APPENDIX XII

ELISA TESTING: BACKGROUNDS IN EQUINE URINE, TEST SENSITIVITY, SAMPLE POOLING, AND SIGNIFICANCE OF NUMBER OF TESTS

by

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SUMMARY

1) All equine urines show significant natural "backgrounds" in ELISA tests.

2) Individual antibodies vary in their affinity for these unidentified "background" materials.

3) The simplest answer to the "background" problem is to develop high affinity antibodies.

4) We have developed a series of high affinity, low background ELISA tests for drugs abused in racing horses.

5) The sensitivity and specificity of many of these tests is sufficient to allow sample pooling.

6) These ELISA tests are calibrated for maximum sensitivity to maximize the forensic usefulness of these tests.

INTRODUCTION

The principal problem in ELISA testing is the existence in horse urine of materials that give rise to substantial backgrounds of apparent drug in most equine urines. The nature of these materials is unknown and their amounts appear to vary from between different urine samples. Additionally, it appears that each anti-drug antibody has its own inherent sensitivity or resistance to these materials, with some antibodies being highly resistant and some extremely sensitive. Preliminary experiments also suggest that this interfering action is much less marked in human urine samples.

In our hands the most satisfactory approach to this problem has been to develop very high affinity antibodies for drugs. This is because high affinity antibodies have high affinity binding sites for drugs, and these binding sites have correspondingly reduced affinities for the unknown interfering materials. In this way the effect of these interfering materials is reduced, and the forensic usefulness of the test is increased.

Based on our experience with the synthesis of over 100 drug haptens and the raising of antibodies to these haptens we have formulated rules of hapten synthesis to give rise to high affinity antibodies. We now present the results of these endeavors for a series of high affinity antibodies and demonstrate the relationship between high affinity, low background and high forensic utility of a panel of ELISA tests for drugs abused in racing horses.(1)

MATERIALS AND METHODS

Horses

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

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

Dosing and Sampling

Authentic drug standards were obtained from appropriate suppliers. All injections were by rapid IV injection into the jugular vein. All urine samples were collected by bladder catheterization, and were stored frozen until assayed.

One Step ELISA Test

The one step ELISA tests were performed as described by Voller et al. (1976) and Yang et al. (1987). Briefly, anti-drug antibody was linked to flat bottom microtiter plates (CoStar) as described by Voller. Similarly, drug hapten was linked to horse radish peroxidase (HRP), as described by Wie et al., (1982), to give rise to a covalently linked drug-HRP complex. 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 180 ul of the drug-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 (tetramethylbenzamine, Kirkegaard and Perry, Gaithersburg, MD) was then added to all wells and their absorbance read at 560 nm on a microwell reader at 0, 5, 10, 15, and 60 minutes after addition of substrate.

To determine natural backgrounds in horse urine 20 ul each of post race urine was analyzed and percentage inhibition of the ELISA test determined for each sample. The fractional inhibitions were then calculated as apparent drug concentration and expressed as a frequency distribution. The highest apparent drug level observed in about 40 urine samples was taken as the peak background level likely to be observed in a day's testing.

RESULTS AND DISCUSSION

Figs. 1, 2 and 3 show the apparent affinity of acepromazine for the anti-acepromazine (generic promazine) antibody, the apparent backgrounds seen in track urines, (Fig. 2) and the duration of detection of acepromazine after a 5 mg dose of this drug (Fig. 3). The data show that the test is highly sensitive to acepromazine, with an I-50 of about 30 picrograms/ml and similarly low backgrounds in equine urine. This ELISA test for acepromazine is therefore an exceptionally sensitive test for this drug in post race urines.

Generic promazine ELISA

Fig. 1 shows the sensitivity of the generic promazine ELISA to the indicated promazine derivatives. Fig. 2 shows the "apparent drug" natural backgrounds in equine urine, while Fig. 3 shows the apparent concentrations of acepromazine in equine urine after administration of 5 mg of acepromazine to a horse.

Similarly, our Etorphine ELISA is highly sensitive to etorphine, (Fig. 4) with an I-50 of about 250 pg/ml for etorphine. The backgrounds in this ELISA are similarly low (Fig. 5) with a mean value of about 250 pg/ml. This test readily detects etorphine or its metabolites in horse urine, even though these samples had been stored for a very long period (Fig. 6). Again, this ELISA test is highly sensitive and specific, with the I-50 for the drug approximately equivalent to or less than the apparent background values.

Etorphine ELISA

Fig. 4 shows the cross reactivity of the etorphine ELISA with the indicated drugs, Fig. 5 shows the apparent etorphine equivalents in 40 post race urines samples while Fig. 6 shows the ability of this ELISA test to detect etorphine in urine from dosed horses.

Fig. 7 and 8 show similar results obtained with our Butorphanol ELISA> This test is very sensitive to Butorphanol having an I-50 for Butorphanol of about 250 pg/ml. The apparent backgrounds in equine urine are also low, showing apparent butorphanol levels of about 400 pg/ml. Although these apparent background levels are somewhat higher than the I-50 for butorphanol, this test has performed well in the field, and yielded several positives.

Fig. 7 shows the cross reactivity of the Butorphanol ELISA with the indicated drugs, while Fig. 8 shows the apparent Butorphanol activity in 40 post race urine samples.

Similarly, our assays for alfentanil, sufentanil and buprenorphine are very sensitive, with I-50's for parent drug of between 100 and 150 picograms/ml, and similarly low backgrounds in equine urine. These four tests are therefore particularly useful in post race equine drug testing.

Table 1
High Potency Low Background ELISA Tests

Drug	I-50	Background	Conc.to Detect.	Maneuver
Promazine	0.03 ng/ml	0.04 ng/ml	4 ng/ml	Pool
Alfentanil	0.11 ng/ml	0.10 ng/ml	4 ng/ml	Pool
Sufentanil	0.15 ng/ml	0.15 ng/ml	4 ng/ml	Pool
Buprenorphine	0.15 ng/ml	0.15 ng/ml	4 ng/ml	Pool
Butorphanol	0.25 ng/ml	0.40 ng/ml	4 ng/ml	Pool
Etorphine	0.25 ng/ml	0.25 ng/ml	1.0 ng/ml	--

This table compares the I-50 values for parent drug, peak background levels seen in about 40 urine samples, minimum concentration to detect/confirm and the ability to use pooled samples.

These data are summarized in Table 1 which shows the relationship between I-50 for parent drug, apparent backgrounds in equine urine and the ability to pool samples. If the I-50 for the test in question is substantially below the threshold concentration of interest for that particular drug, then it is possible to pool samples and very rapidly and economically screen for the presence or absence of drug. For all of these assays with the exception of etorphine it is likely that sample pooling will be an effective strategy since the sensitivity of these tests is much greater than our current ability to confirm these drugs. The only drug for which an exception might be made is etorphine, which because of its great potency is likely to be pharmacologically active at much lower concentrations than the other agents listed and for which pooling of samples is not recommended.

Another approach to the background problem is to screen for the presence of the drug in blood. In response to a specific request we developed an ELISA test for fluphenazine with the goal of detecting abuse of this agent in horses. An antibody to fluphenazine was raised and incorporated into an ELISA test that reacted well with fluphenazine but showed relatively high backgrounds in equine urine. An additional problem was that although this test readily detected parent fluphenazine it did not appear to detect significant amounts of either parent drug or fluphenazine it did not appear to detect significant amounts of either parent drug or fluphenazine metabolites in equine urine. We therefore evaluated the ability of this test to detect parent fluphenazine in equine blood after administration of fluphenazine decanoate, a long acting form of this drug.

As shown in Fig. 11 this ELISA test readily detected the presence of fluphenazine decanoate in equine blood after administration of 12.5 mg of this drug intramuscularly. Blood levels of the drug increased slowly to reach apparent steady state levels in equine blood by 3 days after dosing. The data suggest that when combined with a simple extraction method this test will detect the presence of low levels of fluphenazine in equine blood. These data are in good agreement with data from another laboratory which reports detection of low levels of fluphenazine in the blood of horses for up to two or more weeks after administration of IM doses of this drug.

Fluphenazine ELISA

Fig. 9 shows the cross reactivity of the fluphenazine ELISA with the indicated drugs. Fig. 10 shows the apparent backgrounds in equine urine and Fig. 11 shows the ability of this ELISA to detect low levels of fluphenazine in equine blood when used in combination with a simple extraction step.

A critical factor concerning the effectiveness of these ELISA tests in field trials is stability of these tests. Problems with stability of antibody plates and more particularly the stability of drug-enzyme conjugates can substantially limit the useful life of these plates and therefore their usefulness in the field. As shown in Figs. 12 and 13 the ELISA plates and conjugates used in these tests are stable for at least three months at refrigerator temperatures suggesting that they are sufficiently stable for shipment and storage under most testing conditions.

Figs. 12 and 13 - Shelf Life of WTT ELISA plates and WTT conjugates stored at 4°C.

A final question concerns the number of replicate tests that should be performed before the running of a given test is abandoned, and conversely, the kind of statements that can be made concerning drug use based on different numbers of consecutively negative tests. As developed in more detail elsewhere, the running of a small number of tests (300 to 700) allows one to state that the incidence of drug use is not greater than 1.0% but cannot rule out rates of drug use of less than 1.0%. Similarly, if one tests between 2,000 and 6,000 samples one can say that the rate of drug use is not more than 0.1% but do not rule out rates of drug use less than 0.1%. Finally, if one wishes to rule out sporadic drug use, i.e. rates of drug use of less than one in ten thousand samples one must test between one half and all of the samples submitted, depending on the level of confidence one wishes to place in the test result.

LITERATURE CITED

1. Tobin, T. (1981) Drugs and the Performance Horse, Charles C. Thomas, Publisher, Springfield, IL. pp 363-365.

2. Voller, A., Bidwell, D.W., Bartlett, A. (1976) Bull. Wld. Hlth. Org., 53, 55-56.

3. Yang, J-M, Tai, C.L., Weckman, T.J., Woods, W.E., 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., (1987) Res. Comm. Chem. Pathol. Pharmacol., in press.

