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IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES
X. DETECTION OF PHENYLBUTAZONE IN EQUINE BLOOD AND URINE BY PARTICLE CONCENTRATION FLUOROIMMUNOASSAY AND ELISA

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SUMMARY

We have developed Particle Concentration Fluorescence Immunoassay (PCFIA) and Enzyme Linked Immunosorbent Assay (ELISA) tests for phenylbutazone as part of a panel of immunoassay tests for drugs in racing horses. Since phenylbutazone is a legal drug in most American racing jurisdictions, the primary use for this test is likely to be quantitation of plasma levels of PB. Our PCFIA test has an I-50 for phenylbutazone of about 1 µg/ml, close to the generally used 5 µg/ml plasma limit for phenylbutazone. Our ELISA test is substantially more sensitive to PB, with an I-50 for phenylbutazone of about 10 ng/ml. Both tests are based on our anti-phenylbutazone antibody, which shows limited cross-reactivity with oxyphenbutazone and does not cross-react significantly with other acidic drugs used in horses. Quantitation of phenylbutazone in equine blood and urine by these assays correlates well with gas chromatography/mass spectrometry quantitation of phenylbutazone. Both assays are sufficiently sensitive to detect the presence of phenylbutazone or its metabolites in equine urine for up to 48 hours, and in equine plasma for up to 24 hours after therapeutic doses of this drug. These tests are capable of providing rapid, sensitive, and economical methods for racing jurisdictions to detect and estimate plasma and/or urinary concentrations of phenylbutazone. Other uses include pre-race and pre-purchase screening for unauthorized blood levels of phenylbutazone and screening for phenylbutazone toxicity.

INTRODUCTION

Phenylbutazone (1,2-diphenyl-3,5-dioxo-4-butylpyrazolidine) is a non-steroidal anti-inflammatory drug (NSAID) with analgesic properties. It is widely used in equine veterinary practice in treating soft tissue inflammation associated with musculoskeletal disorders (Gabel et al., 1977; Tobin, 1981).

Because of its widespread availability and its frequent use in racing and other horses, phenylbutazone has become the standard treatment for

musculoskeletal disease and damage in performance horses. Horses are commonly treated with phenylbutazone while they are being trained, but they are generally not allowed to race on pharmacologically effective levels of this drug. This circumstance has given rise to a regulatory level for phenylbutazone to control use of this drug in racing horses. In the United States this level is generally set at about 5 µg/ml of phenylbutazone in plasma (Smith et al., 1987; Tobin et al., 1986).

To enforce such a regulatory level of phenylbutazone in plasma, many jurisdictions quantitate the levels of phenylbutazone in equine plasma and, occasionally, in urine. Currently such quantitation is cumbersome and the methods by which it is carried out are not defined. In general, samples are first screened by a method such as thin layer chromatography (TLC). Then, if evidence suggestive of excessive levels of phenylbutazone develops, the samples are analyzed by more rigorous, quantitative methods, usually high performance liquid chromatography (HPLC).

The method by which samples are best screened for phenylbutazone is not clear. Ideally the method should be inexpensive, specific, and automated. These characteristics are most readily attained by immunoassay, and to this end we raised an antibody and developed an automated immunoassay for phenylbutazone. In this communication we describe our particle concentration fluorescence immunoassay (PCFIA) assay and enzyme linked immunosorbent assay (ELISA) for phenylbutazone and preliminary results of their application to equine phenylbutazone determination.

MATERIALS AND METHODS

Horses

Mature Thoroughbred, half Thoroughbred and Standardbred mares (450-550 kg) were used throughout. The horses were kept at pasture and allowed free access

to food and water. The horses were placed in standard box stalls (17 m^2) approximately 12 hours prior to dosing for acclimatization.

Dosing and Sampling

Phenylbutazone pure drug standard was obtained from Sigma Chemical Co. (St. Louis, MO). The drug was administered as an injectable preparation, Jen-Sal (Jensen-Salsberg Co., Kansas City, KS). A 2 g dose of phenylbutazone was administered by rapid IV injection into the jugular vein.

