Effects of Phenylbutazone and Oxyphenbutazone on Acidic Drug Detection in High Performance Thin Layer Chromatographic Systems

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Interference or "masking" in thin layer chromatography occurs when the presence of one drug on a thin layer plate physically obscures or interferes with the detection of another drug. We investigated the ability of phenylbutazone and oxyphenbutazone to mask or interfere with the detection of acidic drugs of high performance thin layer chromatography. Of 20 acidic drugs called "positive" since 1981 by laboratories affiliated with the Association of Official Racing Chemists, 16 did not comigrate with phenylbutazone or oxyphenbutazone and could not, therefore, be masked by these agents. Three medications (diclofenac, fenoprofen, ibuprofen) were potentially masked by phenylbutazone and one (sulindac) was potentially masked by oxyphenbutazone. These agents were therefore administered to horses to determine whether or not their metabolites would allow their detection. In each case, metabolites of these agents were detectable for at least 24 hr after drug administration and detection was not interfered with by phenylbutazone or oxyphenbutazone. These results suggest that these 20 acidic drugs should be readily detectable in postrace urines of horses in the presence of phenylbutazone either as the parent drug or by virtue of the easily distinguishable metabolites that each agent possesses. There is, therefore, no reason to believe that the agents tested in this study can be effectively masked or interfered with by phenylbutazone or its metabolites in equine urine.

Key Words: Acidic drugs; Drug detection; Masking, Oxyphenbutazone; Phenylbutazone; Thin layer chromatography

INTRODUCTION

Phenylbutazone is a nonsteroidal antiinflammatory drug widely used in the treatment of musculoskeletal problems in racing horses (Tobin, 1981). Because it is commonly used in the treatment of horses in training, blood and urinary residues (Tobin, 1979; Tobin et al., 1982) may appear in both pre- and postrace testing samples taken for analysis in medication control programs (Tobin, 1981).

Some racing chemists hold that residues of phenylbutazone and its metabolites (oxyphenbutazone and γ-hydroxyphenylbutazone) in equine urine can interfere

with the detection of other drugs. Popularly, the phenomenon is called "masking" and phenylbutazone is spoken of as "masking" or interfering with the detection of illegal drugs (Takade and Vassilaros, 1982).

Masking occurs when the presence of a substance on a thin layer chromatographic plate physically obscures or interferes with the detection of a drug (Takade and Vassilaros, 1982). However, the ability of phenylbutazone to "mask" or interfere with the detection of other drugs has been controversial. While some workers hold that phenylbutazone masking is a serious problem, others hold that the problem is trivial and can readily be overcome by the analyst (Takade and Vassilaros, 1982; Tobin, 1983). Further adding to the confusion on "masking" is the fact that until very recently there was no published scientific literature on this subject.

Because of the regulatory and forensic importance of the concept of masking (Tobin, 1983) or interference, we have commenced a study of this problem. In this communication we report on the potential of phenylbutazone (PB) and oxyphenbutazone (OPB) to interfere with high performance thin layer chromatographic (HPTLC) screening for acidic drugs and their metabolites. The other major PB metabolite, γ-hydroxyphenylbutazone (γ-OHPB), occurs in two interchangeable forms (Girod et al., 1957; Haas and Scheibli, 1984), and their role in the masking problem is currently being evaluated.

MATERIALS AND METHODS

Drug Selection and Preparation

Drugs selected as candidates for possible masking by PB and OPB were those called "positive" by the Association of Official Racing Chemists (AORC) laboratories for the calendar years 1981, 1982, and 1983 (Johnston, 1982, 1983, 1984). From this master list of 58 drugs, the 20 acidic drugs were selected for study. One miligram of each drug standard was made up in 100 ml of methanol and, depending on the detectability of the drug, from 100 to 200 μl of each standard was used for spotting directly on the HPTLC plates.

Standard solutions of PB and OPB were made up by adding 5 mg of PB or OPB to 10 ml of an antioxidant stabilizing methanol solution (20 mg butylated hydroxytoluene and 2 g ascorbic acid in 100 ml methanol) (Smith, 1984). Aliquots of these solutions were added to water or equine urine to give concentrations of 25 μg PB and 50 μg OPB/ml. These concentrations were near the highest values reported for PB and OPB in 200 urines from thoroughbreds racing in Kentucky (Houston et al., 1983a,b, 1984).

For each of the 20 acidic drugs studied, aliquots of the methanol standards were added to water or equine urine to give concentrations of from 10 to 20 μ/ml either alone or along with PB and OPB.

Standard solutions of sulindac, sulindac sulfide (Merck, Sharp & Dohme), sulindac sulfone (Merck, Sharp & Dohme), sodium diclofenac, calcium fenoprofen, ibuprofen, piroxicam, naproxen, and meclofenamic acid were made up by adding 2 mg of drug to 10 ml of methanol. Urine standards of each drug were prepared by adding 100 μl of the methanol solutions (20 μg of drug) to 1 ml of the pretreatment urine.

For each drug, 100 μl of the methanol solutions were used for spotting directly on the HPTLC plates.

In all cases, the aliquots of the methanol standards were evaporated carefully to dryness under a stream of nitrogen before addition of the urine.