4. Wie, S.I., Hammock, B.D. (1982) Anal. Biochem., 125, 168-176.

Fig. 1

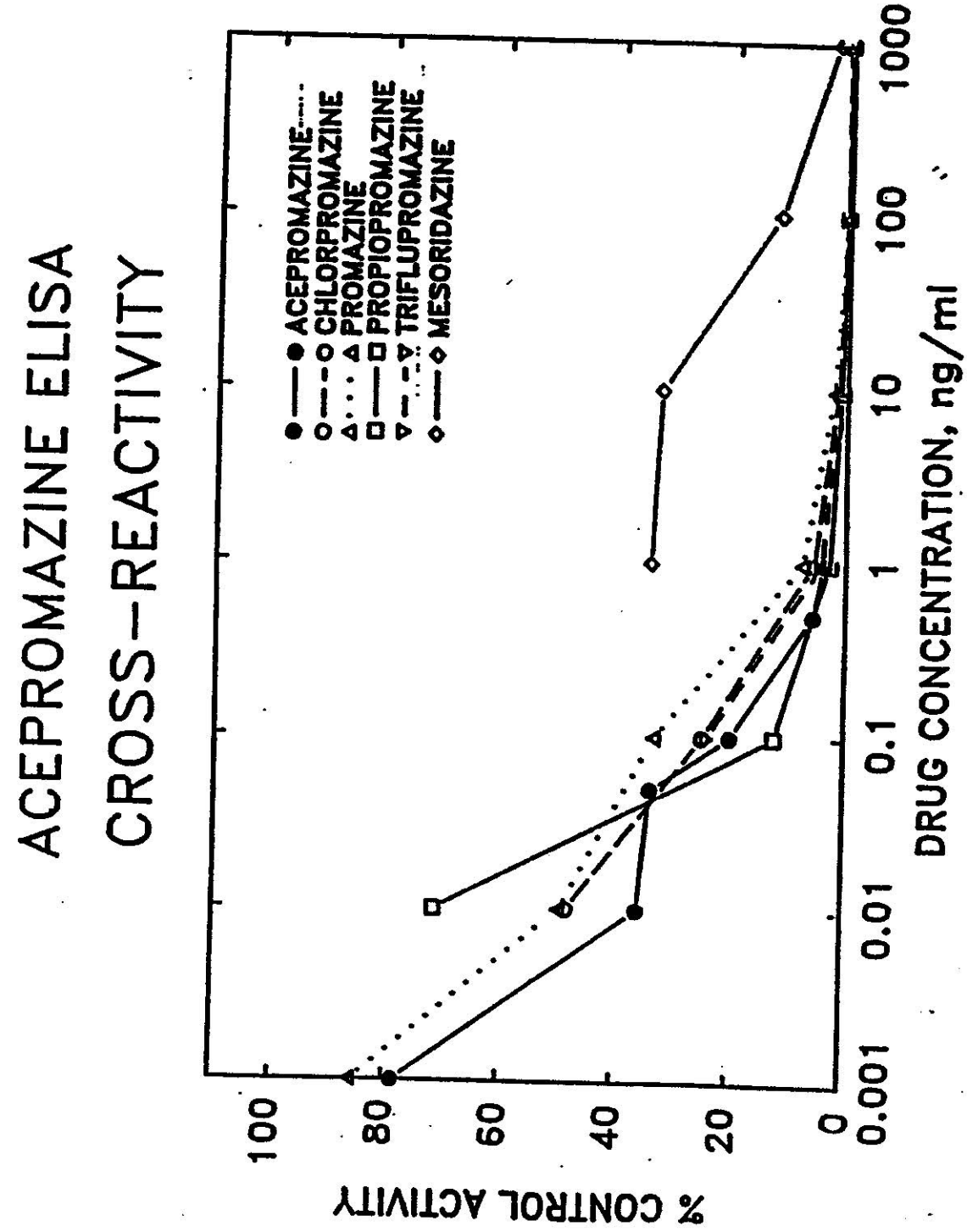

ACEPROMAZINE ELISA
CROSS-REACTIVITY
ACEPROMAZINE
CHLORPROMAZINE
PROMAZINE
PROPIOPROMAZINE
TRIFLUPROMAZINE
MESORIDAZINE
% CONTROL ACTIVITY
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
DRUG CONCENTRATION, ng/ml

Fig. 2

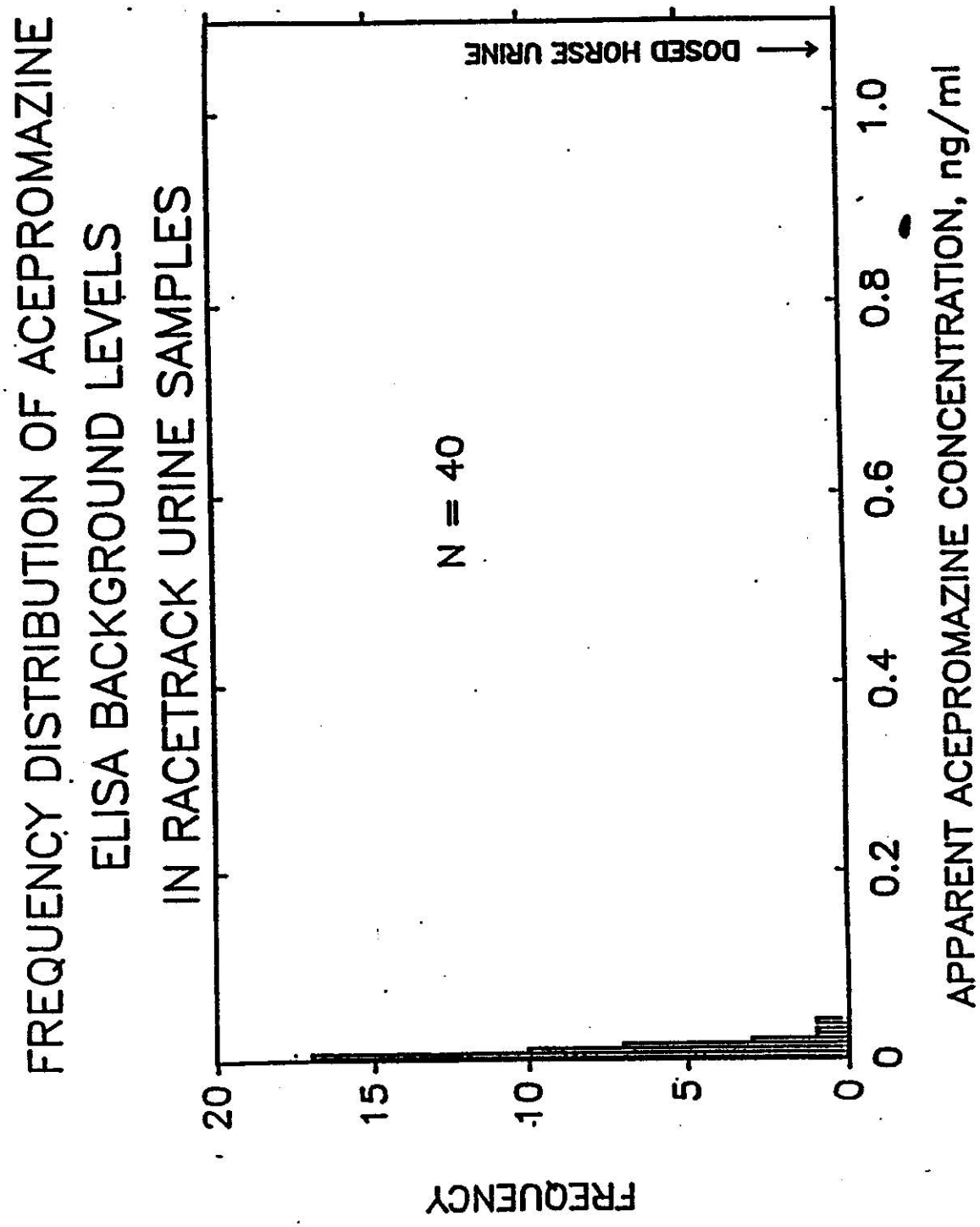

FREQUENCY DISTRIBUTION OF ACEPROMAZINE
ELISA BACKGROUND LEVELS
IN RACETRACK URINE SAMPLES
N = 40
DOSED HORSE URINE
FREQUENCY
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1.0
APPARENT ACEPROMAZINE CONCENTRATION, ng/ml

