Effects of Phenylbutazone and Oxyphenbutazone on Acidic-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 acidic drugs by high-performance thin-layer chromatography was investigated. Of 20 acidic drugs called 'positive' since 1981 by laboratories affiliated with the Association of Official Racing Chemists, 16 did not co-migrate with phenylbutazone or oxyphenbutazone and could not therefore be masked by them.

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 see if they could be detected as their metabolites. Metabolites of fenoprofen, ibuprofen, and sulindac were detectable for at least 24 hours and detection was not interfered with by phenylbutazone or oxyphenbutazone. The metabolite of diclofenac was not as readily detected. However, although the parent drug exactly co-migrated with phenylbutazone, it reacted with a visualization reagent much more strongly and more rapidly than phenylbutazone—allowing it to be detected up to 48 hours when phenylbutazone was present.

These results suggest that these 20 acidic drugs should be readily detectable in post-race urine of horses in the presence of phenylbutazone and oxyphenbutazone, either as the parent drugs or as easily distinguishable metabolites. There is therefore no reason to believe that they can be effectively masked by phenylbutazone or its metabolites in equine urine.

Introduction

Phenylbutazone is a non-steroidal anti-inflammatory drug (NSAID) widely used to treat musculoskeletal problems in racehorses.[1] Because phenylbutazone is commonly used to treat horses in training, residues may appear in both pre- and post-race blood and urine samples taken for analysis in medication-control programs.[2–3]

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.[4]

Masking occurs when a substance on a thin-layer chromatographic (TLC) plate physically obscures (interferes with) the detection of a drug.[4] However, the ability of phenylbutazone to mask 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.[4–5] The confusion has been aggravated because, until very recently, there was no published scientific literature on the subject.

Because of the regulatory and forensic importance of the concept of masking,[5] we are studying this problem. This paper deals with the potential of phenylbutazone and oxyphenbutazone to interfere with high-performance thin-layer chromato-

graphic (HPTLC) screening for acidic drugs and their metabolites.

The other major phenylbutazone metabolite (γ-hydroxyphenylbutazone) occurs in two interchangeable forms[6–7] and their role in masking is being evaluated now.

Materials and Methods

Drug selection and preparation

Drugs selected as candidates for possible masking by phenylbutazone and oxyphenbutazone were those called 'positive' by the Association of Official Racing Chemists laboratories for the calendar years 1981 through 1983.[8–9] From this master list of 58 drugs, the 20 acidic drugs were selected for study. Standard solutions (100 or 200 μg/ml) of these 20 acidic drugs (and of sulindac sulfide and sulindac sulfone) were prepared in methanol. From 100 to 200 μl of these solutions (depending on detectability) were used for spotting as HPTLC standards.

Standard solutions of phenylbutazone and oxyphenbutazone (500 μg/ml) were prepared in a methanolic antioxidant solution (20 mg butylated hydroxytoluene and 2 g ascorbic acid in 100 ml methanol).[10] Portions of these solutions were used to spike water or equine urine at concentrations of 25 μg/ml for phenylbutazone and 50 μg/ml for oxyphenbutazone. These concentrations were near the highest values reported for phenylbutazone and oxyphenbutazone in 200 urines from thoroughbreds racing in Kentucky.[11–13]

For each of the 20 acidic drugs studied portions of the methanol standards were used to spike water or equine urine (at 10 to 40 μg/ml) either alone or along with phenylbutazone and oxyphenbutazone.

The aliquots of all methanol standards were evaporated carefully to dryness under a stream of nitrogen before adding the urine.

Dosing and sampling

Five horses (female; mixed breed) were dosed orally by stomach tube (1 drug per horse; 2 g each) with: sulindac (Merck, Sharp & Dohme, Rahway, NJ); diclofenac sodium (Ciba-Geigy, Summit, NJ); ibuprofen (Upjohn, Kalamazoo, MI); fenoprofen calcium (Eli Lilly, Indianapolis, IN); piroxicam (Pfizer, Groton, CT). Urine samples were taken by bladder catheter before treatment and post-dose (at 2, 4, 8, 12, 24, 36, 48, and 72 h).