All urine samples were collected by bladder catheterization. Blood samples were collected by venipuncture into vacuum blood tubes containing potassium oxalate and sodium fluoride for plasma or into serum separation tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ). The blood tubes were centrifuged to collect the plasma or serum. All samples were stored frozen until assayed.

Urine samples were filtered through Spin-X^{TM} microcentrifuge filters (Costar^{R}, Cambridge, MA) and diluted (1:5) with 0.01 M phosphate buffered saline, pH 7.4 (PBS) buffer prior to assay. Plasma samples were analyzed directly.

Anti-phenylbutazone Antibody

Rabbit anti-phenylbutazone antibody was developed by derivatizing phenylbutazone linking it to bovine serum albumin and raising antibodies as described by McDonald et al. (1987).

Cross-Reactivity Studies

A series of in vitro cross-reactivity studies were undertaken with a number of phenylbutazone metabolites and congeners. Pure drug standards were obtained as follows: oxyphenbutazone from Ciba Pharmaceuticals (Summit, NJ), flunixin from Schering Laboratories (Bloomfield, NJ), furosemide from Sigma Chemical Co. (St. Louis, MO), naproxen from Syntex Laboratories (Palo Alto, CA) and meclofenamic acid from Warner-Lambert Co. (Ann Arbor, MI).

Particle Concentration Fluorescence Immunoassay (PCFIA)

PCFIA studies were performed as previously described (Jolley *et al.*, 1984; McDonald *et al.*, 1987; Yang *et al.*, 1987) on a Pandex Fluorescence Concentration Analyzer (Pandex, Mundelein, IL). The basic functional unit in the Pandex PCFIA is a 96 well plate with a filter base in each plate. To each well is added 20 μl of phenylbutazone-B-phycoerythrin (phenylbutazone-BPE), 20 μl of anti-phenylbutazone antibody, and 20 μl of blank, standard, or test sample. The system is allowed to equilibrate for 10 minutes after which a second antibody is added. The second antibody consists of goat anti-rabbit antibody bound to latex beads. The system is allowed to react for another 10 minutes and then the fluid is drawn out of the system through the filter membrane. The reaction system is washed with 80 μl of phosphate buffer to resuspend the latex particles, and the system again drawn down with the vacuum. The filtration step has the effect of concentrating the latex beads 1000-fold, thereby increasing the sensitivity of the method. After the wash step, the fluorescence of the particles at 545 and 575 nm is measured. The mean response from control horse urines is usually about 25,000-30,000 arbitrary fluorescence units/well. Automation of the PCFIA system (Prange *et al.*, 1988) is readily available by means of the Baxter/Pandex Screen Machine (Baxter Healthcare Corp., Pandex Division, Mundelein, IL).

Phenylbutazone was derivatized and the anti-phenylbutazone antibody raised at the University of Kentucky. All PCFIA test reagents were supplied by and are commercially available from International Diagnostic Systems Corporation (St. Joseph, MI).

Enzyme-Linked Immunosorbent Assay

For the ELISA assay rabbit anti-phenylbutazone antibody was produced as previously described (Woods *et al.*, 1986; Tai *et al.*, 1988). Phenylbutazone

was linked to horseradish peroxidase (HRP) (Pradelles et al., 1985) to give rise to a covalently bound phenylbutazone-HRP complex.

The assay is based on competition between free drug in the standard, test, or control sample and phenylbutazone-HRP conjugate for the anti-phenylbutazone antibody. After an incubation period, TMB Microwell Peroxidase substrate solution (Kirkegaad and Perry, Gaithersburg, MD) is added and the optical density measured. The optical density increases with increasing phenylbutazone-HRP bound, which in turn is inversely proportional to the amount of phenylbutazone in the sample. Thus lower optical density implies higher drug concentration.