Fig. 3

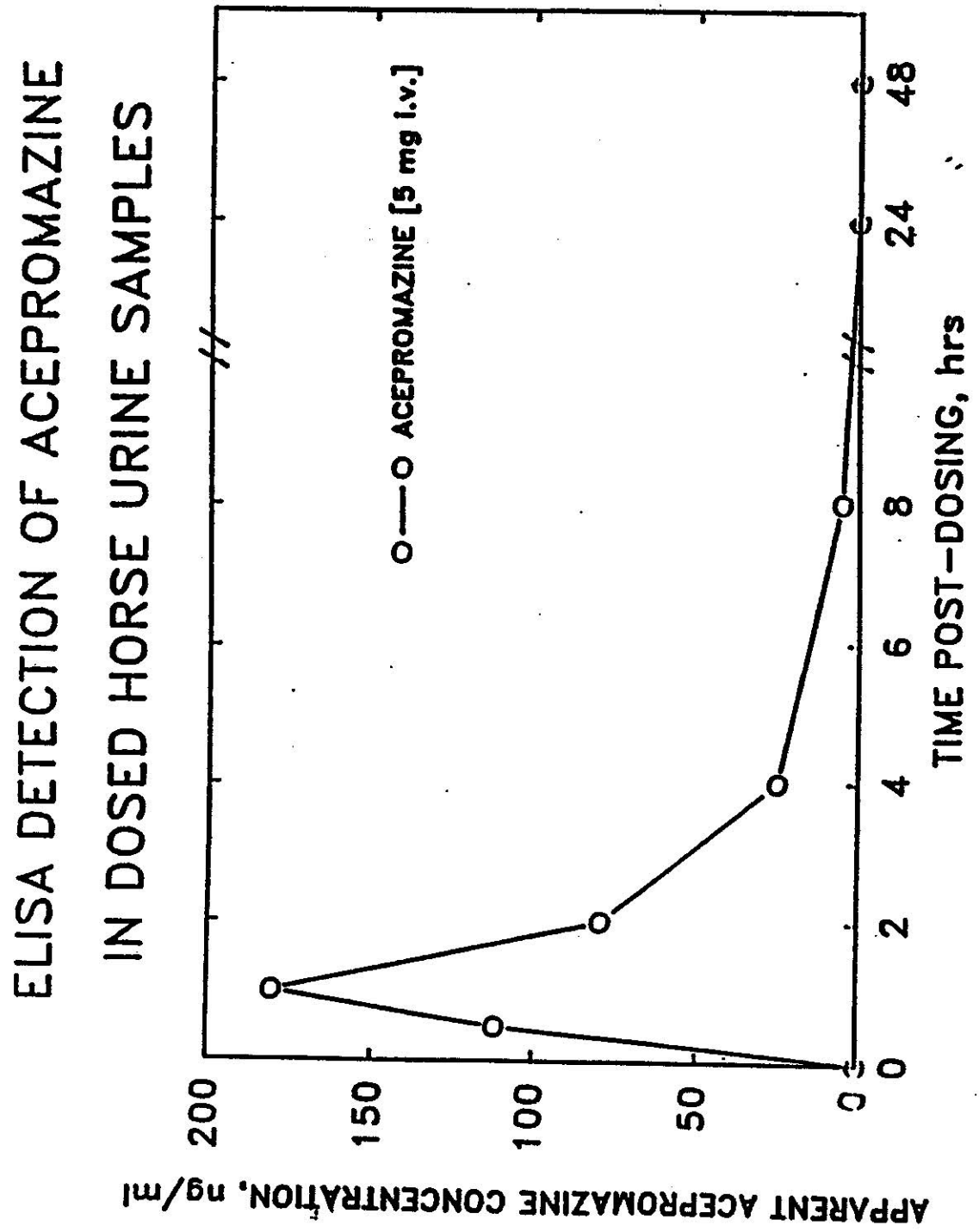

ELISA DETECTION OF ACEPROMAZINE
IN DOSED HORSE URINE SAMPLES
O—O ACEPROMAZINE [5 mg I.V.]
APPARENT ACEPROMAZINE CONCENTRATION, ng/ml
200
150
100
50
0
0
2
4
6
8
24
48
TIME POST-DOSING, hrs

Fig. 4

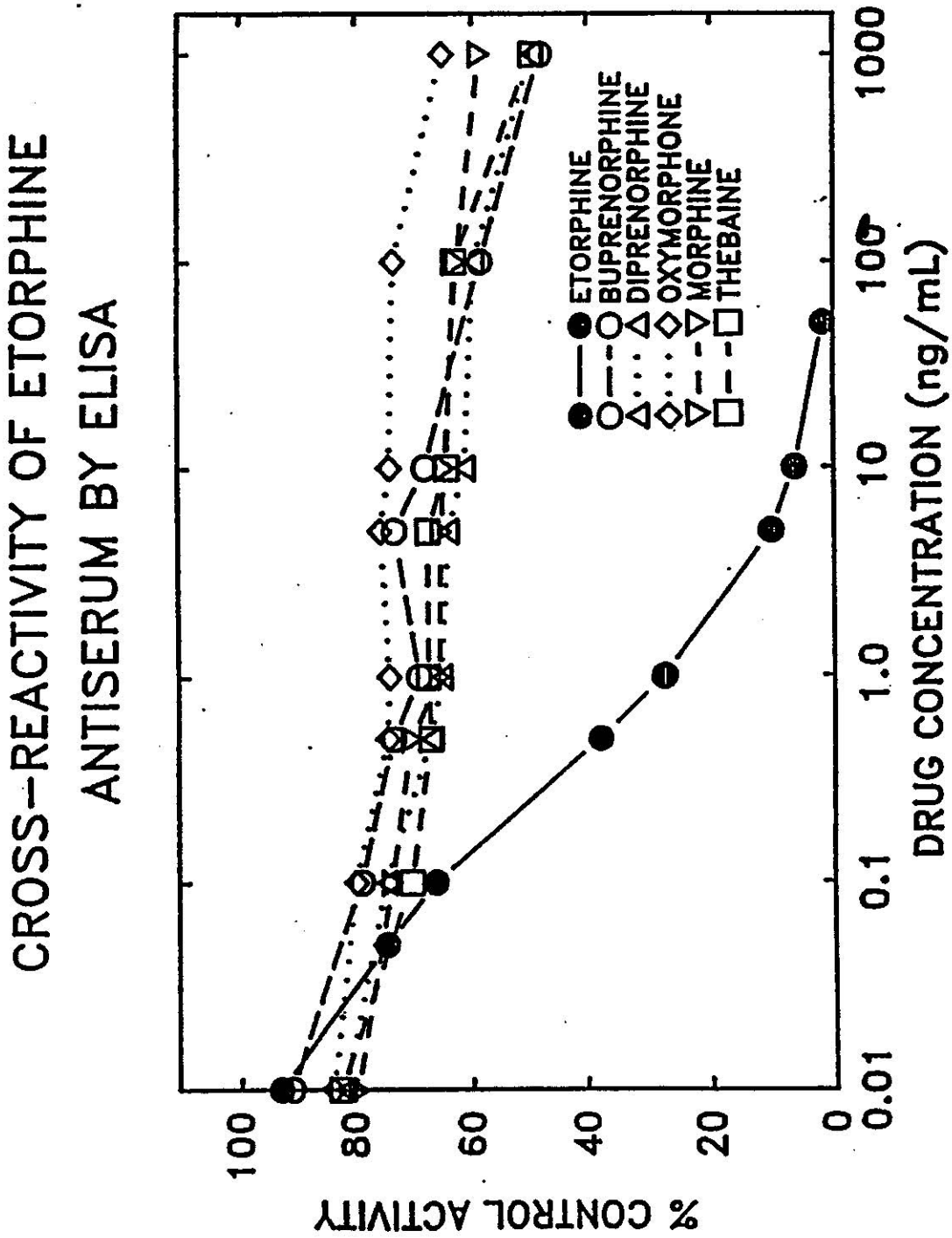

CROSS-REACTIVITY OF ETORPHINE
ANTISERUM BY ELISA
% CONTROL ACTIVITY
100
80
60
40
20
0
0.01
0.1
1.0
10
100
1000
DRUG CONCENTRATION (ng/mL)
ETORPHINE
BUPRENORPHINE
DIPRENORPHINE
OXYMORPHONE
MORPHINE
THEBAINE

Fig. 5

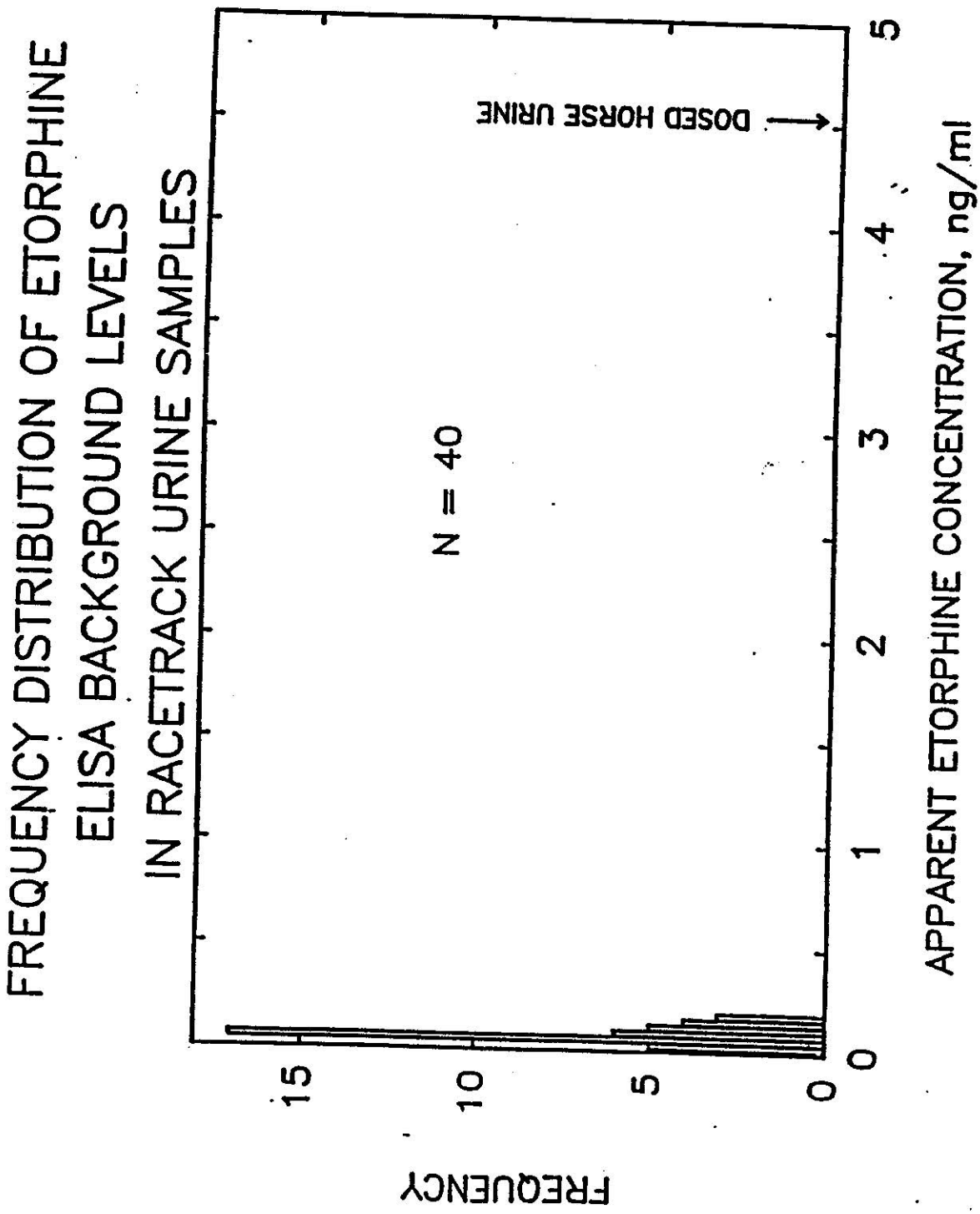

FREQUENCY DISTRIBUTION OF ETORPHINE
ELISA BACKGROUND LEVELS
IN RACETRACK URINE SAMPLES
N = 40
DOSED HORSE URINE
FREQUENCY
0
5
10
15
0
1
2
3
4
5
APPARENT ETORPHINE CONCENTRATION, ng/ml