Urine samples taken from Kentucky track horses post-race and certified by the Kentucky Equine Drug Testing Laboratory to contain naproxen or meclofenamic acid were also examined.

Drug extraction

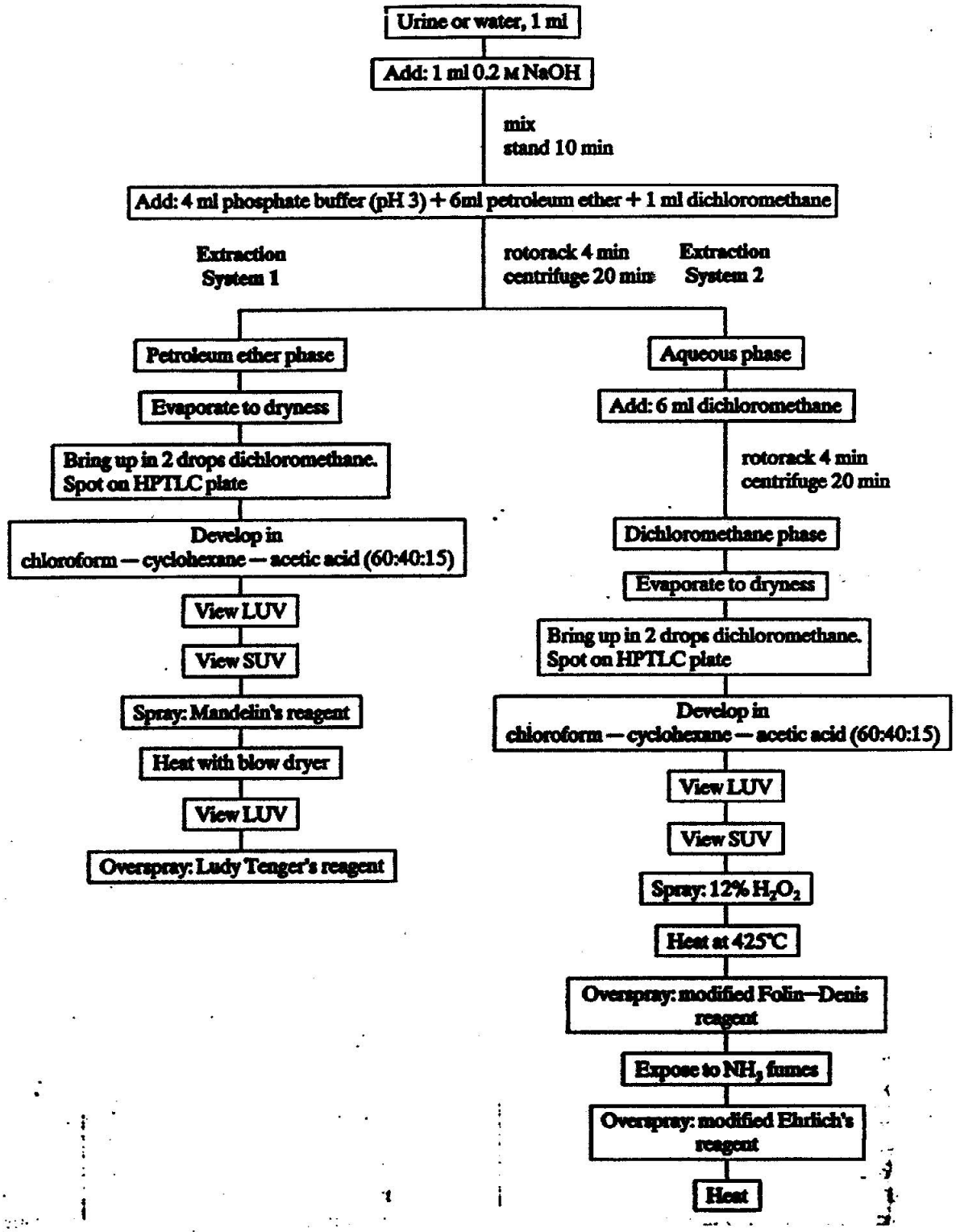
All extractions followed the methods used in the Kentucky Equine Drug Testing Program [Figure 1]. To water or urine (1 ml) was added 0.2 M sodium hydroxide (1 ml) and the 'hydrolysis' was allowed to continue 10 minutes. To the hydrolysate were added: acid phosphate buffer (saturated monobasic potassium phosphate, KH_2PO_4, in water titrated to pH 3.0 with concentrated phosphoric acid, H_3PO_4; 4 ml); petroleum ether (6 ml); dichloromethane (1 ml)—in that order. The sample was shaken on a rotorack (4 min), centrifuged (500g; 20 min), and the petroleum ether phase transferred to a clean tube for evaporation to dryness under a stream of nitrogen. The residue was dissolved in dichloromethane (2 drops) and the entire sample then spotted on a thin-layer plate. This was Extraction System 1.