Dosing and Sampling

Four horses (female, mixed breed) were dosed, one drug per each horse, orally by stomach tube with 2 g each sulindac (Merck, Sharp & Dohme; Rahway, NJ), sodium diclofenac (Ciba-Geigy; Summit, NJ), ibuprofen (Upjohn; Kalamazoo, MI), calcium fenoprofen (Lilly; Indianapolis, IN), and piroxicam (Pfizer; Groton, CT). Urine samples were taken by bladder catheter pretreatment and at 2, 4, 8, 12, 24, 36, 48, and 72 hr postdose.

Urine samples taken from Kentucky track horses postrace and certified by the Kentucky Equine Drug Testing Laboratory to contain naproxen or meclofenamic acid were also examined by the acid extraction HPTLC method.

Drug Extractions

All drug extractions followed the methods used in the Kentucky Equine Drug Testing Program. To 1 ml of water or the urine sample was added 1 ml of 0.2 N sodium hydroxide (NaOH), and the "hydrolysis" was allowed to proceed 10 min. To the hydrolysate were added 4 ml acid phosphate buffer, pH 3.0 (saturated potassium phosphate, monobasic, KH_2PO_4, in water titrated to pH 3.0 with concentrated phosphoric acid, H_3PO_4), 6 ml petroleum ether, and 1 ml dichloromethane (DCM) in that order. The sample was shaken on a rotorack for 4 min and centrifuged at 500g for 20 min, and the organic phase was transferred to a clean tube for evaporation to dryness under a stream of nitrogen. The residue was dissolved in 2 drops DCM, and the entire sample was then spotted on the thin layer plate. This method was referred to as Extraction System No. 1 (Figure 1).

To the aqueous phase from above was added 6 ml DCM. The sample was rotoracked 4 min and centrifuged at 500g for 20 min, and the aqueous fraction was discarded. The DCM fraction was then evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 2 drops DCM. The entire sample was spotted on a second thin layer plate. This method was referred to as Extraction System No. 2 (Figure 1).

Thin Layer Chromatography Procedures

The thin layer chromatographic (TLC) techniques used were those currently employed in the Kentucky Equine Drug Testing Program. Whatmann HP-KF high performance silica gel plates, 10 × 10 cm², 200-μm layer thickness were used throughout. These plates were stored in a desiccator with anhydrous calcium sulfate. For spotting, methanol aliquots of each drug were dried under nitrogen and then dissolved in 2 drops of DCM. These DCM standard solutions, or the urine or water extracts redissolved in DCM, were then spotted on the plate using drawn-out Pasteur pipettes. Spots were applied in incremental fashion and dried between each movement using a blow dryer. Spots were placed 15 mm above the bottom of the plate
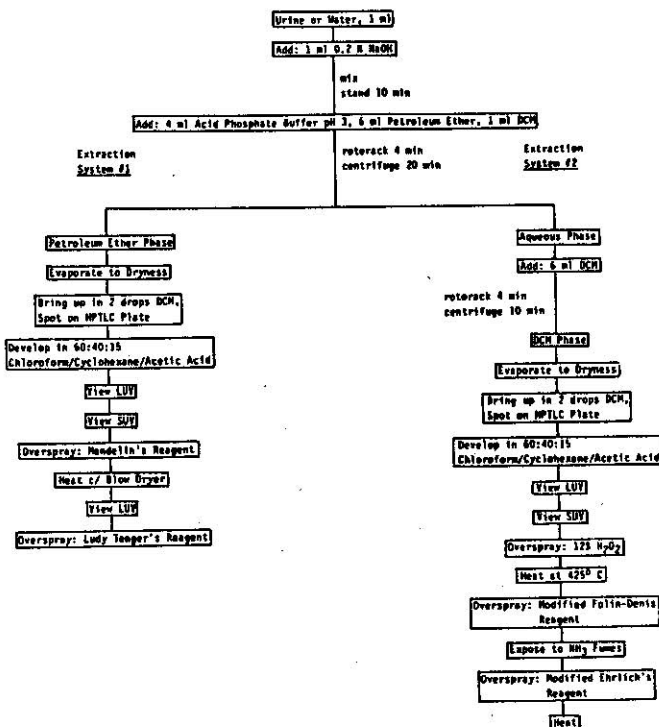
FIGURE 1.   Flow chart of acid drug detection by HPTLC including developing systems and visualization techniques.

and 5 mm apart, to give up to 19 spots/plate. Plates were developed by allowing the solvent to rise 40 mm above the bottom of the plate. The solvent system used for both extraction methods was chloroform:cyclohexane:acetic acid (60:40:15).

Visualization Techniques

The HPTLC plate No. 1 (Extraction System No. 1) was first viewed under long-wave ultraviolet light (LUV) (365 nm) in a view box immediately after removal of the plate from the developing tank (LUV visualization was activated by the acetic acid, which evaporated on drying). Subsequently, the plate was viewed under short-wave ultraviolet light (SUV) (254 nm). The plate was then oversprayed with Mandelin's Reagent (Table 1) followed by brief drying under a blow dryer and viewing under LUV. Thereafter, the plate was oversprayed with Ludy Tenger's Reagent (Table 1).