Fig. 6

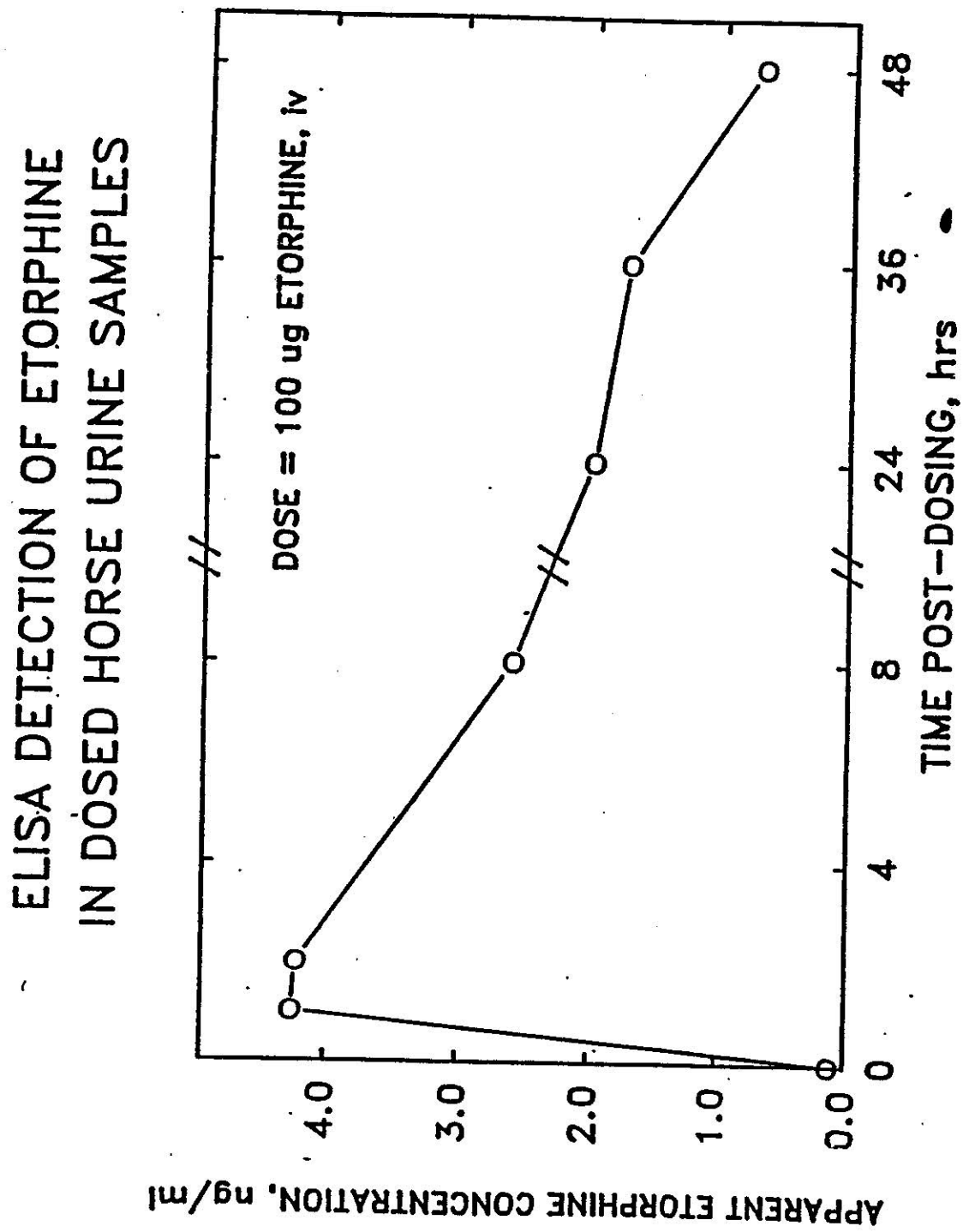

ELISA DETECTION OF ETORPHINE
IN DOSED HORSE URINE SAMPLES
DOSE = 100 ug ETORPHINE, iv
APPARENT ETORPHINE CONCENTRATION, ng/ml
0.0
1.0
2.0
3.0
4.0
0
4
8
24
36
48
TIME POST-DOSING, hrs

Fig. 7

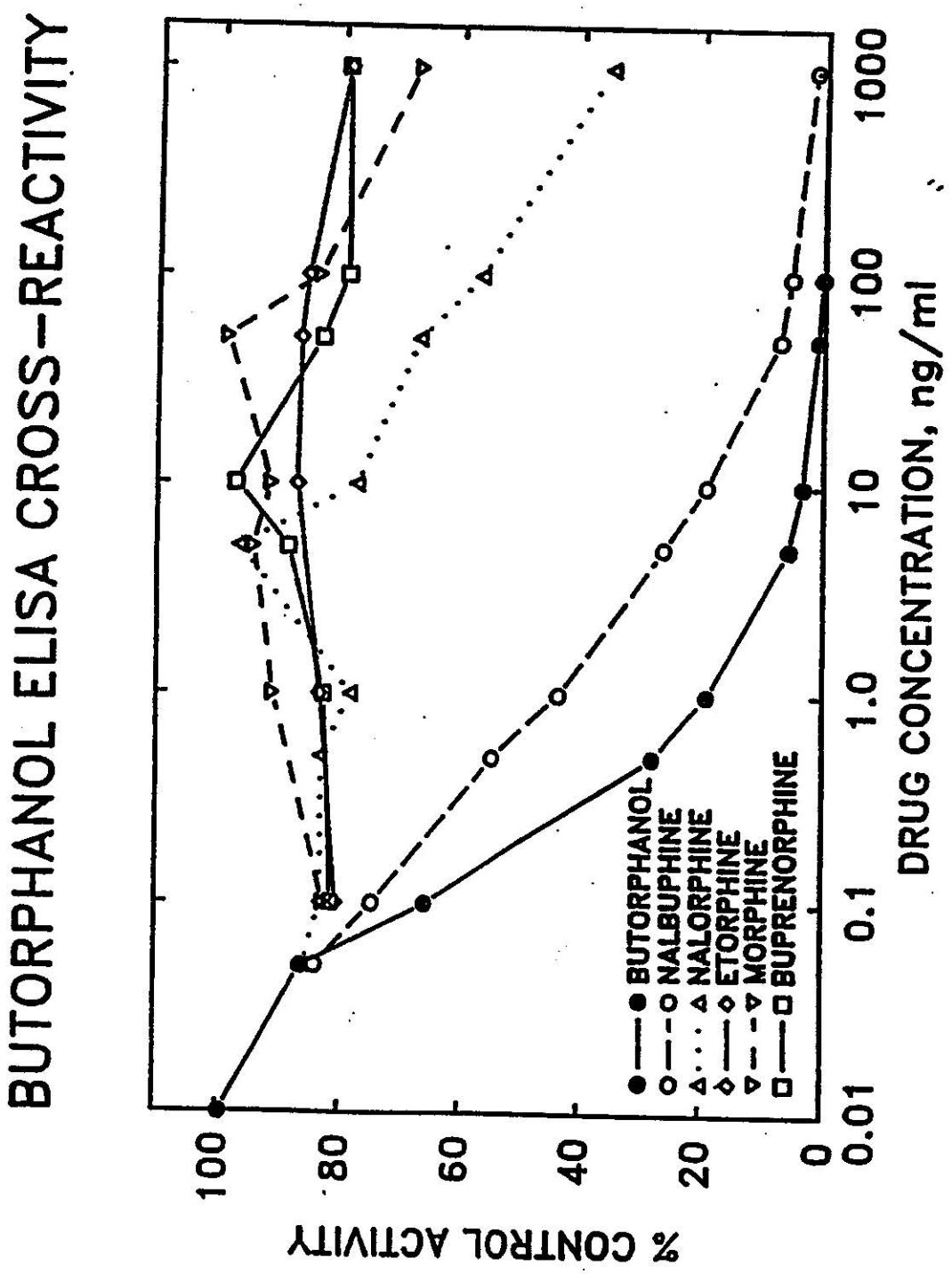

BUTORPHANOL ELISA CROSS-REACTIVITY
% CONTROL ACTIVITY
100
80
60
40
20
0
0.01
0.1
1.0
10
100
1000
DRUG CONCENTRATION, ng/ml
BUTORPHANOL
NALBUPHINE
NALORPHINE
ETORPHINE
MORPHINE
BUPRENORPHINE

