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

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Abstract

The ability of phenylbutazone and oxyphenbutazone to 'mask', or interfere with, the detection of basic drugs by high-performance thin-layer chromatography (HPTLC) was investigated. Of 54 basic drugs called 'positive' since 1981 by laboratories affiliated with the Association of Official Racing Chemists, 42 did not co-migrate with phenylbutazone or oxyphenbutazone and could not therefore be masked by them. When oxyphenbutazone was added to urine samples (at 75 μg/ml), subjected to extraction for basic drugs, then run in our routine HPTLC systems no significant 'spots' due to oxyphenbutazone appeared. Masking by oxyphenbutazone therefore did not and could not occur in our test systems. When phenylbutazone was added to urine samples (at 30 μg/ml) and run in the routine HPTLC systems, phenylbutazone spots were visible under ultraviolet light and with certain sprays for visualizing basic drugs. But these spots did not interfere with routine thin-layer testing for basic drugs. It was concluded that phenylbutazone and oxyphenbutazone had no significant ability to interfere with detection of the parent forms of these basic drugs under the conditions used in these experiments.

Introduction

Phenylbutazone is a non-steroidal anti-inflammatory drug widely used to treat musculoskeletal problems in racehorses.[1] Some racing chemists hold that residues of phenylbutazone and its metabolites (oxyphenbutazone and γ-hydroxyphenylbutazone) can 'mask', or interfere with, the detection of other drugs in equine urine. Masking occurs when a substance on a thin-layer chromatographic (TLC) plate physically obscures (interferes with) the detection of a drug.[2] However the ability of phenylbutazone to mask other drugs has been controversial.

Because of the regulatory and forensic importance of the concept of masking[3] we are studying this problem. This paper deals with the potential of phenylbutazone and oxyphenbutazone to co-extract with prohibited basic drugs from urine and interfere with their detection by high-performance thin-layer chromatography (HPTLC).

The other major phenylbutazone metabolite, γ-hydroxyphenylbutazone, occurs in two interchangeable forms.[4–5] We are currently examining the role of these two forms of γ-hydroxyphenylbutazone in masking and will present these results at a later time.

Basic drugs include most of the stimulants, depressants, anesthetics, and tranquilizers used as illegal medications. This work is an experimental approach to the question of whether phenylbutazone or its metabolites can interfere with the detection of these drugs—particularly stimulant and narcotic drugs.

Materials and Methods

Drugs

The 54 drugs selected for study were those basic drugs called 'positive' by Association of Official Racing Chemists laboratories for the calendar year 1981 and thereafter as such information became available. Standard solutions of each drug (100 μg/ml) were prepared in methanol and, depending on the detectability of the drug, from 20 to 100 μl was used for spotting.

Drug extractions

Extractions followed the pattern used in the Kentucky Equine Drug Testing Program. To urine (1 ml) were added dichloromethane (4 ml) and concentrated ammonium hydroxide (4 drops). The sample was shaken on a rotorack (5 min), centrifuged (500g; 20 min), and the aqueous fraction removed. The dichloromethane fraction was evaporated to dryness under a stream of nitrogen and dissolved in dichloromethane (2 drops). The entire sample was spotted on a thin-layer plate. For work with spiked samples 1 μg/ml of the drug in question was added to the initial urine sample. In all initial screening experiments 1 ml of urine was used. In later — more critical — experiments [Tables 4–5] 3 ml of urine was used to allow comparison with routine race-track testing.

TLC procedures

The TLC techniques used were those current in the Kentucky Equine Drug Testing Program. Whatman HP-KF high-performance silica gel plates (10 × 10 cm; 200μm layer thickness) were used throughout. For spotting, methanol aliquots of the drug standard solutions were dried under nitrogen, then dissolved in dichloromethane (2 drops). Solutions were spotted using drawn-out Pasteur pipettes. Spots were applied incrementally and dried between each application using a hair dryer. Spots were placed 15 mm above the bottom of the plates and 5 mm apart to give a line of up to 19 spots per plate. Plates were developed by allowing the solvent to rise 60 mm above this line.