Mass Spectrometry

Gas chromatography-mass spectrometry (GC/MS) was performed on a Hewlett Packard GC Model 5890 equipped with a 12 m HP-1 methyl silicone capillary column and a Hewlett Packard Model 5970 Mass Selective Detector (Hewlett Packard, Palo Alto, CA). GC oven temperature profile was: 70°C (1 min. hold), 20°C/min to 280°C. After centrifugation, each 1 mL plasma sample was placed in a centrifuge tube along with 4 mL of saturated KH_2PO_4 solution, 6 mL dichloromethane, and 10 µg hexestrol as internal standard. The tubes were capped and mixed thoroughly, and the organic layers transferred to clean tubes. Samples were evaporated to dryness under a stream of nitrogen and reconstituted with 50 ul ethylacetate. Extracted plasma samples were injected in splitless mode.

RESULTS

A standard curve for the phenylbutazone PCIFA test shows that addition of 100 ng/ml phenylbutazone to the system produces about an eighty percent inhibition of fluorescence counts (Figure 1). Increasing concentration of phenylbutazone increases the inhibition in a log-linear manner, with half-maximal inhibition occurring at approximately 1 µg/ml phenylbutazone.

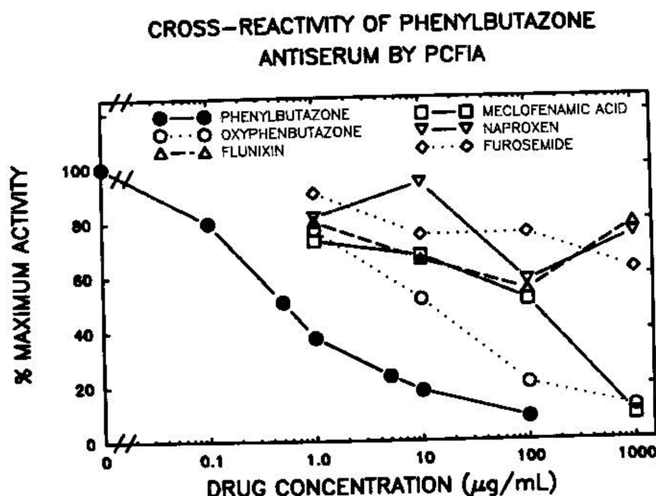

Figure 1. The figure shows the percentage of maximal PCFIA activity and cross-reactivity observed in the presence of the indicated concentrations of phenylbutazone or other drugs in PBS buffer. I-50 for phenylbutazone in this PCFIA system is approximately 1.0 µg/ml. All data points are expressed as a percentage of fluorescence with no phenylbutazone added to the system, which is assigned a value of 100%.

The ability of various phenylbutazone congeners and metabolites to inhibit the phenylbutazone antibody in the PCFIA system was also investigated. As shown in Figure 1 oxyphenbutazone cross-reacts with the phenylbutazone antibody with an I-50 of approximately 10 µg/ml. None of the other drugs investigated, flunixin, naproxen, furosemide nor meclofenamic acid, in concentrations up to 100 µg/ml, cross-reacted significantly with the phenylbutazone antibody.

The ability of the phenylbutazone test to detect phenylbutazone in the blood and urine of horses dosed with this drug was evaluated. Phenylbutazone was administered intravenously at a level of 2 g to each of two horses. At this dose, inhibition of the PCFIA fluorescence was essentially complete for plasma samples taken from 15 minutes through 8 hours post-dose (Figure 2). By 24 hours after dosing, the system was only about 40% inhibited, and plasma

levels of the drug had returned to control values by 52 hours post-dose. The inhibition pattern in urine was similar, though more prolonged. After a 2 g dose of phenylbutazone, inhibition of the PCFIA reaction was essentially complete for urine samples from 30 minutes through 12 hours after dosing. Inhibition had decreased to 65% by 24 hours post-dose, and about 45% by 48 hours post-dose. Fluorescence intensity had returned to control levels for urine by 72 hours after dosing (Figure 3).

