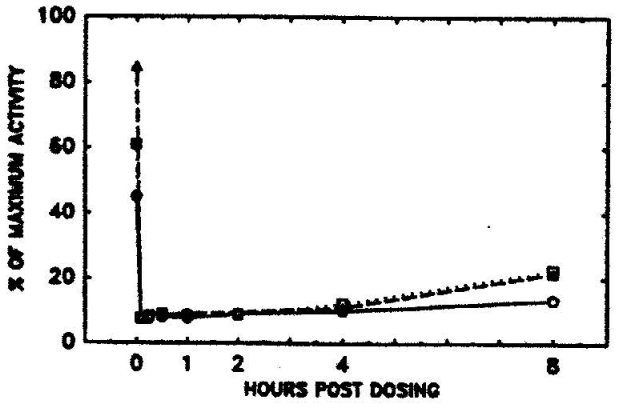


Figure 5. *ELISA inhibitory activity in equine urine after 50 mg/horse IV administration of morphine.*

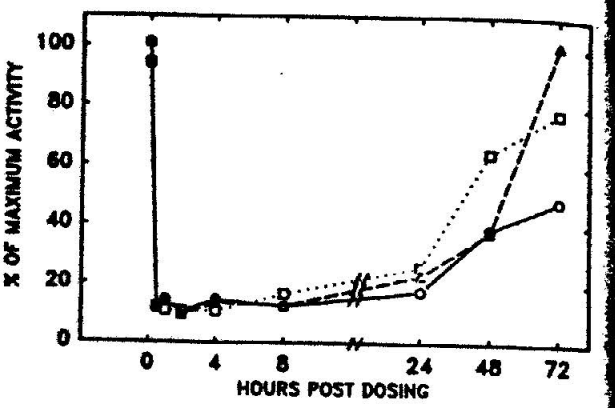
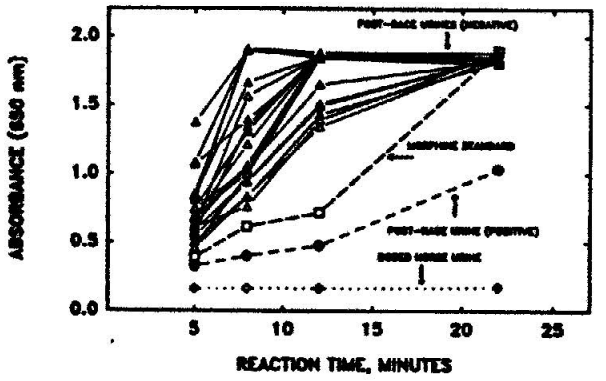


Figure 6. *One step ELISA reactions in a series of post-race urine samples.*



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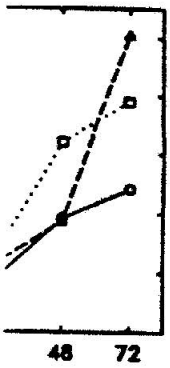

Table I. *Cross reactivity of anti-morphine antibody with other narcotic analgesics in PCFIA.*

Conc (ng/ml)	Drug					
	Morphine	Oxymorphone	Hydromorphone	Levorphanol	Nalorphine	Codeine
0	100	100	100	100	100	100
0.1	69	75	55.0	79	-	-
0.5	34	-	28.0	53	-	-
1.0	28	68	17.0	46	68	69
10.0	14.6	31	6.6	16	-	-

All data points are expressed as a percentage of fluorescence in pre-drug samples, which were arbitrarily assigned a value of 100%.

Table II. *Detection of morphine and its analogs in equine plasma by PCFIA.*

Time Post-Dose	Drug (Dose/Horse)				
	Morphine (10 mg)	Morphine (1 mg)	Oxymorphone (3 mg)	Hydromorphone (8 mg)	Hydromorphone (2 mg)
CONTROL	100	100	100	100	100
15 min.	-	-	41.7	43.6	41.2
30 min.	20.2	36.7	45.0	33.2	33.6
1 hr.	-	-	41.0	28.1	33.3
2 hrs.	14.6	41.8	37.8	32.0	40.0
4 hrs.	-	-	42.0	-	-
6 hrs.	17.2	43.0	-	-	-

All data points are expressed as a percentage of fluorescence in pre-drug samples, which were arbitrarily assigned a value of 100%.

Table III. *Cross-reactivity of morphine ELISA with congeners in NASRC Quality Assurance Program dosed horse urine samples.*

Drug administered	Dose	Route	Time Post-dose	ELISA value
Codeine	30mg	IV	2 hr	0.193
Hydromorphone	10mg	IM	2 hr	0.211
Levorphanol	10mg	IV	2 hr	0.226
Oxymorphone	5mg	IM	2 hr	0.262
Post-Race Urines (X±SEM), n=28				0.562 ± 0.015

The control value is the mean of the values obtained in the simultaneous analysis of post-race urine samples in which no narcotic analgesics were detected.