The HPTLC plate No. 2 (Extraction System No. 2) was also first viewed "wet" under LUV followed by viewing under SUV. The plate was then oversprayed with 12% hydrogen peroxide (H_2O_2) followed by heating face-down on a hot plate at 425°C. After cooling, the plate was oversprayed with modified Folin-Denis Reagent (Table 1). The plate was then exposed to ammonia (NH_3) fumes by being placed in a tank

TABLE 1   Visualization Reagents (Blake, 1985)

Ludy Tenger's Reagent
    1 g Bismuth subcarbonate (JT Baker, Phillipsburg, NJ)
    6 g Sodium iodide (Mallinckrodt, St. Louis, MO)
    3 ml Hydrochloric acid, concentrated
    100 ml Distilled water
Flask A: 1 g bismuth subcarbonate was dissolved by swirling in 3 ml concentrated hydrochloric acid.
Flask B: 6 g sodium iodide was dissolved in 50 ml distilled water in a 100-ml flask.
Concents of Flask A were poured slowly into Flask B with mixing. Flask B was then slowly, and with
    continuous swirling, filled to the 100-ml mark with distilled water. Formation of a black precipitate,
    due to too rapid addition of water, must be avoided.

Ehrlich's Reagent, modified
    100 mg 4-Dimethylaminocinnamaldehyde (Aldrich Chem; Milwaukee, WI)
    50 ml 6 N Hydrochloric acid
    50 ml Methanol
The 100 mg 4-dimethylaminocinnamaldehyde was dissolved in a mixture of 50 ml 6 N hydrochloric acid
    and 50 ml methanol.

Mandelin's Reagent
    1 g meta-Ammonium vanadate (Fisher, Pittsburg, PA)
    100 ml 50% Sulfuric acid
Concentrated sulfuric acid was diluted 1:1 with distilled water to prepare 50% sulfuric acid; 1 g meta-
    ammonium vanadate was dissolved in 100 ml of the 50% sulfuric acid.

Folin-Denis Reagent, modified (Blake et al., 1978)
    10 g Sodium tungstate, dihydrate (JT Baker, Phillipsburg, NJ)
    2 g 12-Molybdosilicic acid (Pfaltz & Bauer, Stamford, CT)
    5 ml Phosphoric acid
    100 ml Water, distilled
The 10 g sodium tungstate, dihydrate, and 2 g 12-molybdosilicic acid were added to a mixture of 5 ml
    phosphoric acid and 50 ml distilled water and allowed to reflux 2 hr. After cooling, the solution was
    diluted to 100 ml with distilled water.

with a beaker of ammonium hydroxide. The plate was air dried and then oversprayed with modified Ehrlich's Reagent (Table 1), followed by heating on a hot plate.

Measurement and Data Recording

All spots visualized in each of the HPTLC systems were circled with a soft lead pencil at each visualization step, and the plates were photocopied. In addition, the appearance, color, and R_f of each spot were observed and recorded. The R_f is the ratio between the migration distance of a particular spot and the total migration distance of the solvent front.

RESULTS

Phenylbutazone and Oxyphenbutazone

The R_fs of PB, OPB, and the 20 acidic parent drugs tested are shown in Table 2. Only one significant spot was observed for PB, that having an R_f 0f 0.69. Two spots

TABLE 2   Mean R_f values $\pm$ SDM of the Acidic Drugs

Drug	Mean R_f Values $\pm$ SDM	Number of Plates Observed
PB	0.69 $\pm$ 0.01	26
OPB$_1$ (major)	0.30 $\pm$ 0.01	26
OPB$_2$ (minor)	0.10 $\pm$ 0.01	9
Meclofenamic acid	0.71 $\pm$ 0.02	4
Ibuprofen[a]	0.71 $\pm$ 0.01	19
Diclofenac[a]	0.70 $\pm$ 0.02	11
Fenoprofen[a]	0.69 $\pm$ 0.01	12
Naproxen	0.66 $\pm$ 0.02	4
Ketoprofen	0.65 $\pm$ 0.03	8
Salicylic acid	0.59 $\pm$ 0.02	8
Thiosalicylic acid	0.59 $\pm$ 0.04	6
Diflunisal	0.58 $\pm$ 0.02	6
Zomepirac	0.49 $\pm$ 0.02	8
Tolmetin	0.45 $\pm$ 0.03	5
Piroxicam	0.42 $\pm$ 0.01	2
Sulindac[b]	0.30 $\pm$ 0.01	10
Bumetanide	0.25 $\pm$ 0.02	6
Ethacrynic acid	0.24 $\pm$ 0.03	6
Theophylline	0.17 $\pm$ 0.02	4
Caffeine	0.16 $\pm$ 0.02	4
Flunixin	0.14 $\pm$ 0.01	4
Furosemide	0.14 $\pm$ 0.01	4
Theobromine	0.07 $\pm$ 0.01	4

[a] Drugs potentially masked by phenylbutazone.
[b] Drugs potentially masked by oxyphenbutazone.

were usually present for OPB after extraction. The larger OPB spot with a R_f of 0.30 was observed by direct spotting or after extraction, whereas a much smaller spot (R_f = 0.10) was only seen occasionally after extraction. The R_fs for the PB and OPB spot as well as for the one spot for each of the 20 drugs tested were very reproducible under the conditions outlined under Methods.

The responses of parent acidic drugs to the visualization techniques are shown in Tables 3A and 3B. The PB spiked at 25 μg/ml in water or urine extracted well in both extraction systems and resulted in a circular to oval spot.

The OPB spiked at 50 μg/ml in water or urine extracted well in System No. 1 and somewhat less well in System No. 2. The major OPB spot (R_f = 0.30) was circular when extracted out of water and out of urine in System No. 1. The OPB spots were usually obscured by urine background in System No. 2. The major and minor OPB spots yielded similar color reactions in both systems with the difference that the minor OPB spot was usually less intense.