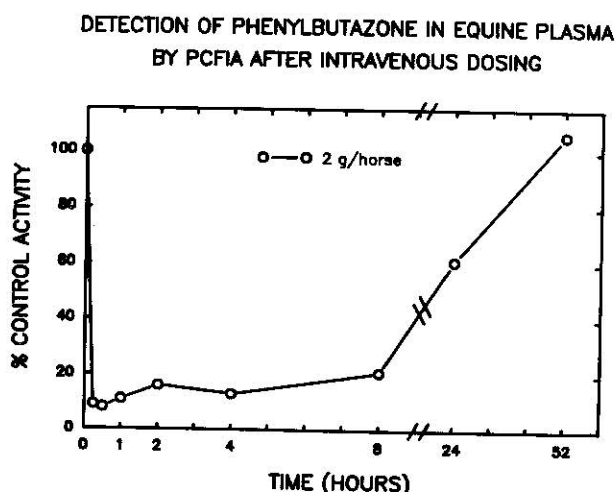

Figure 2. The inhibition of the PCFIA reaction by plasma was measured after administration of 2g phenylbutazone IV. Inhibition was evident throughout the first 24 hours after dosing. Control activity is the reading for the pre-dose plasma sample.

The ELISA format of this assay shows broadly similar reactivity with the exception that the ELISA assay is far more sensitive to phenylbutazone and oxyphenbutazone than the PCFIA test. As shown in Figure 4, half maximal inhibition of the ELISA test occurs at about 10 ng/ml of phenylbutazone as compared with about 800 ng/ml in the PCFIA format. Similarly the ELISA format is also much more sensitive to oxyphenbutazone than the PCFIA format. As with

DETECTION OF PHENYLBUTAZONE IN EQUINE URINE
BY PCFIA AFTER INTRAVENOUS DOSING

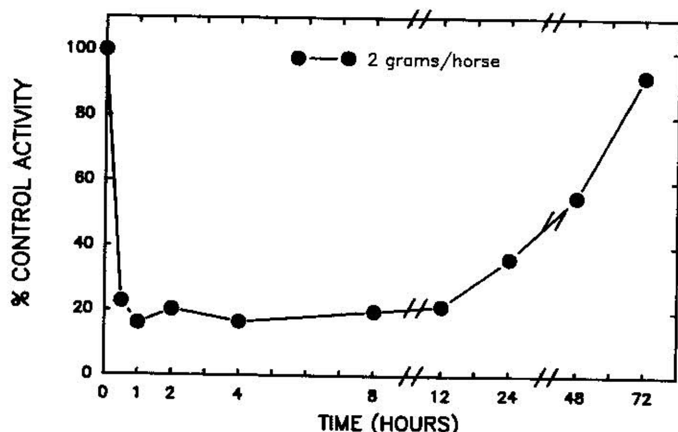

Figure 3. The inhibition of the PCFIA reaction by urine after a of 2g phenylbutazone IV dose was measured. Inhibition was evident for the first 24 hours after dosing. Control activity is the reading for the pre-dose urine sample.

CROSS-REACTIVITY OF PHENYLBUTAZONE
ANTISERUM BY ELISA

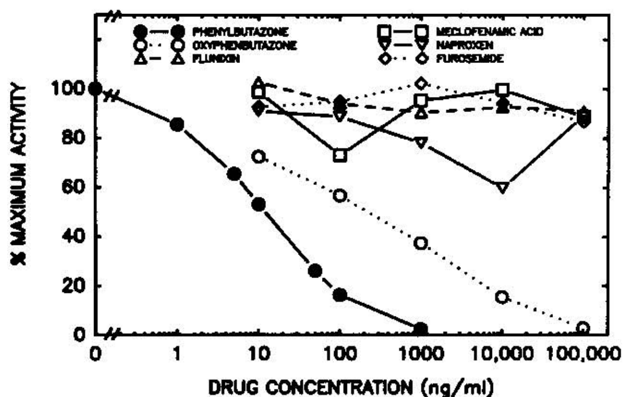

Figure 4. The percentage of maximal ELISA activity and cross-reactivity observed in the presence of the indicated concentrations of phenylbutazone or other drugs in PBS buffer was measured. I-50 for phenylbutazone in this ELISA system is about 10 ng/ml. All data are expressed as a percentage of optical density with no phenylbutazone added to the system.

the PCFIA format, the other commonly used acidic drugs did not react to any significant degree with this antibody in the ELISA format.