Solvent systems and visualization

Three systems were used to develop plates, corresponding to the screening systems used for basic drugs in the Kentucky Equine Drug Testing Program [Figure 1]. In System 1 [the 'phenothiazine' system; Table 1] plates were developed in chloroform–methanol (9 : 1). Initial visualization was by short-wave ultraviolet light (SUV; 254 nm) in a view box. The plates were sprayed with a phenothiazine location reagent consisting of ferric chloride (250 mg), ethanol (50 ml), and concentrated sulfuric acid (50 ml), then heated at low heat on a hotplate. The plates were then sprayed with Dragendorff's reagent (Whatman Co.) and exposed to nitrogen dioxide (by adding concentrated nitric acid to a beaker containing copper pellets, in a developing tank).

In System 2 [the 'benzocaine' system; Table 1] the plates were developed in ethyl acetate–methanol–acetic acid (8 : 1 : 1). After SUV visualization they were exposed to nitrogen dioxide, sprayed with *N*-1-naphthylethylenediamine location reagent, then with ninhydrin, and heated.

In System 3 [the 'narcotic' system; Table 1] the plates were developed in chloroform–methanol–propionic acid (80 : 15 : 5). After SUV visualization the plates were sprayed with a modified Folin–Denis reagent and exposed to ammonia fumes (by placing them in a tank with a beaker of concentrated ammonia solution).

Measurement and data recording

All spots were circled with a soft lead pencil at each visualization step, and the plates photocopied. The appearance, color, and R_f of each spot were recorded.

Results and Discussion

Interference by oxyphenbutazone

We assumed that approximately 75 μg/ml was the highest concentration of oxyphenbutazone likely to be found in a post-race urine sample[6–8] and determined that, from such a sample, 0.36 μg of oxyphenbutazone would be transferred through the standard extraction process onto an HPTLC plate. When this amount (0.36 μg) was spotted directly on HPTLC plates and

Figure 1 *Flow chart of basic-drug detection by HPTLC, including developing systems and visualization*