Detection as Parent Drug

The 20 acidic drugs studied included diuretics, methylxanthine stimulants, and several types of nonsteroidal antiinflammatory drugs (NSAIDs). The NSAIDs in-

TABLE 3A HPTLC Visualizations and Overspray Reactions of Acidic Drugs (NSAIs)

| | | | | | | REACTIONS | | | | | |
| | | | | | | System No. 1 | | | System No. 2 | | |
Drug	R_f	Visible	LUV	SUV	Mandelin + Δ	LUV Post-Mandelin	Ludy Tenger	H_2O_2 + Δ	Folin-Denis	NH_3	Ehrlich + Δ
Pyrazolon derivatives											
PB	0.69	—	—	Clear	Brown	Dark	Brown, dark	Brown	Gray	Gray	Pink, purple
OPB	0.30	—	—	Clear	Gold	Dark	Red	Brown	Gray	Gray	Pink
Oxicams											
Piroxicam	0.42	Yellow	Green	Clear	White	Green	White	—	—	Faint gray	Faint gray
Fenamates and analogues											
Meclofenamic acid	0.71	—	Bright blue	Clear[a]	Dark violet	Blue	Faint pale	Brown	Gray	Gray	Pink, brown
Flunixin	0.14	—	Dark	Clear	—	—	Orange	Brown	Gray	Gray	Faint orange
Acetic acid derivatives											
Tolmetin	0.45	—	Dark	Clear	Red	Dark	Orange	—	—	Gray	—
Zomepirac	0.49	—	Dark	Clear	Pink	Dark	Orange	Tan	Gray	Faint gray	Faint purple
Sulindac	0.30	Yellow	Dark	Clear	White	Orange	Orange	Tan	Gray	Gray	Gray
Diclofenac	0.70	—	—	Clear	Brick red	Dark	—	Tan	Brown	Gray	Pink
Propionic acid derivatives											
Naproxen	0.66	—	—	Clear[b]	Violet	Dark	Faint pale	Brown	Gray	Gray	Gray
Ketoprofen	0.65	—	—	Clear	Tan	Deep yellow	Pale orange	Brown	Gray	Gray	Gray
Ibuprofen	0.71	—	—	Faint	White	Pale yellow	Faint pink	Brown	Gray	Faint gray	Faint pink
Fenoprofen	0.69	—	—	Faint	White	Pale yellow	—	—	Gray	Gray	Gray

NA, not applicable (not extracted in system); —, no reaction, no color; Δ, heat.
[a] Crescent in presence of PB.
[b] Triangle in presence of OPB.

TABLE 3B HPTLC Visualization and Overspray Reactions of Acidic Drugs (Salicylates, Diuretics, Methyxanthines)

| | | | | | | REACTIONS | | | | | |
| | | | | System No. 1 | | | | | System No. 2 | | |
Drug	R_f	Visible	LUV	SUV	Mandelin + Δ	LUV Post-Mandelin	Ludy Tenger	H_2O_2 + Δ	Folin-Denis	NH_3	Ehrlich + Δ
Salicylates											
Salicylic acid	0.59	—	Pale blue	Clear	Blue-pink	Pale	Pale orange	NA	NA	NA	NA
Diflunisal	0.58	—	Green	Clear	Blue-gray	Green	Pale	Tan	Gray	Gray	Gray
Thiosalicylic acid	0.59	—	Blue	Diffuse	NA	NA	NA	—	Gray	Gray	—
Diuretics											
Furosemide	0.14	—	Blue	Clear	NA	NA	NA	—		—	Pink
Ethacrynic acid	0.24	—	—	Small	—	—	—	Tan	Bluish white	Gray	Gray
Bumetanide	0.25	—	Bright blue	Clear	Lavender	—	Faint pale	Yellow	Gray	Gray	Bright purple
Methylxanthines											
Caffeine	0.16	—	—	Clear	—	—	Pink	—	—	Yellow	Orange
Theobromine	0.07	—	—	Clear	NA	NA	NA	—	—	—	—
Theophylline	0.17	—	—	Clear	—	—	Pink[a]	—	—	Gray	—

NA, not applicable (not extracted in system); —, no reaction, no color; Δ, heat.
[a] Extracts poorly in System No. 1.

cluded oxicams, fenamates, acetic acid derivatives, propionic acid derivatives, and salicylates.

The Oxicams

Piroxicam, spiked at 20 μg/ml in water or urine, extracted well in Systems No. 1 and 2. The R_f position in the window between PB and OPB and the green LUV spot clearly distinguished piroxicam from PB or OPB.

The Fenamates

Meclofenamic acid was clearly detectable in both systems and appeared as a narrow crescent just above PB. The bright blue LUV spot and the dark crescent just above PB after treatment with Mandelin's Reagent distinguished meclofenamic acid from PB.

In System No. 1, flunixin yielded a readily distinguishable spot above the minor OPB spot. Flunixin's reaction with Ludy Tenger's Reagent characterized this drug.

The Acetic Acid Derivatives

The drugs studied from this group were tolmetin, zomepirac, sulindac, and diclofenac. Tolmetin and zomepirac were distinguishable from PB and OPB by virtue of their R_f positions and reaction with Mandelin's Reagent.