Because of the high sensitivity of the ELISA test we diluted all plasma and urine samples 100 fold prior to assay for phenylbutazone. Figure 5 shows the plasma levels of phenylbutazone detected in a horse after IV dosing with 2 g of phenylbutazone with optical density values plotted on the left hand axis. Consistent with previous experience with phenylbutazone, apparent phenylbutazone levels in these samples peaked at about 30 μg/ml shortly after drug administration and then declined thereafter to about 1 μg/ml at twenty four hours after drug administration.

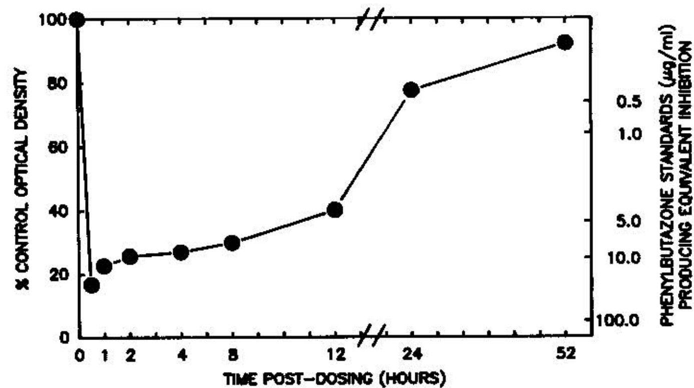

Figure 5. ELISA detection of phenylbutazone in equine plasma after 2 gram IV dosing. Inhibition of the ELISA reaction was observed for 24 hours after dosing. Control optical density is the reading for the pre-dose sample.

Similarly when we evaluated the ability of this test to detect phenylbutazone administration in equine urine, the apparent concentrations of phenylbutazone and its metabolites in this sample remained above 10 µg/ml until about twenty four hours after dosing, at which time the apparent concentration of phenylbutazone and its metabolites was about 39 µg/ml. By 48 hours after dosing the apparent concentrations of phenylbutazone and its metabolites had declined to the equivalent of about 1 µg/ml of phenylbutazone and no phenylbutazone equivalents were detectable at 72 hours after the last dose of phenylbutazone (Figure 6). These data are very similar to the PCFIA data presented in Figure 3, showing a good correlation between the two methods.

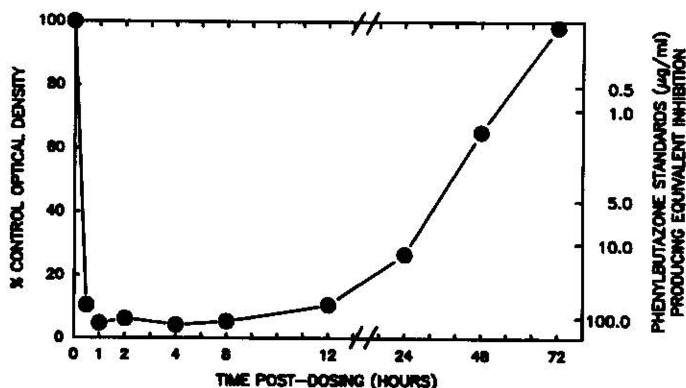

Figure 6. ELISA detection of phenylbutazone in equine urine after 2 gram IV dosing. Inhibition of the ELISA reaction was observed for 24 hours after dosing. Control optical density in the reading for the pre-dose sample.