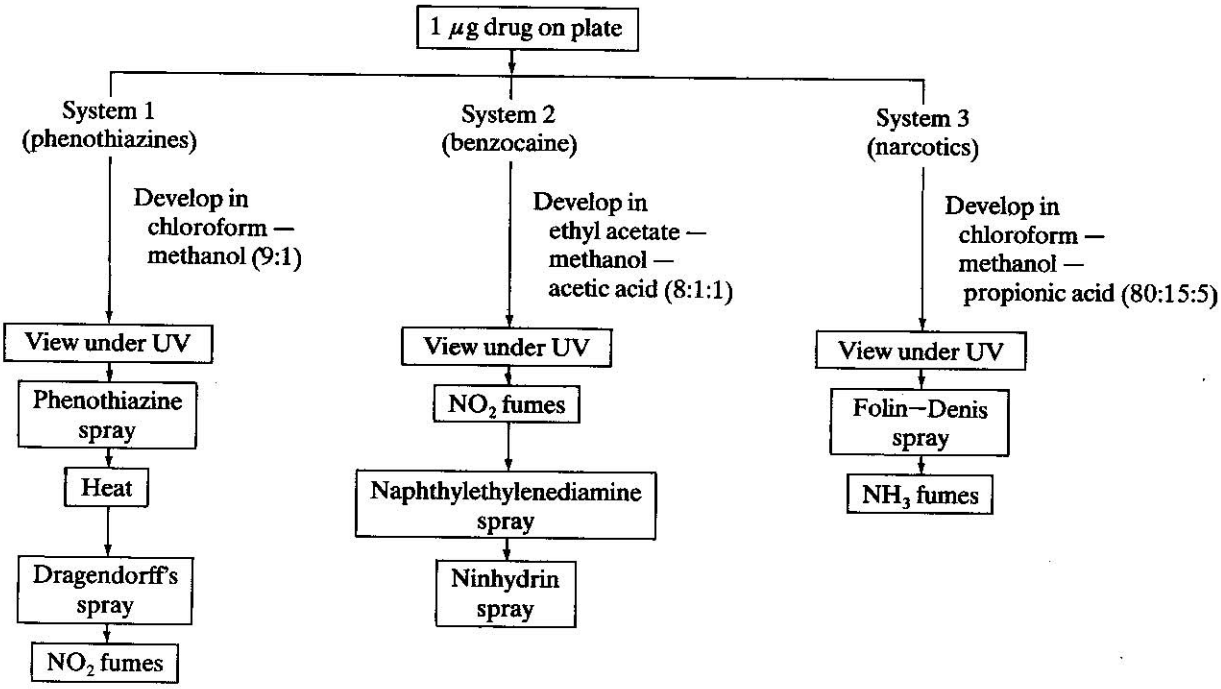

Table 1 *Basic-drug HPTLC systems*

System	Solvent	Typical detected compounds
1	Chloroform — methanol (9:1)	Phenothiazines
2	Ethyl acetate — methanol — acetic acid (8:1:1)	Benzocaine; clenbuterol
3	Chloroform — methanol — propionic acid (80:15:5)	Narcotic analgesics

run in each solvent system no spots were detected during the spray visualizations. When urine samples containing added oxyphenbutazone (75 μg/ml) were extracted and run in Systems 1 and 3 no detectable interfering spots were observed during the spray sequences. However, when this concentration of oxyphenbutazone was extracted and run in System 2 a spot yielding a blue color with an R_f very close to that of benzocaine was observed. This oxyphenbutazone spot might be confused with benzocaine (at least until sprayed) but this would lead to a false positive and would not be masking.

Thus we concluded that under the conditions of these tests the chances of masking from oxyphenbutazone were minimal.

Interference by phenylbutazone

In a survey of horses racing in Kentucky[6–8] urinary phenylbutazone concentrations of 0.1 to 30.50 μg/ml were found. Although 30-μg/ml concentrations were rare and depended primarily on the pH of the urine sample, they can nevertheless occur in post-race urine samples in Kentucky. We therefore evaluated the effects of 15- and 30-μg/ml concentrations of phenylbutazone on the detection of basic drugs.

Most drugs in this survey did not co-chromatograph with phenylbutazone and could be eliminated from consideration [Table 3]. Thus in System 2 only benzocaine is likely to cause detection problems [Table 5]. However, the color reaction

Table 2 R_f *values of phenylbutazone and oxyphenbutazone in basic-drug HPTLC systems*

System	Phenylbutazone		Oxyphenbutazone	
	R_f	*n*	R_f	*n*
1	0.83 ± 0.03	9	0.65 ± 0.05	8
	0.76 ± 0.03*	6	0.52 ± 0.06*	8
2	0.89 ± 0.02	5	0.88 ± 0.05	4
3	0.97 ± 0.00	6	0.84 ± 0.01	2

* *Small secondary spot found in System 1 only*

of benzocaine on spraying with naphthylethylenediamine is sufficiently distinct that phenylbutazone at the concentrations found in Kentucky urine samples is unlikely to cause detection problems.

In System 1 phenylbutazone gives rise to two spots: one (R_f = 0.83) is due to phenylbutazone itself; the other (R_f = 0.76) is due to a breakdown product [Table 2]. These spots may potentially interfere with five drugs which chromatograph in the same general region [Table 5].

However, the color reactions of these drugs with visualization reagents are different from those of the phenylbutazone spots. The spot with the higher R_f does not react with the phenothiazine visualization reagent and thus does not interfere with phenothiazine. Beyond this, phenothiazine is an anthelmintic agent and unlikely to be used to affect the performance of horses or, indeed, to be a prohibited medication in the United States. Further, it is from a family of agents which are usually detected as metabolites. Since metabolites commonly have smaller R_f values than parent drugs, they are even less likely to be interfered with.