Sulindac extracted well in both systems and yielded a spot that comigrated with the major OPB spot (Figure 2). Sulindac reacted with several of the visualization reagents, but these reactions were obscured by OPB spiked at 50 μg/ml in urine.

Diclofenac extracted well in both systems but yielded a spot that comigrated with PB (Figure 2). Diclofenac at 10 μg/ml in urine was characterized by its brick-red color reaction with Mandelin's Reagent.

The Propionic Acid Derivatives

The drugs from this category studied were naproxen, ketoprofen, ibuprofen, and fenoprofen. Naproxen was characterized by a triangular spot that reacted with Mandelin's Reagent and appeared just below PB.

Ketoprofen extracted well in both systems and was distinguished by appearing just below PB, reacting with Mandelin's Reagent observable under LUV after heating.

Ibuprofen and fenoprofen extracted poorly in both systems. When spiked in urine at 40 μg/ml, only faint spots that closely migrated with PB were observed (Figure 2). None of the reactions of ibuprofen and fenoprofen was sufficiently distinctive to allow their identification in the presence of PB.

The Salicylates

The salicylates examined in this study were salicylic acid, diflunisal, and thiosalicylic acid. All three salicylates had identical R_f values and were visual in the window between PB and OPB. Therefore, the drugs were not masked by PB or OPB.

The Diuretics

The diuretics examined were furosemide, ethacrynic acid, and bumetanide. Fuorsemide did not extract in System No. 1, but extracted well in System No. 2. Furo-
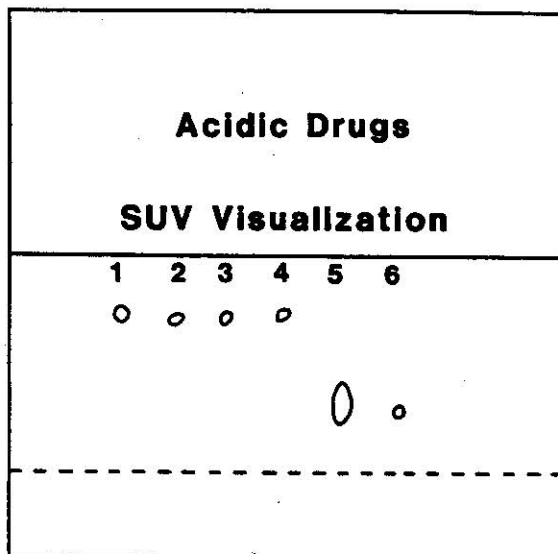
FIGURE 2. Thin layer chromatogram of PB, OPB, and comigrating acidic parent drugs. The DCM solutions of the drugs were spotted directly onto the plate at about 50 μg drug per spot. The sample numbers correspond to: (1) PB, (2) diclofenac, (3) fenoprofen, (4) ibuprofen, (5) OPB, (6) sulindac. The plate was developed in chloroform:cyclohexane:acetic acid (60:40:15) and visualized under shortwave UV. The spots were marked with a soft lead pencil. On this plate, the following R_fs were observed: PB = 0.74, OPB = 0.33, diclofenac = 0.71, fenoprofen = 0.71, ibuprofen = 0.74, sulindac = 0.29.

semide was best distinguished from PB and OPB by the R_f position and the blue LUV spot.

Ethacrynic acid (10 μg/ml) extracted well in both systems and yielded a small spot below the major OPB spot. Ethacrynic acid yielded a characteristic reaction with the Folin-Denis Reagent when the plate was heated to 425°C.

Bumetanide (10 μg/ml) extracted well in both systems with a bright blue LUV spot below the major OPB spot. Bumetanide was best distinguished from PB and OPB by the LUV spot and its reactions with Mandelin's Reagent and Ehrlich's Reagent.

The Methylxanthines

The methylxanthines included in this study were caffeine, theobromine, and theophylline. Caffeine extracted well in both systems and was best distinguished from PB and OPB by the R_f position and by its reactions with Ludy Tenger's Reagent and Ehrlich's Reagent.

Theobromine and theophylline did not extract in System No. 1. Both of these drugs extracted well in System No. 2, but neither drug reacted well with any of the visualization reagents.
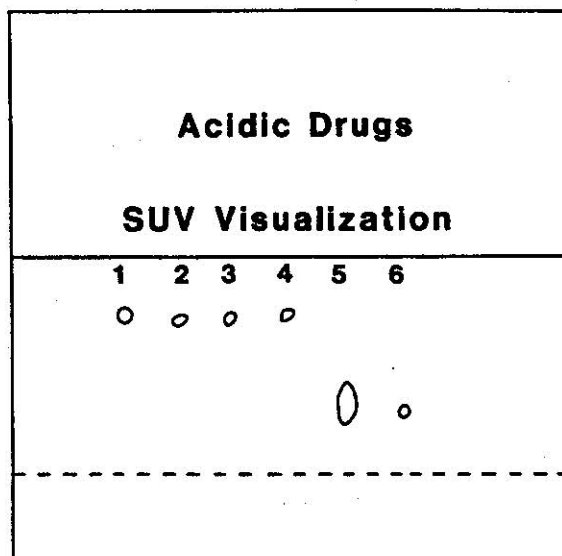
FIGURE 2.   Thin layer chromatogram of PB, OPB, and comigrating acidic parent drugs. The DCM solutions of the drugs were spotted directly onto the plate at about 50 μg drug per spot. The sample numbers correspond to: (1) PB, (2) diclofenac, (3) fenoprofen, (4) ibuprofen, (5) OPB, (6) sulindac. The plate was developed in chloroform:cyclohexane:acetic acid (60:40:15) and visualized under shortwave UV. The spots were marked with a soft lead pencil. On this plate, the following R_fs were observed: PB = 0.74, OPB = 0.33, diclofenac = 0.71, fenoprofen = 0.71, ibuprofen = 0.74, sulindac = 0.29.