Fig. 8

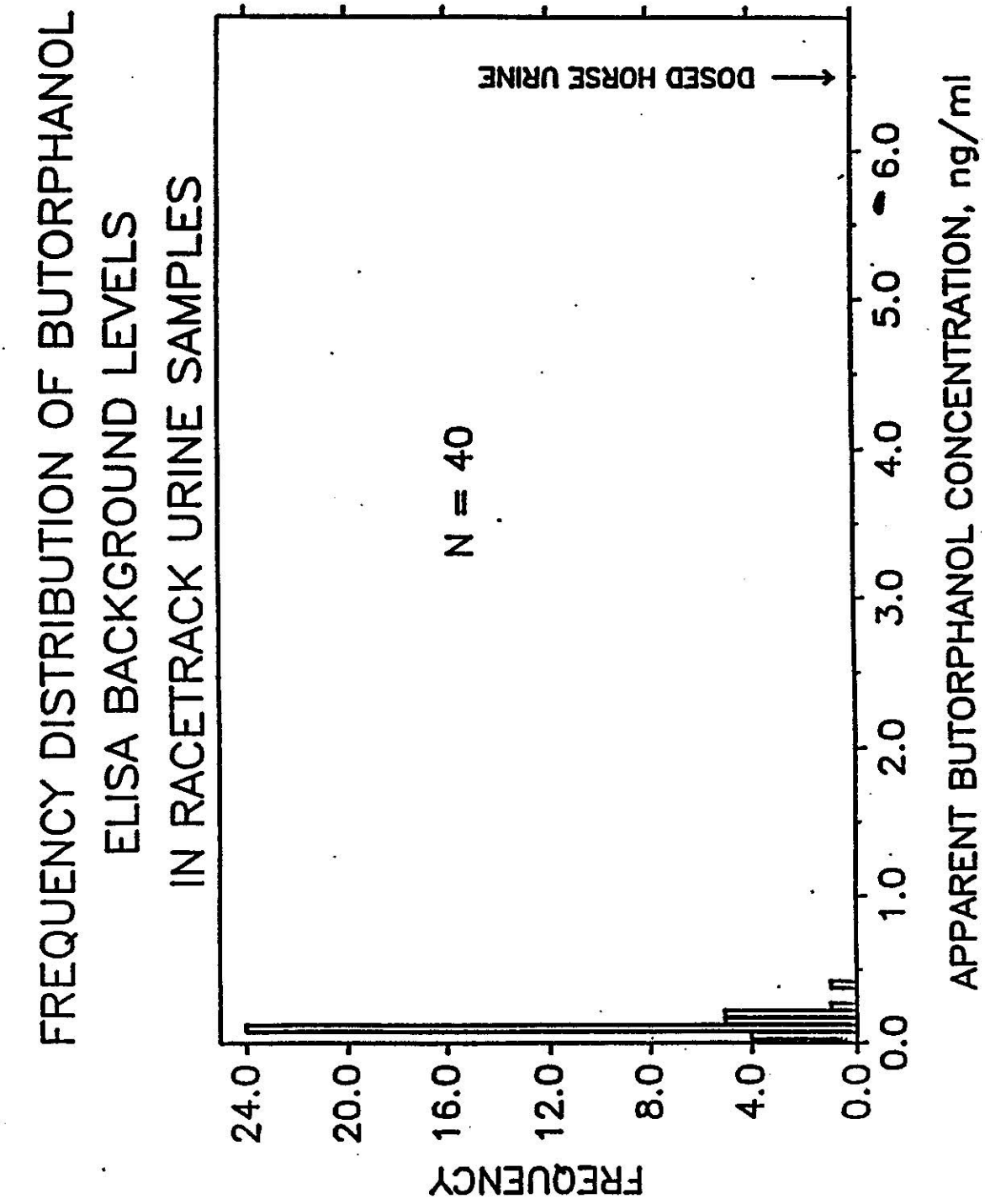

FREQUENCY DISTRIBUTION OF BUTORPHANOL
ELISA BACKGROUND LEVELS
IN RACETRACK URINE SAMPLES
DOSED HORSE URINE
N = 40
FREQUENCY
24.0
20.0
16.0
12.0
8.0
4.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
APPARENT BUTORPHANOL CONCENTRATION, ng/ml

Fig. 9

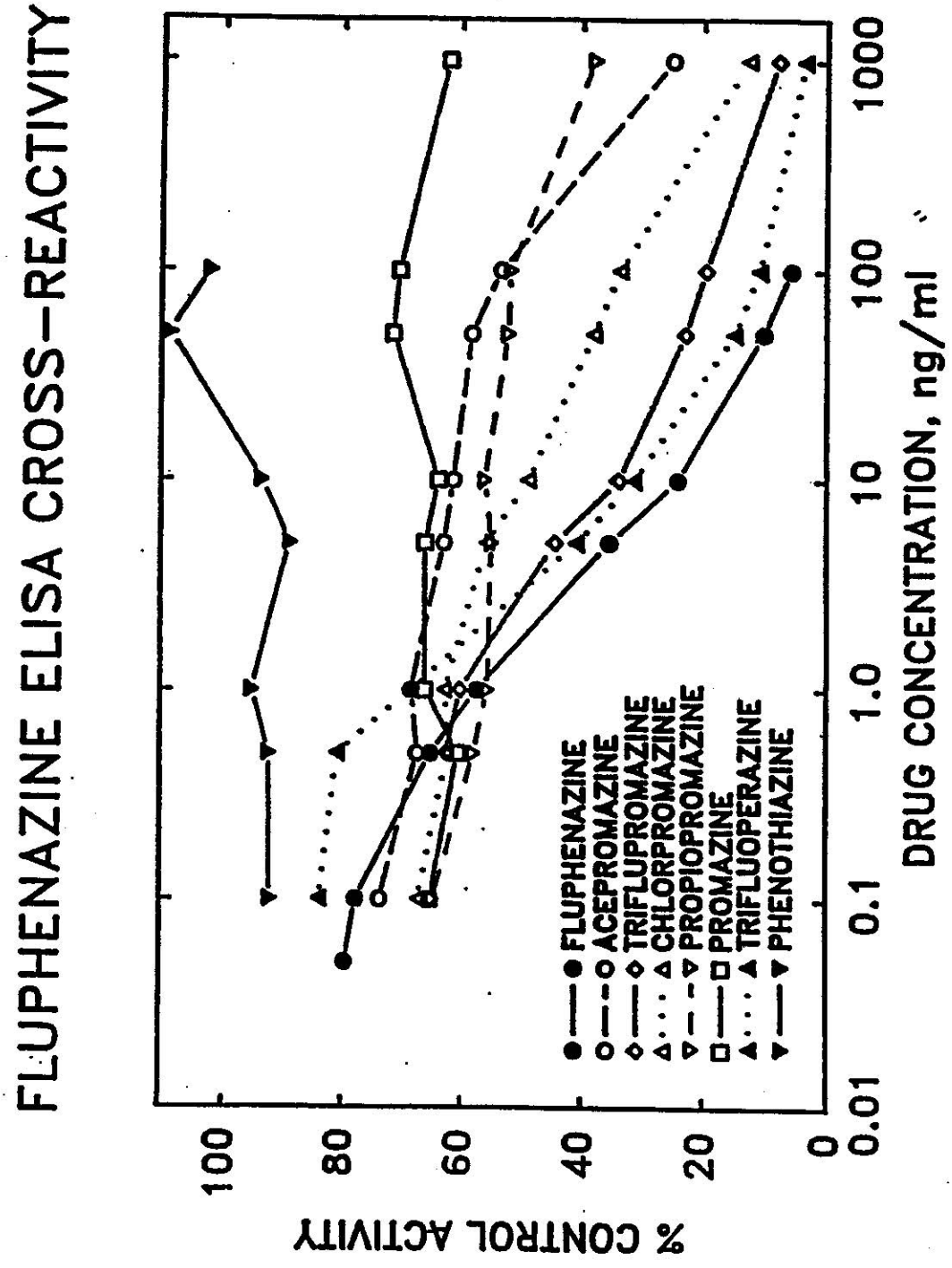

FLUPHENAZINE ELISA CROSS-REACTIVITY
% CONTROL ACTIVITY
100
80
60
40
20
0
0.01
0.1
1.0
10
100
1000
DRUG CONCENTRATION, ng/ml
FLUPHENAZINE
ACEPROMAZINE
TRIFLUPROMAZINE
CHLORPROMAZINE
PROPIOPROMAZINE
PROMAZINE
TRIFLUOPERAZINE
PHENOTHIAZINE

Fig. 10

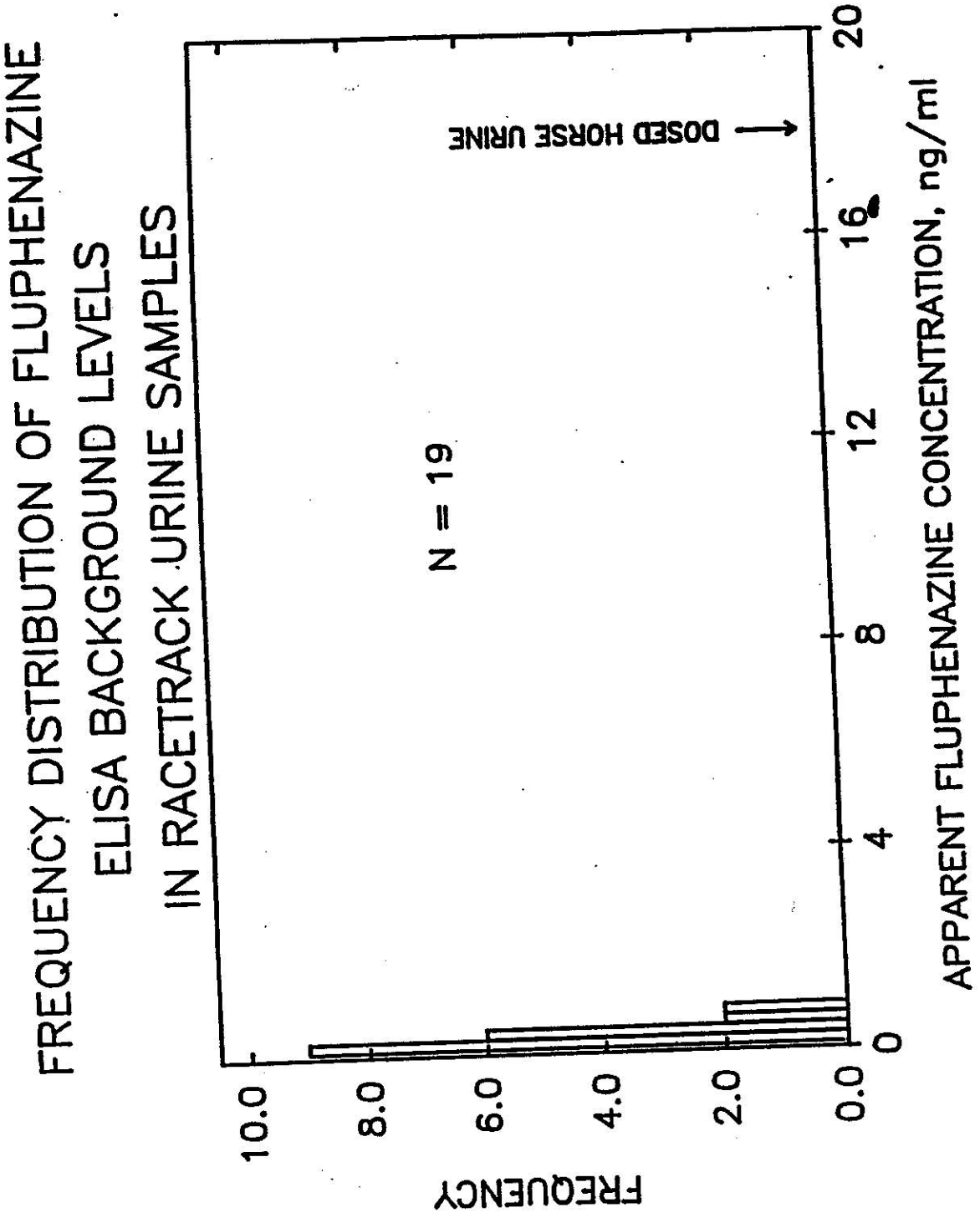

FREQUENCY DISTRIBUTION OF FLUPHENAZINE
ELISA BACKGROUND LEVELS
IN RACETRACK URINE SAMPLES
N = 19
DOSED HORSE URINE
FREQUENCY
10.0
8.0
6.0
4.0
2.0
0.0
0
4
8
12
16
20
APPARENT FLUPHENAZINE CONCENTRATION, ng/ml