The other four agents in Table 5 may co-chromatograph with the unidentified breakdown product of phenylbutazone. These agents are bupivacaine, lidocaine, diazepam, and benzphetamine. Of these four drugs only benzphetamine would probably be detected as metabolites. However, the spots are distinguishable from the minor phenylbutazone spot by their R_f values. Beyond this, the breakdown product of phenylbutazone does not react with Dragendorff's reagent and thus does not interfere with the color test for these drugs.

Figure 2 *Thin-layer chromatogram of phenylbutazone and high-*R_f *basic drugs in System 1. Urine samples were spiked with drugs, extracted, and spotted on HPTLC plates which were developed and visualized using System 1. The sample numbers correspond to:*

1 blank urine
2 phenylbutazone spike (30 μg/ml)
3 diazepam spike (4 μg/ml)
4 phenylbutazone + diazepam spike
5 lidocaine spike (4 μg/ml)
6 phenylbutazone + lidocaine spike
7 phenothiazine spike (4 μg/ml)
8 phenylbutazone + phenothiazine spike
9 benzphetamine spike (4 μg/ml)
10 phenylbutazone + benzphetamine spike
11 bupivacaine spike (4 μg/ml)
12 phenylbutazone + bupivacaine spike
13 diazepam direct (4 μg)
14 lidocaine direct (4 μg)
15 phenothiazine direct (4 μg)
16 benzphetamine direct (4 μg)
17 bupivacaine direct (4 μg)
18 phenylbutazone direct (4.8 μg)
19 phenylbutazone direct (30 μg).

Spots were marked at the end of the visualization sequence when the phenylbutazone breakdown product does not show. Except for phenothiazine, none of the drugs co-chromatographed with phenylbutazone itself.

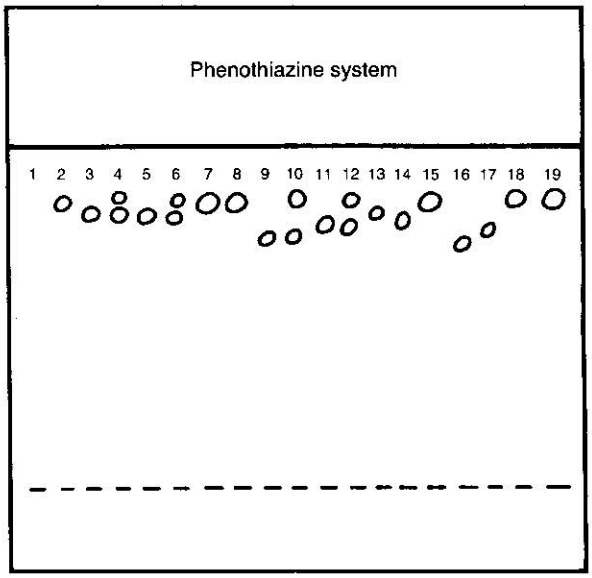

Table 3 *Drugs not masked by phenylbutazone or oxyphenbutazone, based on R_f values*

System 1

Acepromazine (0.19)
Alphaprodine (0.38)
Amitriptyline (0.38)
Amphetamine (0.12)
Chlorobutanol (0.25)
Chlorpheniramine (0.07)
Chlorpromazine (0.24)
Diphenhydramine (0.22)
Dipyrone (0.02)
Doxylamine (0.04)
Guaifenesin (0.40)
Heptaminol (0.00)
Mazindol (0.08)
Mephentermine (0.32)
Mepyramine (0.12)
Methenamine (0.33)
Methocarbamol (0.37)
Methylphenidate (0.28)
4-Methyl-5-thiazoleethanol (0.40)
Nefopam (0.35)
Nicotine (0.34)
Nicotinamide (0.24)
Oxazepam (0.41)
Paracetamol (0.28)
Pemoline (0.27)
Phenyltoloxamine (0.41)
Promazine (0.21)
Tetracaine (0.36)
Trichlormethiazide (0.29)
Trimethoprim (0.22)
Tripelennamine (0.18)
Xylazine (0.28)