Table IV. *ELISA screening of post-race urine samples followed by mass spectral analysis.*

Sample Date	# Urine Samples	# Flagged by ELISA	# Positive by GC/MS Analysis	Drug Identified
10-3,4-87	34	5	3	Oxymorphone
10-4-87	16	1	1	Oxymorphone
10-11-87	8	1	1	Oxymorphone
10-17-87	36	3	2	Oxymorphone
10-17,18-87	27	3	1	Oxymorphone
10-20-87	21	4	4	Oxymorphone
10-27-87	24	1	1	Hydromorphone
Totals/ 9 Days Racing	166	18	13	

Discussion

A.K. EUGSTER If I understood you right, you had 5 samples you could not confirm on mass spec. Do you have any idea why they were positive?

MARIE GREEN We did most of the testing for the flagged positives. The samples that were not confirmed, we either did not have enough urine to confirm them or the mass spectral analysis was not of good quality and we did not want to call positives on them. So, I suspect there probably was an opioid in there but it could not be confirmed by GC/MS. It was either low sample amount or poor spectral data for those five samples.

LEE STURMA So our confirmation rate is probably greater than the 72% indicated.

MG Yes, for the ELISA it is. Of course, we have the problem confirming it on GC/MS.

AE Is there a possibility, as a follow-up on my question, that it could be a cross-reaction with endogenous compounds?

MG There is that possibility.

RANDY LEAVITT Is this the ELISA kit available from IDS?

LS It is, indeed.

RL We have had the opportunity to play around with this kit a little bit and it is rather impressive in terms of the cross-reactivity data. In fact, we were able to take an extract of buprenorphine and get a positive test using this kit. However, you should maybe point out that nor-codeine and nor-morphine will not cross-react with this particular antibody.

BOB SMITH Two months ago there was a very interesting paper published in the journal *Nature*. It recorded the first time the identification of both morphine and codeine as natural components of the vertebrate brain. It also showed all the enzymes responsible for synthesizing the morphine molecule are endogenous. My question is, do you see any evidence of the natural levels of these two substances as impacting upon your assay approach?

LS The endogenous levels of morphine and codeine in the horse, that is an interesting question, and I would like to refer to someone wiser in neurochemistry than I. Is there someone in the audience that would like to respond?

C.L. Chen Your question is from the brain is a peptide, a morphine-like substance. There are many kinds that have same receptor for morphine receptors.

BS I am talking distinctively about morphine and

codeine and not the other peptides with morphine or codeine-like activity. The paper showed the natural presence of these materials together with a number of their precursors, the enzyme responsible for their formation. In other words, the genetic operators you find in plants that dissemble these hormones are also naturally present in mammals.

TOM TOBIN That information comes as a surprise, but not a complete surprise because Dr. Pedro Cueatracasas has shown that in bovine milk and also in human milk, small levels of morphine are found. They attributed that to morphine in hay plant origin. The work that Dr. Smith reports; has this been repeated?

BS I am not aware that it has been repeated but to have been received in the august columns of *Nature*, I think it is a fair indication of reliability of the work. It has been extensively reviewed by the authorities in the field and it seems to me to be fairly authoritative.

TT It becomes apparent that levels of morphine that will occur in herbivores from plant origin and presumably also from mammalian origin, will be natural constituents in urine. It is quite clear that the rules of racing in most of North America will have to be rewritten on this basis because they tend to start at the phrase "foreign to the natural horse." From what Dr. Smith says, the opiates are now natural to the natural horse. We will all have to put this in our pipes and smoke it.

Footnotes

[a]Alltech-Applied Science, State College, PA.
[b]Lilly Company, Indianapolis, IN.
[c]Spin-X™, Costar®, Dynatech, Chantilly VA.
[d]Immulon Removawells®, Dynatech, Chantilly, VA.
[e]TMB Microwell Peroxidase Substrate, Kirkegaard and Perry, Gaithersburg, MD.
[f]International Diagnostic Systems, St. Joseph, MI.
[g]Anti-morphine antibody and reagents supplied by International Diagnostics Systems, St. Joseph, MI Drug standards supplied by the Illinois Racing Board Laboratory, Elgin, IL.
[h]Pandex FCA 15-010-1, Pandex, Mundelein, IL.
[i]Hewlett-Packard GC Model 5890, MS-5970 detector, Hewlett-Packard, Palo Alto, CA.
[j]HP-1, Hewlett-Packard, Palo Alto, CA.
[k]Sigma Chemicals, St. Louis, MO.
[l]E Merck, F-254 Silica gel-60 plates, Alltech Applied Science, Deerfield, IL.