Since the PCFIA method is the most readily automated of the two methods described, we compared the ability of PCFIA to quantitate phenylbutazone spiked into serum with quantitation of these samples by GC/MS. As shown in Figure 7, there was a very good correlation between the values obtained by the two methods, with a correlation coefficient of about 0.99 between the two methods.

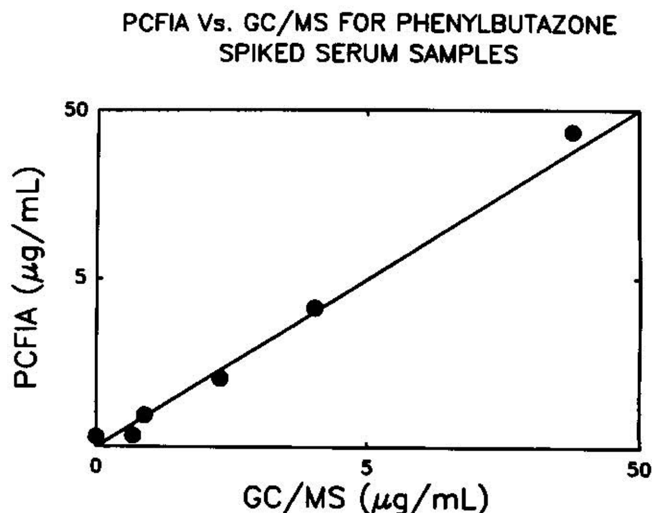

Figure 7. PCFIA vs. GC/MS for phenylbutazone spiked serum samples. The vertical axis displays the PCFIA readings obtained when equine serum samples were spiked with phenylbutazone. The correlation between the two methods was r = 0.99.

DISCUSSION

Phenylbutazone is the most frequently and widely used therapeutic agent in horses of all breeds and types (Tobin et al., 1986). It is the horse's equivalent of aspirin, and is recommended for treatment of soft tissue damage. As athletic animals, horses are prone to soft tissue inflammation, and phenylbutazone is therefore widely administered. Phenylbutazone is

so commonly used in horses that its use may in fact be considered part and parcel of good horsemanship (Tobin, 1981).

The widespread use of phenylbutazone in the training of horses is specifically recognized in most North American rules of racing. The Association of Racing Commissioners International suggests 5 µg/ml as the plasma level of phenylbutazone that should be permitted in racing horses, and most major race jurisdictions adhere to this rule. This rule is enforced by plasma level quantitation, and there is, therefore, a need for simple and rapid method of quantitating plasma levels of phenylbutazone.

Beyond the regulation of phenylbutazone in racing, there is a compelling medical need for a simple test for the phenylbutazone in the blood and urine of horses. This is because while phenylbutazone is the most commonly used drug in horses, it is also a relatively toxic drug in horses. Phenylbutazone is unusual among equine medications in that it shows dose dependent kinetics, apparently inhibiting its own metabolism (Tobin et al., 1986). This leads to phenylbutazone accumulation and to marked toxicity. Phenylbutazone overdose can cause the death of a horse in five days or less. Because of this, there exists a need for a simple, sensitive test for phenylbutazone to allow horsemen, horse owners, and veterinarians to monitor blood or urinary concentrations of phenylbutazone in their horses.

Other potential uses for an assay such as the one described here are in pre-race testing and the pre-purchase testing of horses. For pre-race testing of horses, especially in jurisdictions such as New York that nominally do not permit horses to run on phenylbutazone, an assay such as the one described here would permit horsemen or racing authorities to determine within minutes the phenylbutazone status of their horses. For racing jurisdictions outside of North America with no allowable level of

phenylbutazone this test could also be a very useful pre-competition screening test.