The aqueous phase was re-extracted by adding dichloromethane (6 ml), shaking on a rotorack (4 min), and centrifuging (500g; 20 min). The aqueous fraction was then discarded. The dichloromethane fraction was evaporated to dryness under a stream of nitrogen and the residue dissolved in dichloromethane (2 drops). The entire sample was spotted on a second thin-layer plate. This was Extraction System 2.

Thin-layer chromatography 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; 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, then dissolved in dichloromethane (2 drops). These dichloromethane standard solutions, or the urine or water extracts redissolved in dichloromethane, were spotted on the plates using drawn-out Pasteur pipettes. Spots were applied incrementally and dried between each application using a blow dryer. Spots were

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



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 40 mm above this line. The developing solvent used with both extraction methods was chloroform–cyclohexane–acetic acid (60:40:15).

Visualization techniques

The HPTLC plate from Extraction System 1 was viewed under long-wave ultraviolet light (LUV; 365 nm) in a view box immediately after the plate was removed from the developing tank (before the acetic acid, which activates visualization, had evaporated). The plate was then viewed under short-wave ultraviolet light (SUV; 254 nm), sprayed with Mandelin's reagent [Table 1], dried briefly with a blow dryer, viewed again under LUV, and oversprayed with Ludy Tenger's reagent [Table 1].

Table 1 *Visualization reagents*

Ludy Tenger's reagent

Flask A: 1 g bismuth subcarbonate is dissolved by swirling in 3 ml concentrated hydrochloric acid.

Flask B: 6 g sodium iodide is dissolved in 50 ml distilled water. (Flask B is graduated.)

The contents of Flask A are poured slowly into Flask B with mixing. Flask B is then slowly, and with continuous swirling, filled to the 100-ml mark with distilled water. Formation of a black precipitate, due to the too rapid addition of water, must be avoided.

Ehrlich's reagent (modified)

100 mg 4-dimethylaminocinnamaldehyde is dissolved in a mixture of 50 ml 6 M hydrochloric acid and 50 ml methanol.

Mandelin's reagent

Concentrated sulfuric acid is diluted 1:1 with distilled water. 1 g ammonium metavanadate is dissolved in 100 ml of this sulfuric acid.

Folin–Denis reagent (modified[24])

10 g sodium tungstate dihydrate and 2 g 12-molybdosilicic acid (Pfaltz & Bauer, Stamford, CT) are added to a mixture of 5 ml phosphoric acid and 50 ml distilled water and refluxed for 2 hours. After cooling, the solution is diluted to 100 ml with distilled water.

The HPTLC plate from Extraction System 2 was also first viewed 'wet' under LUV followed by viewing under SUV. The plate was then sprayed with hydrogen peroxide (12%) followed by heating face-down on a hotplate (425°C). After cooling, the plate was oversprayed with modified Folin–Denis reagent [Table 1]. It was then exposed to ammonia fumes (by being placed in a tank with a beaker of concentrated ammonia solution), air-dried, and oversprayed with modified Ehrlich's reagent [Table 1], followed by heating on a hotplate.