System 2

Clenbuterol (0.33)
Phenylpropanolamine (0.23)
Procaine (0.05)

System 3

Apomorphine (0.17)
Butorphanol (0.22)
Levorphanol (0.25)
Morphine (0.15)
Nalbuphine (0.17)
Naloxone (0.51)
Pentazocine (0.49)

Table 4 *Drugs possibly masked by oxyphenbutazone, based on R_f values*

System 1 (Oxyphenbutazone, 0.52 and 0.65)

Benzidine (0.58)
Benzphetamine (0.75)
Bupivacaine (0.78)
Diazepam (0.72)
Dimethyl sulfoxide (0.48)
Doxapram (0.63)
Lidocaine (0.77)
Mepivicaine (0.52)
Phenothiazine (0.84)
Tetramisole (0.47)

System 2 (Oxyphenbutazone, 0.88)

Benzocaine (0.82)

System 3 (Oxyphenbutazone, 0.84)

Fentanyl (0.74)

Table 5 *Drugs possibly masked by phenylbutazone, based on R_f values*

System 1 (Phenylbutazone, 0.76 and 0.83)

Benzphetamine (0.75)
Bupivacaine (0.78)
Diazepam (0.72)
Lidocaine (0.77)
Phenothiazine (0.84)

System 2 (Phenylbutazone, 0.89)

Benzocaine (0.82)

In summary therefore, oxyphenbutazone (in the concentrations found in post-race urines of racehorses in Kentucky) *did not* extract by the basic-drug procedure we use in high enough amounts to result in significant thin-layer spots. Because of this, oxyphenbutazone cannot interfere with routine thin-layer testing for any basic drug under our test conditions.

Phenylbutazone (in the concentrations found in post-race urines in Kentucky) *did* extract by the basic-drug procedure in amounts yielding observable spots on the HPTLC plates. Although these spots chromatographed in the same areas as the spots of six drugs on the test list, the drugs were always distinguishable from phenylbutazone by their R_f values or by their reactions with sprays. Therefore, under the conditions of these tests, there was no evidence that phenylbutazone can interfere with the detection of basic drugs.

Acknowledgments

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oratories (Wilmington, DE); McNeil Pharmaceutical (Spring House, PA).

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Discussion

BLACKMAN Dr Tobin, I wish I could share your confidence. You selected fifty-four drugs from North America. I believe that's self-selecting against your problem in that there are a number of jurisdictions there where phenylbutazone is allowed. As a result, you will be selecting against those drugs that may interfere. Secondly, I believe it's naive to think that only those drugs that are found in North America may, in fact, be used. Would you care to comment?

TOBIN Well, you can help me in my research. If you can nominate a drug that is masked, or indeed name more than one, I'll be happy to test it. Jerry and I have consistently asked for guidance on individual drugs that people considered to be masked. We have never had a candidate suggested to us. Secondly, we were concerned about the state of the art in testing in North America. Your observation is quite correct that some jurisdictions permit phenylbutazone. However, some of the larger jurisdictions—for example, New York which does fifty per cent of all drug tests done in the United States—do not permit the use of phenylbutazone. And we felt that as a reasonable starting hypothesis, we would look at the drugs reported as 'positives' by AORC labs. And certainly on the basic-drug side we had virtually no problem whatsoever, and on the acidic-drug side, with a little skill, we could circumvent any possible problems. So perhaps to throw the question back... Can you nominate some drug candidates for me that you believe would give me a problem?

BLACKMAN Thank you for the question. I'm presenting it as a theoretical possibility, Tom, and I think it's one that we ought to be aware of.

TOBIN Thank you.

BEAUMIER Tom, many of us are using immunoassay techniques for detection of, say, fentanyl or etorphine. Have you seen any cross-reactivity with phenylbutazone and some of the antibodies?

TOBIN We haven't tested for that, Pierre. We have just started within the last two months or so doing immunoassays. I have no information on that whatsoever.