Fig. 11

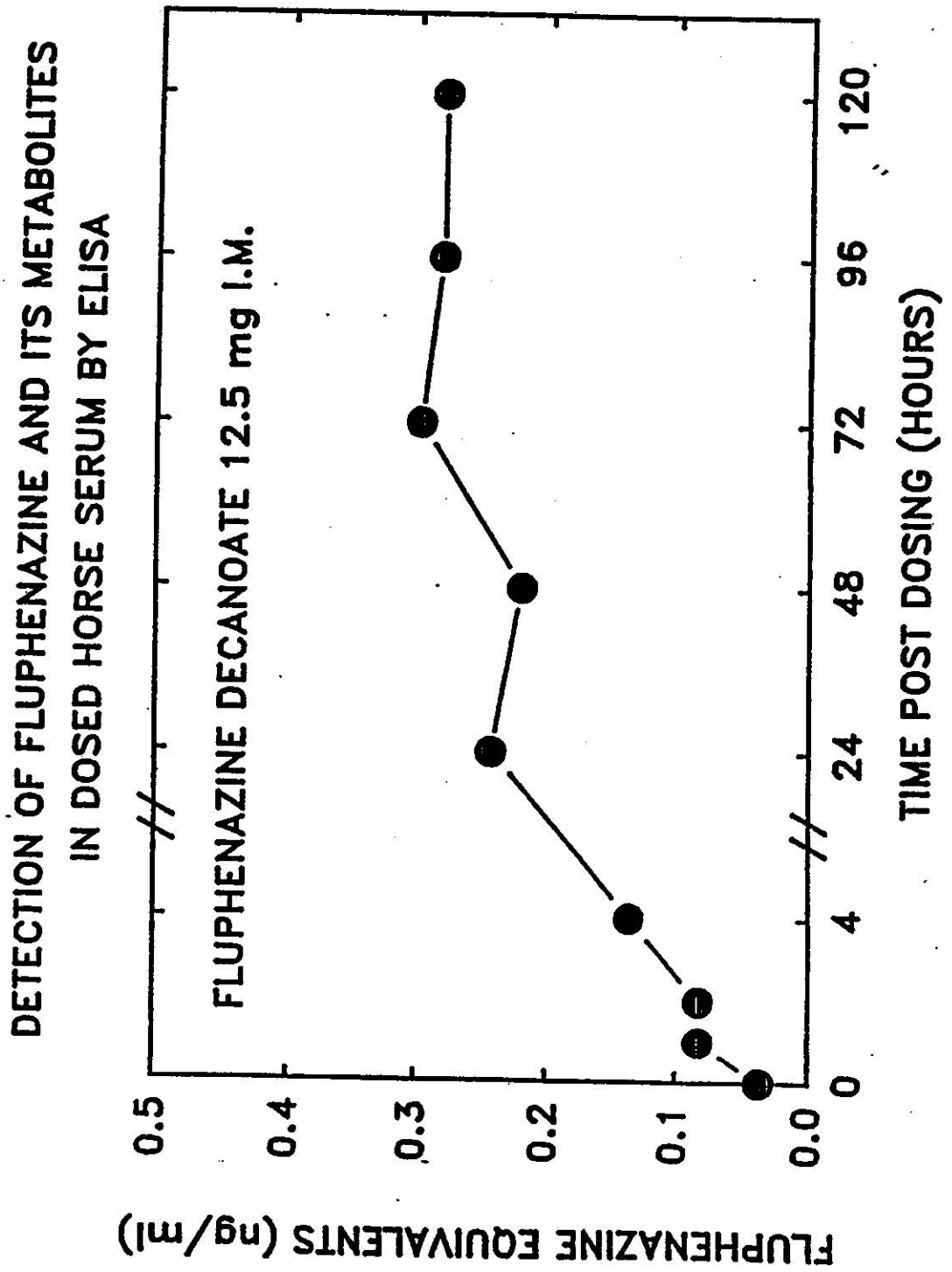

DETECTION OF FLUPHENAZINE AND ITS METABOLITES
IN DOSED HORSE SERUM BY ELISA
FLUPHENAZINE DECANOATE 12.5 mg I.M.
FLUPHENAZINE EQUIVALENTS (ng/ml)
0.0
0.1
0.2
0.3
0.4
0.5
0
4
24
48
72
96
120
TIME POST DOSING (HOURS)

Fig. 12

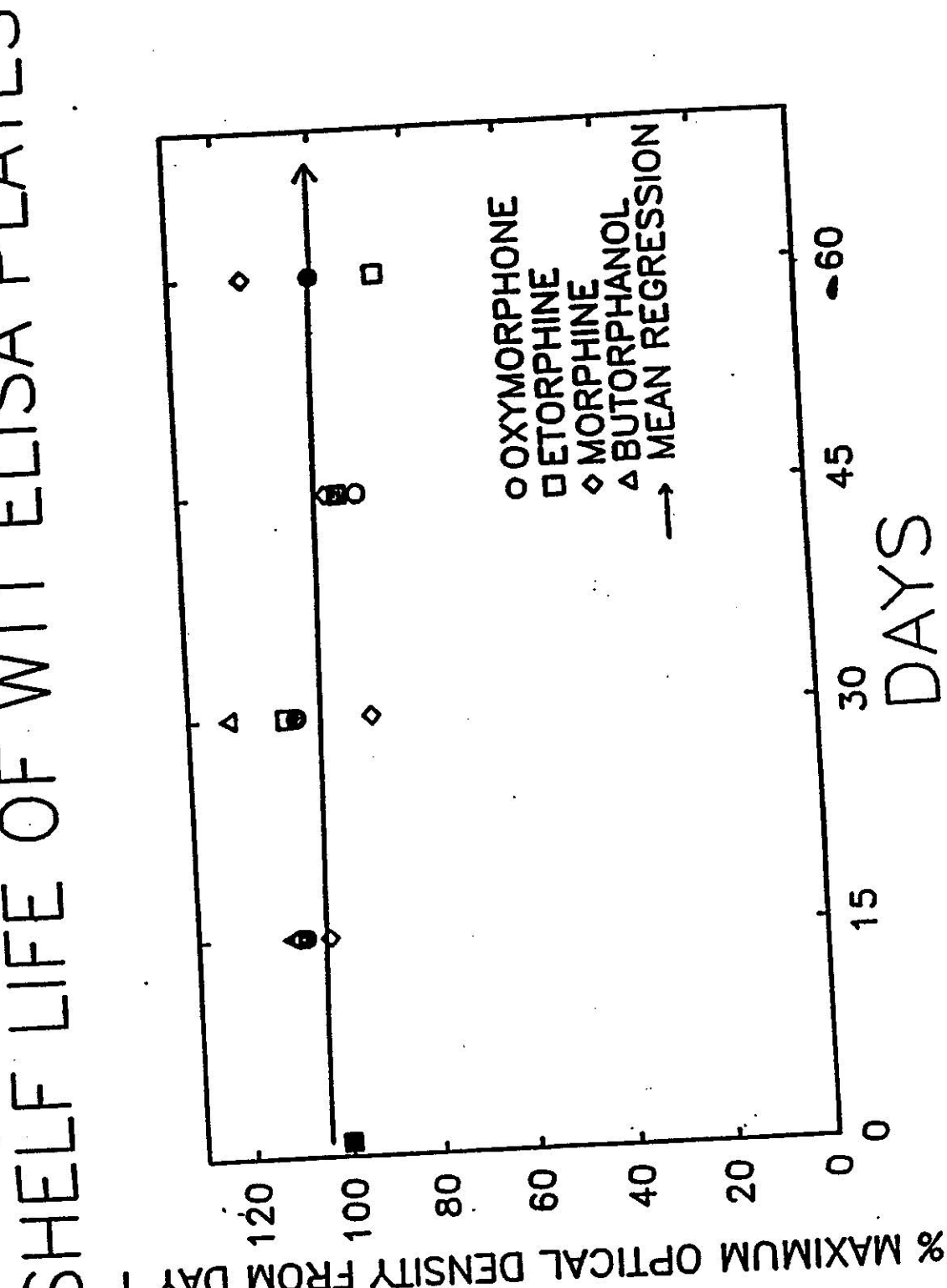

SHELF LIFE OF WTT ELISA PLATES
% MAXIMUM OPTICAL DENSITY FROM DAY 1
120
100
80
60
40
20
0
0
15
30
45
60
DAYS
OXYMORPHONE
ETORPHINE
MORPHINE
BUTORPHANOL
MEAN REGRESSION