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

Phenylbutazone and oxyphenbutazone

The R_f values of phenylbutazone, oxyphenbutazone, and the 20 acidic parent drugs tested are shown in Table 2. Only one significant spot was observed for phenylbutazone (R_f=0.69). For oxyphenbutazone just one spot (R_f=0.30) was observed by direct spotting but after extraction a second, much smaller spot (R_f=0.10) was also often seen. The 20 drugs studied gave only one spot each. All R_f values were highly reproducible under the conditions outlined.

The responses of parent acidic drugs to the visualization techniques are shown in Table 3. Phenylbutazone added to water or urine (25 μg/ml) extracted well in both extraction systems and resulted in a circular-to-oval spot appearing dark under SUV. In System 1 phenylbutazone reacted with Mandelin's reagent to give a brown spot which was dark under LUV. This spot visibly darkened when treated with Ludy Tenger's reagent. In System 2 phenylbutazone turned brown after treatment with hydrogen peroxide and heat. This spot turned grey when oversprayed with Folin–Denis reagent and exposed to ammonia fumes. Treatment with Ehrlich's reagent and heat resulted in a pink-to-purple spot.

Oxyphenbutazone added to water or urine (50 μg/ml) extracted well in System 1 but not quite

Table 2 R_f *values of acidic drugs*

Drug	Mean R_f ± SD	Number of plates observed
Phenylbutazone	0.69 ± 0.01	26
Oxyphenbutazone		
(major)	0.30 ± 0.01	26
(minor)	0.10 ± 0.01	9
Meclofenamic acid	0.71 ± 0.02	4
Ibuprofen*	0.71 ± 0.01	19
Diclofenac*	0.70 ± 0.02	11
Fenoprofen*	0.69 ± 0.01	12
Naproxen	0.66 ± 0.02	4
Ketoprofen	0.65 ± 0.03	8
Salicylic acid	0.59 ± 0.02	8
Thiosalicylic acid	0.59 ± 0.04	6
Diflunisal	0.58 ± 0.02	6
Zomepirac	0.49 ± 0.02	8
Tolmetin	0.45 ± 0.03	5
Piroxicam	0.42 ± 0.01	2
Sulindac†	0.30 ± 0.01	10
Bumetanide	0.25 ± 0.02	6
Ethacrynic acid	0.24 ± 0.03	6
Theophylline	0.17 ± 0.02	4
Caffeine	0.16 ± 0.02	4
Flunixin	0.14 ± 0.01	4
Furosemide	0.14 ± 0.01	4
Theobromine	0.07 ± 0.01	4

* *Drugs potentially masked by phenylbutazone*
† *Drugs potentially masked by oxyphenbutazone*

as well in System 2. The major oxyphenbutazone spot (R_f = 0.30) was circular and appeared dark under SUV when extracted from water or urine in System 1. Oxyphenbutazone SUV spots were usually obscured by urine background in System 2. The minor oxyphenbutazone spot (R_f = 0.10) was not visible under SUV. The major and minor oxyphenbutazone spots gave similar color reactions in both systems except that the minor oxyphenbutazone spot was usually less intense. In System 1 oxyphenbutazone reacted with Mandelin's reagent to give a bright gold which turned brown on heating. This spot was dark under LUV. Treatment with Lady Tenger's reagent resulted in a red spot. In System 2 the oxyphenbutazone spots became tan after treatment with hydrogen peroxide and heat. They turned grey when oversprayed with Folin–Denis reagent and exposed to ammonia fumes. Treatment with Ehrlich's reagent and heat resulted in orange-to-red spots.

Detection as parent drug

The 20 acidic drugs studied included diuretics, methylxanthine stimulants, and several types of NSAIDs: these included oxicams, fenamates, acetic and propionic acid derivatives, and salicylates.