Pre-purchase examination of horses represents another potential use for this test, especially in the enzyme-linked immunosorbent assay (ELISA) format (Tobin et al, 1988). In this format the test could be used "on site" by veterinarians and horse sales companies to determine the medication status of animals offered for sale. This is important because a small dose of phenylbutazone can render a lame horse "sound" for the purposes of effecting a sale, and thereby misrepresent the condition and value of an animal. A simple and effective screening tests for phenylbutazone such as described here will enable the buyer to protect himself against undesirable medication practices on the part of the sellers.

The immunoassay described in this communication appears to fill these needs for a simple, fast, and sensitive phenylbutazone assay. The antibody is sufficiently sensitive to phenylbutazone to readily detect its presence in both plasma and urine. The assay, however, is not highly sensitive, as would be appropriate to test for an illegal drug, but is rather a modestly sensitive antibody suitable for use with a therapeutic medication such as phenylbutazone. With an I-50 for phenylbutazone in the PCFIA format of about 1 μg/ml, this assay is sufficiently sensitive to detect racetrack residues of this drug, and also to detect its presence in equine urine for approximately two days after treatment with this agent. Since the therapeutic effect of phenylbutazone is over within about twenty four hours, this sensitivity is sufficient for its effective regulatory use, but not so sensitive as to create a regulatory problem.

This antibody appears to be very suitable for regulatory use. It cross-reacts poorly with oxyphenbutazone, being 10 times less sensitive to this agent. Since oxyphenbutazone is cleared relatively rapidly from plasma

and does not attain high levels in plasma (Tobin et al.), the probability of significant interference from plasma levels of oxyphenybutazone is small.

The antibody also reacts poorly or not at all with other acidic drugs that are likely to be found in high concentrations in equine plasma. The antibody does not recognize furosemide, flunixin, or naproxen. It cross-reacts somewhat with meclofenamic acid, but is much less reactive with meclofenamic acid than, for example with oxyphenbutazone. Based on the cross-reactivities presented in Figures 1 and 4 there is little likelihood of significant interference in equine plasma from these agents.

When the assay was used to follow the plasma levels of phenylbutazone after the standard dose (2 g/1,000 lbs) recommended by the American Association of Equine Practitioners, it readily detected the presence of phenylbutazone in these samples for about twenty four hours, approximately the duration of the therapeutic effect of phenylbutazone. On the other hand, the drug or its metabolites were detected for a longer period in horse urine, consistent with the tendency of parent drug and, more especially, metabolites to persist longer in the urine than in blood.

An important use for these assays for phenylbutazone is likely to be for rapid estimation of the quantity of phenylbutazone present in the blood of horses prior to racing. Since this application is generally likely to require a high throughput of samples, we evaluated this application of the test in both the PCFIA and the ELISA format. As shown in Figure 7 the PCFIA format readily detects and accurately quantitates the presence of phenylbutazone in equine plasma samples. This spiking approach, however, leaves unanswered the degree of interference likely to be found from drug metabolites, although the concentrations of oxyphenbutazone found in equine plasma are small and unlikely to interfere significantly with the PCFIA quantitation of phenylbutazone. Similarly, work with the ELISA format of

this test showed that when it was used to evaluate the plasma levels of phenylbutazone in post-race samples from horses racing in Kentucky, there was a very good correlation between serum concentrations of phenylbutazone and the concentrations determined by GC/MS. However, further work is required on the use of this test as a quantitative screen for phenylbutazone in pre- and post-race testing situations.

In summary therefore, we have raised an antibody to phenylbutazone and incorporated this antibody into PCFIA and ELISA tests for phenylbutazone in horses. These tests are rapid, sensitive, and readily adaptable to automation. Their initial use is likely to be as a screening test to determine whether or not horsemen are in compliance with the 5 μg/ml plasma tolerance level for phenylbutazone in racing horses widely used in North America. Outside of this, they can be used to screen for the presence of phenylbutazone in the blood or urine of horses where detectable levels of phenylbutazone are not permitted. Such circumstances include racing jurisdictions which do not allow the presence of phenylbutazone, horses offered for sale and, most importantly, horses that are suspected of having been overdosed with phenylbutazone.

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