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Ethacrynic acid (10 μg/ml) extracted well in both systems and yielded a small spot below the major OPB spot. Ethacrynic acid yielded a characteristic reaction with the Folin-Denis Reagent when the plate was heated to 425°C.

Bumetanide (10 μg/ml) extracted well in both systems with a bright blue LUV spot below the major OPB spot. Bumetanide was best distinguished from PB and OPB by the LUV spot and its reactions with Mandelin's Reagent and Ehrlich's Reagent.

The Methylxanthines

The methylxanthines included in this study were caffeine, theobromine, and theophylline. Caffeine extracted well in both systems and was best distinguished from PB and OPB by the R_f position and by its reactions with Ludy Tenger's Reagent and Ehrlich's Reagent.

Theobromine and theophylline did not extract in System No. 1. Both of these drugs extracted well in System No. 2, but neither drug reacted well with any of the visualization reagents.

Detection by Metabolites

The drugs sulindac, diclofenac, fenoprofen, and ibuprofen comigrated with PB and OPB, so these drugs were administered to horses, and the R_f values of their major metabolites were determined. A sample HPTLC plate of these drugs and their metabolites is shown in Figure 3. The visualization responses of sulindac, diclofenac, fenoprofen, ibuprofen, piroxicam, and their metabolites are shown in Table 4. Figure 4 shows the duration of detectability by HPTLC of these drugs and their metabolites.

Sulindac

Sulindac and two of its metabolites extracted well in both systems yielding bright yellow spots that appeared as dark spots under LUV and SUV. The parent drug comigrated with the major OPB spot, and the sulfide metabolite spot appeared just below the PB spot. The OPB completely obscured the parent sulindac spot during
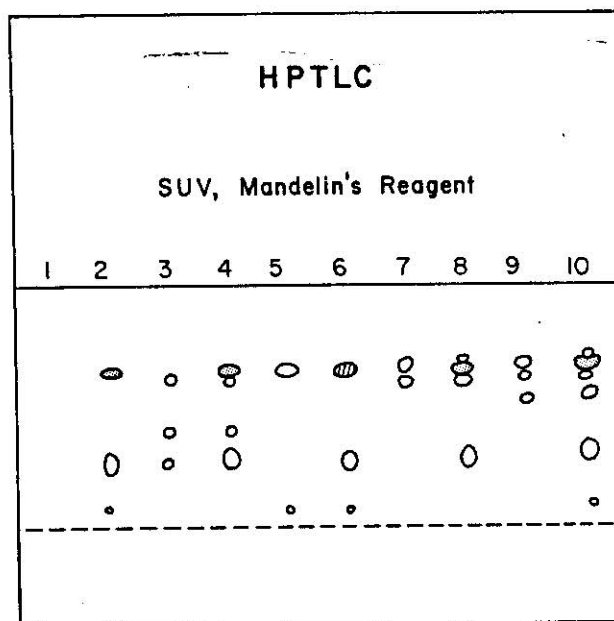

FIGURE 3. Thin layer chromatogram of PB, OPB, comigrating drugs and their metabolites. The DCM solutions of extracts (System No. 1) of horse urine 8 hr postdose, with and without added PB; OPB were spotted on the plate. The plate was developed and was visualized by SUV and Mandelin's Reagent. The sample numbers correspond to: (1) blank urine; (2) blank urine plus added PB, OPB; (3) sulindac and metabolites; (4) sulindac, metabolites plus added PB, OPB; (5) diclofenac and metabolites; (6) diclofenac, metabolite plus PB, OPB; (7) fenoprofen and metabolite; (8) fenoprofen, metabolite plus added PB, OPB; (9) Ibuprofen and metabolites; (10) ibuprofen, metabolites plus added PB, OPB. Darkened spots are PB, cross-hatched spot is diclofenac and PB. Dotted line is spotting origin.

reactions with visualization reagents, but PB and the sulindac sulfide spots were always separate. The sulfone metabolite spot was located between PB and OPB and was easily observable (Figure 3). The yellow sulindac parent and sulfide spots were observed in urine up to 24 hr postdose, whereas the sulfone spot was visible up to 48 hr postdose (Figure 4).

Diclofenac

Diclofenac extracted well in both systems, whereas its small metabolite spot was seen only in System No. 1. This diclofenac metabolite was often obscured by the minor OPB spot (Figure 3). Diclofenac parent reacted rapidly with Mandelin's Reagent to give a very distinctive brick-red color, whereas the brown Mandelin's reaction of PB occurred more slowly. The immediate Mandelin's reaction of diclofenac allowed the parent drug to be detected up to 48 hr postdose (Figure), even in the presence of comigrating PB (Figure 3).