Fig. 13

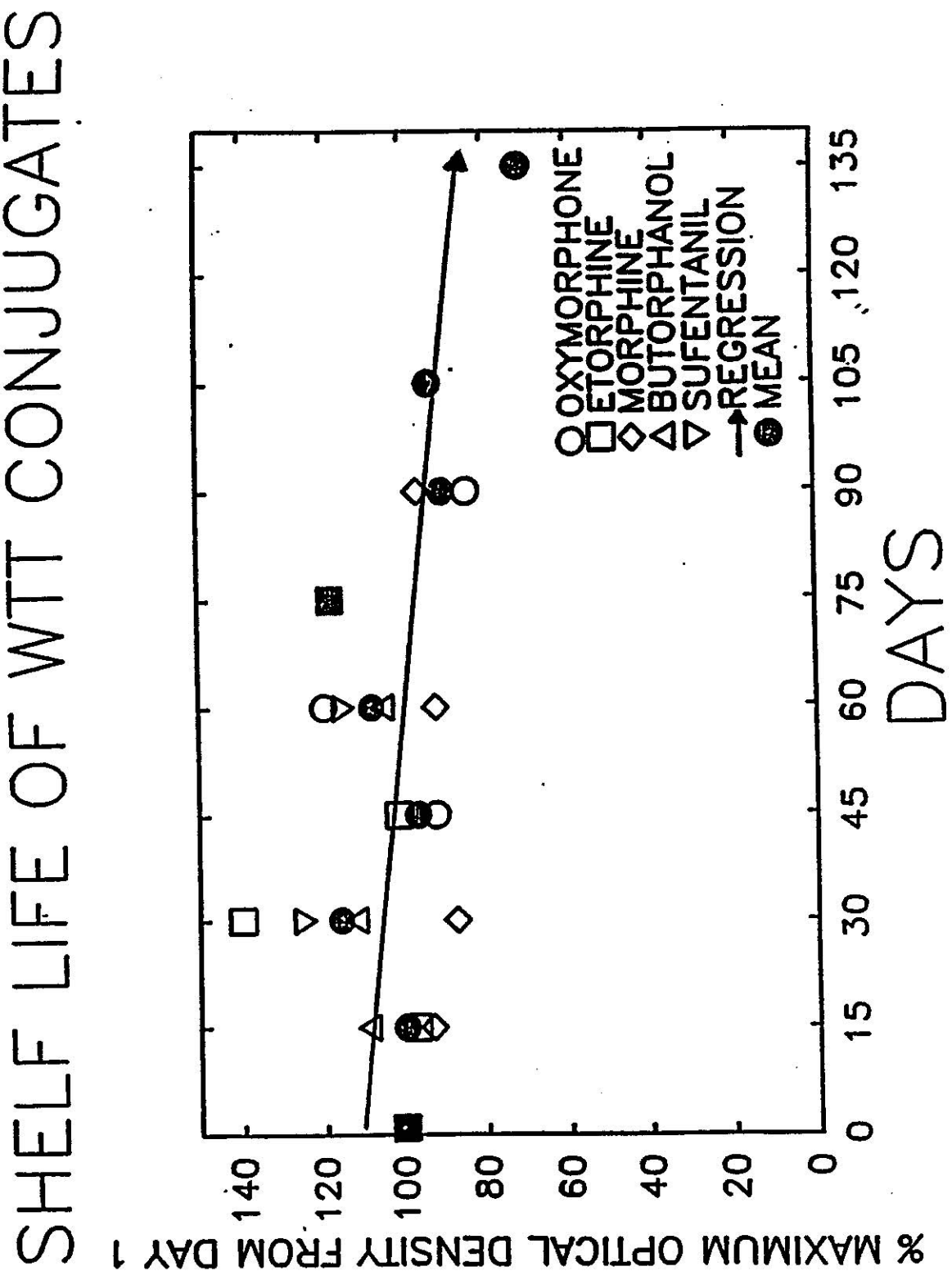

SHELF LIFE OF WTT CONJUGATES
% MAXIMUM OPTICAL DENSITY FROM DAY 1
0
20
40
60
80
100
120
140
0
15
30
45
60
75
90
105
120
135
DAYS
OXYMORPHONE
ETORPHINE
MORPHINE
BUTORPHANOL
SUFENTANIL
REGRESSION
MEAN

Fig. 10

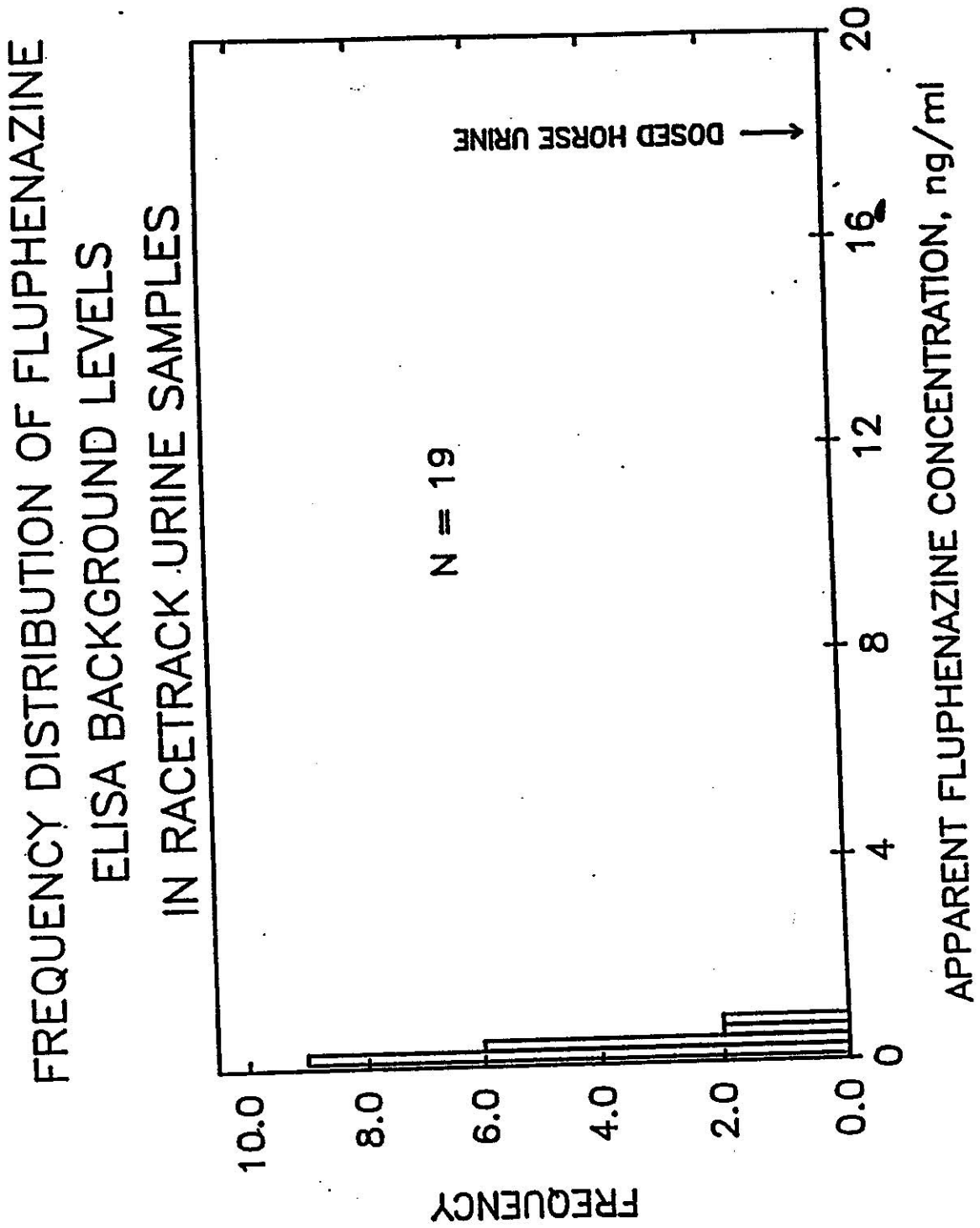

FREQUENCY DISTRIBUTION OF FLUPHENAZINE
ELISA BACKGROUND LEVELS
IN RACETRACK URINE SAMPLES
N = 19
DOSED HORSE URINE
FREQUENCY
10.0
8.0
6.0
4.0
2.0
0.0
0
4
8
12
16
20
APPARENT FLUPHENAZINE CONCENTRATION, ng/ml

Fig. 11

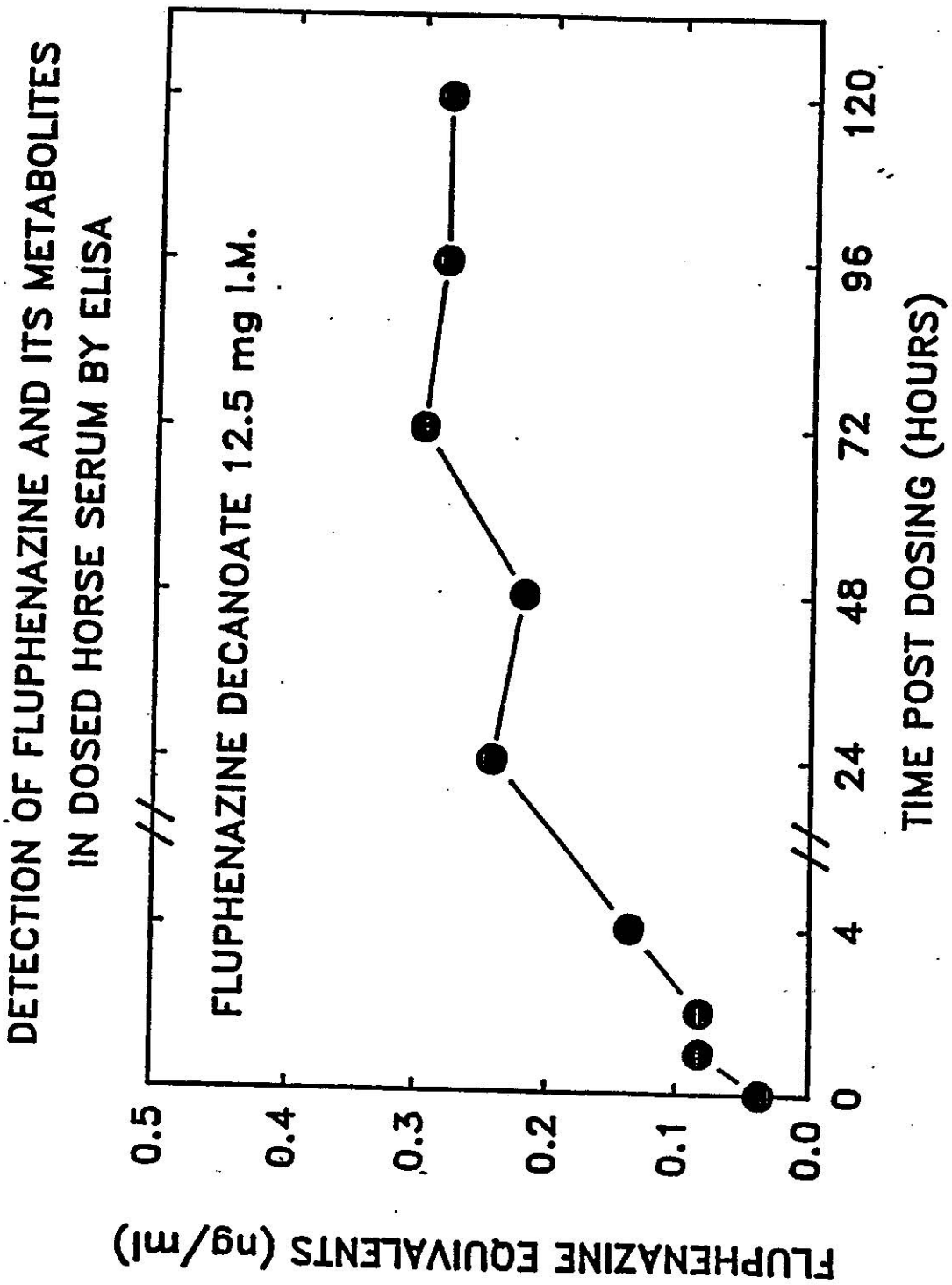

DETECTION OF FLUPHENAZINE AND ITS METABOLITES
IN DOSED HORSE SERUM BY ELISA
FLUPHENAZINE DECANOATE 12.5 mg I.M.
FLUPHENAZINE EQUIVALENTS (ng/ml)
0.0
0.1
0.2
0.3
0.4
0.5
0
4
24
48
72
96
120
TIME POST DOSING (HOURS)

Fig. 10

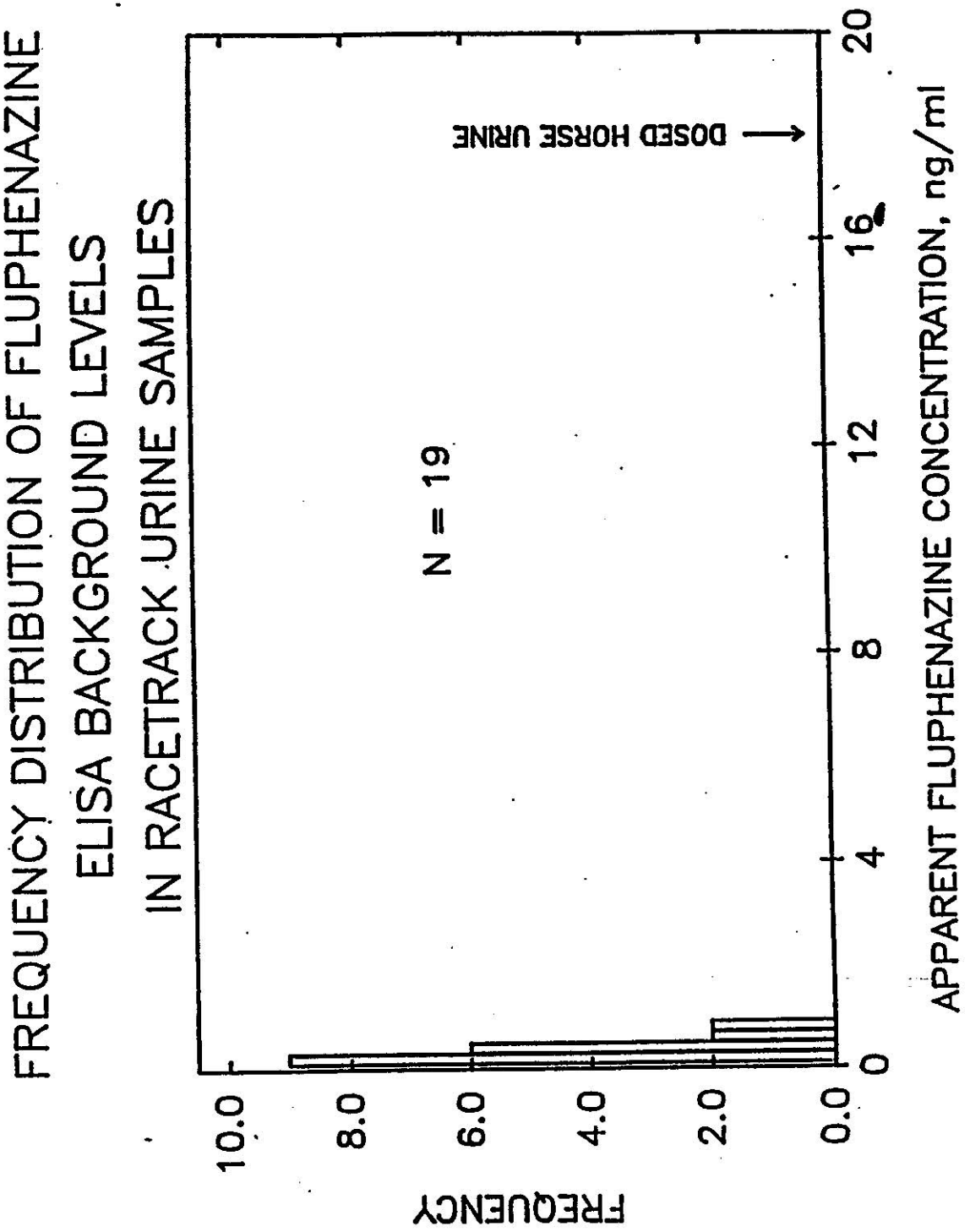
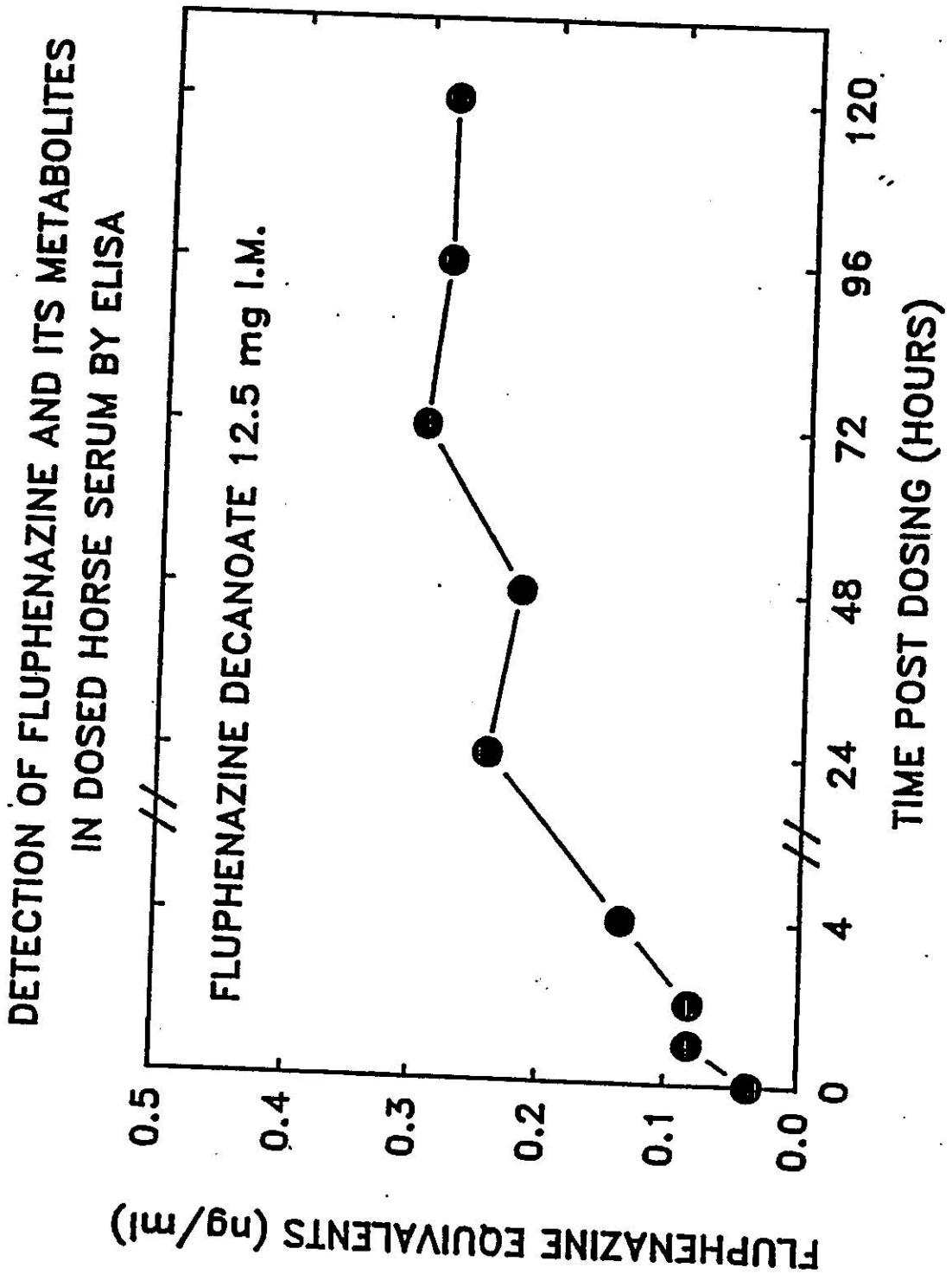

FREQUENCY DISTRIBUTION OF FLUPHENAZINE
ELISA BACKGROUND LEVELS
IN RACETRACK URINE SAMPLES
N = 19
DOSED HORSE URINE
FREQUENCY
10.0
8.0
6.0
4.0
2.0
0.0
0
4
8
12
16
20
APPARENT FLUPHENAZINE CONCENTRATION, ng/ml

Fig. 11


DETECTION OF FLUPHENAZINE AND ITS METABOLITES
IN DOSED HORSE SERUM BY ELISA
FLUPHENAZINE DECANOATE 12.5 mg I.M.
FLUPHENAZINE EQUIVALENTS (ng/ml)
0.0
0.1
0.2
0.3
0.4
0.5
0
4
24
48
72
96
120
TIME POST DOSING (HOURS)