Fenoprofen

A fenoprofen metabolite appeared below PB, as an easily visible coral pink spot after Mandelin's Reagent and heat (Figure 3). This spot first appeared at 8 hr postdose and was easily distinguishable in the presence or absence of PB for up to 48 hr postdose (Figure 4).
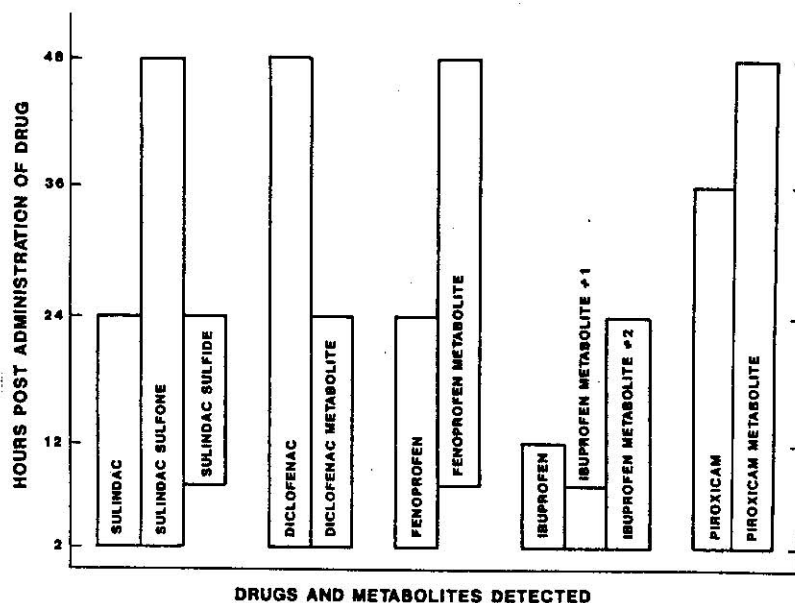

FIGURE 4.   Duration of detectability by HPTLC of acidic drugs and their metabolites. Horses were dosed orally with 2 g of the parent drug. Urine samples were taken at 2, 4, 8, 12, 24, 36, 48, and 72 hr postadministration. The urine samples were extracted, and the drugs and metabolites were detected by HPTLC.

TABLE 4 Visualization Responses

| | | | | | | | REACTIONS | | | | |
| | | | | | | SYSTEM No. 1 | | | | SYSTEM No. 2 | |
DRUG	R_f	VISIBLE	LUV	SUV	MANDELIN + Δ	LUV POST-MANDELIN	LUDY TENGER	H_2O_2 + Δ	FOLIN-DENIS	NH_3	EHRLIC
PB	0.69	—	—	Clear	Brown	Dark	Brown, dark	Brown	Gray	Gray	Pink
OPR	0.30	—	—	Clear	Gold	Dark	Red	Brown	Gray	Gray	Pink
Sulindac	0.30	Yellow	Dark	Clear	White	Orange	Orange	Tan	Gray	Gray	Gray
Sulindac sulfide	0.67	Yellow	Dark	Clear	Yellow	—	Orange	Yellow	Gray	Dark yellow	Yellow
Sulindac sulfone	0.42	Yellow	Dark	Clear	Yellow	Dark	Orange	Yellow	Grayish yellow	Dark yellow	Yellow
Diclofenac	0.70	—	—	Clear	Brick red	Dark	—	Tan	Grayish yellow	Dark yellow	Yellow
Diclofenac metabolite	0.09	—	—	Clear	Grey	—	—	—	Brown	Gray	Pink
Fenoprofen	0.69	—	—	Faint	White	Pale yellow	—	—	Gray	Gray	Pink
Fenoprofen metabolite	0.64	—	—	—	Coral	—	Gray	—	Gray	Gray	Gray
Ibuprofen	0.71	—	—	Faint	White	Pale yellow	Faint pink	Brown	Brown	Faint gray	Faint pink
Ibuprofen metabolite No. 1	0.50	—	—	—	White	Pale yellow	Faint pink	Gray	Gray	Faint pink	—
Ibuprofen metabolite No. 2	0.65	—	—	—	—	Pale yellow	—	—	—	—	—
Piroxicam	0.44	Yellow	Green	Clear	White	Green	White	Brown	Gray	Faint gray	Faint gray
Piroxicam metabolite	0.13	Yellow	—	Clear	—	White	—	Gray	Deep gray	Gray	Gray

Ibuprofen

Ibuprofen had two urine metabolites appearing as distinctive pale-yellow spots under UV after treatment with Mandelin's Reagent. Metabolite No. 1 appeared well below PB, but could be seen only up to 8 hr postdosing (Figure 4). Ibuprofen metabolite No. 2 appeared just below PB and was apparent up to 24 hr postdosing.

Other Drugs

Piroxicam was clearly visible as a green LUV spot for up to 36 hr postdose, while a piroxicam metabolite appeared in System No. 2 as a very distinctive dark-gray spot up to 48 hr postdose (Figure 4).

Both meclofenamic acid and naproxen were found unchanged postrace in urine samples. Meclofenamic acid appeared as a distinctive dark crescent above PB after treatment with Mandelin's Reagent. Naproxen was seen as a triangular spot located just below PB have a lavender reaction with Mandelin's Reagent. A possible meclofenamic acid metabolic was occasionally seen. No distinctive naproxen metabolite was observed.

DISCUSSION

Of the 20 acidic drugs in this study, 16 drugs had locations on the plate far enough removed from PB or OPB that their SUV spots could be observed in the presence of PB and OPB. Most of these drugs also had color reactions with certain of the visualization reagents that further distinguished the spots. Those drugs that, because of their R_f locations and color reactions, were easily detectable in urine samples spiked with at least 10 µg/ml of the drug and high levels of PB and OPB were piroxicam, tolmetin, zomepirac, naproxen, flunixin, furosemide, meclofenamic acid, bumetanide, ethacrynic acid, ketoprofen, and caffeine. The salicylates were not masked by PB or OPB, whereas theobromine and theophylline could not be detected by the acidic extraction procedures.

The four drugs that, because of comigration with PB or OPB, were found to have a potential for masking difficulties were diclofenac, sulindac, ibuprofen, and fenoprofen. The distinctive quality of diclofenac in the presence of PB is that diclofenac reacted immediately with Mandelin's Reagent to give the characteristic brick-red color, whereas PB is much slower to react to give its brown color. However, after an oral dose of 2 g diclofenac, diclofenac can be detected for up to 48 hr in the presence of a high concentration (25 µg/ml) of added PB. Young et al. (1981) reported that parent diclofenac was readily detected by TLC in equine urine at a level greater than 1 µg/ml 6 hr postdose, when administered at a therapeutic dosage (400 mg/horse orally).

Sulindac was found to be detectable as the highly colored (yellow) sulfone metabolite for up to 48 hr postdose, even though the parent drug may be totally obscured by OPB. Up to 24 hr postdose, the parent sulindac was found to be visible (yellow) and distinctive in the presence of OPB before the plate was subjected to visualization treatments. The presence of the visible sulfide metabolite seen from 8 to 24 hr postdose may be an added confirmation to the detection of sulindac in the urine.

Of the 20 drugs examined, ibuprofen and fenoprofen appeared to have the highest potential for masking by PB. Fenoprofen has been reported to have one major urinary metabolite in man, 4-hydroxyfenoprofen (Nash, 1979). Nash reported that when fenoprofen calcium was administered orally in man, about 45% occurred in the urine as fenoprofen and 55% as 4-hydroxyfenoprofen.

Fenoprofen was most easily detected by the metabolite occurring below and separated from the PB spot. Whereas the parent fenoprofen spot was detectable soon after administration (2–8 hr postdose), this spot was poorly visible after 8 hr postdose and barely visible in the presence of PB at 24 hr postdose. However, the fenoprofen metabolite was quite apparent as a colored (coral pink) spot from 8 to 48 hr postdose, and visualization of this spot was not diminished by the PB spot located above it.

Ibuprofen has been reported to have two major metabolites, found in horse urine, which were detectable by TLC (Evans et al., 1976; Lambert et al., 1979). Evans et al. (1976) reported that only about 3–6% of administered ibuprofen was found in the urine as the parent drug, the balance occurring as the two metabolites. In rats, dogs, baboons, and man, these metabolites are 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid and 2-[4-(2-carboxylpropyl)phenyl]propionic acid (Adams et al., 1967; Mills et al., 1973; Hutt and Caldwell, 1983).

Detection of ibuprofen was also aided by presence of metabolites, although not as much as fenoprofen. For up to 12 hr postdose, the presence of the ibuprofen parent drug caused the added PB to appear as a crescent curled under the small indistinctive ibuprofen parent drug. Of the two ibuprofen metabolites observed, only metabolite No. 2 was very useful in detection of ibuprofen. This metabolite was observable for up to 24 hr and was located in the window between PB and OPB. Ibuprofen still remains as a somewhat difficult drug to detect by these visualization methods; however, its detection does not appear to be hampered by the presence of PB.

It may be noted that even though a parent drug was not masked by the presence of PB, if this drug occurred predominately as a metabolite in the urine, PB could still interfere with the detection process. For instance, piroxicam has been reported to have one metabolite, 5-hydroxypiroxicam, in the horse and this metabolite occurs in urine at about 100-fold the concentration of the parent drug after oral dosing (Fenwick and Todi, 1985). For that reason, urines from horses dosed with, or known to have been administered on a racetrack with, piroxicam, meclofenamic acid, and naproxen were examined by our HPTLC acidic extraction method.

The piroxicam parent was found to be easily detected in urines for up to 36 hr after a 2 g oral dose of the drug. A piroxicam metabolite was also quite evident up to 48 hr postdose. Neither the parent nor the metabolite is interacted with by either PB or OPB. Meclofenamic acid and neproxen were both easily seen as the parent drugs in urine samples taken from Kentucky track horses postrace, and the urine samples were found to contain those drugs by the Kentucky Equine Testing Laboratory. These last two drugs have urine metabolites, but are more often seen as the parent drugs (Blake, 1985).

Of these drugs examined from horses administered with 2 g orally of the parent drug, it was found the metabolites play an important role in the detection of the

urine metabolites. It was also seen that piroxicam, naproxen, and meclofenamic acid, while having urine metabolites, occur in urine samples as the parent drug in high enough concentrations to be detected postrace after prerace dosing.

The detection of diclofenac, which comigrated with PB, was found not to be aided by the presence of an easily detectable metabolite. However, it was found that after administration orally of 2 g diclofenac sodium, the parent drug could be seen for up to 48 hr after dosing, if the observer were careful to examine the plate immediately after treatment with the visualization reagent. Therefore, the detection of this drug in the presence of PB is, as for all forensic chemistry, dependent on the skill and experience of the analyst.

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