Oxicams Piroxicam added to water or urine (20 μg/ml) extracted well in Systems 1 and 2. The R_f position in the window between phenylbutazone and oxyphenbutazone and the green LUV spot clearly distinguished piroxicam from phenylbutazone and oxyphenbutazone.

Fenamates Meclofenamic acid was clearly detectable in both systems (R_f = 0.71) appearing as a narrow crescent just above phenylbutazone. The bright blue LUV spot and the dark crescent just above phenylbutazone after treatment with Mandelin's reagent distinguished meclofenamic acid from phenylbutazone.

In System 1 flunixin yielded a readily distinguishable spot (R_f = 0.14) above the minor oxyphenbutazone spot. Flunixin's reaction with Lady Tenger's reagent characterized this drug.

Acetic acid derivatives The drugs studied from this group were: tolmetin; zomepirac; sulindac; diclofenac. Tolmetin and zomepirac were distinguishable from phenylbutazone and oxyphenbutazone by their R_f values and reactions with Mandelin's reagent.

Sulindac extracted well in both systems yielding a spot (R_f = 0.30) which co-migrated with the major oxyphenbutazone spot (R_f = 0.30) [Figure 2]. The yellow sulindac spot was clearly seen in visible light. Sulindac reacted with several of the visualization reagents but these reactions were obscured by oxyphenbutazone added to urine (50 μg/ml).

Diclofenac extracted well in both systems but yielded a spot (R_f = 0.70) which co-migrated with phenylbutazone (R_f = 0.69) [Figure 2]. Diclofenac in urine (10 μg/ml) was characterized by its brick-red reaction with Mandelin's reagent.

Propionic acid derivatives The drugs studied in this category were: naproxen; ketoprofen; ibuprofen; fenoprofen. Naproxen was characterized by a triangular

Table 3 *HPTLC visualization of acidic drugs*

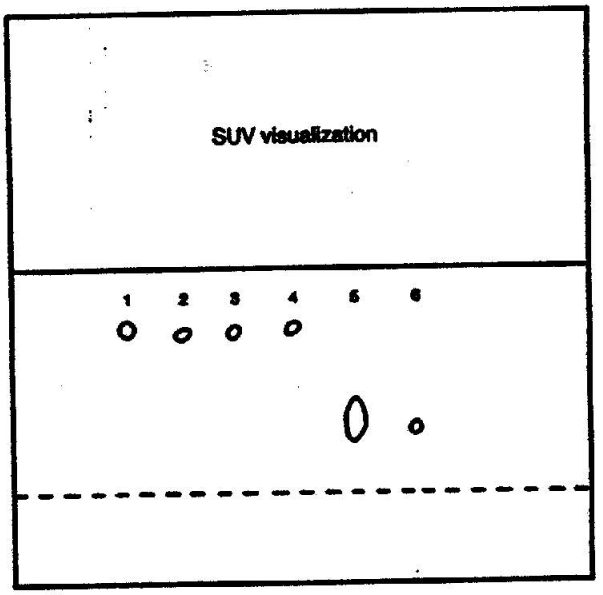
Drug	R_f	Visible	System 1 LUV	System 1 SUV	System 1 Mandelin + heat	System 1 LUV post-Mandelin	System 1 Ludy Tenger	System 2 H_2O_2 + heat	System 2 Folin–Denis	System 2 Ammonia	System 2 Ehrlich + heat
Pyrazolon derivatives											
Phenylbutazone	0.69	—	—	Clear	Brown	Dark	Brown, dark	Brown	Grey	Grey	Pink, purple
Oxyphenbutazone	0.30	—	—	Clear	Gold	Dark	Red	Brown	Grey	Grey	Pink
Oxicams											
Piroxicam	0.42	Yellow	Green	Clear	White	Green	White	—	—	Faint grey	Faint grey
Fenamates and analogues											
Meclofenamic acid	0.71	—	Bright blue	Clear*	Dark violet	Blue	Faint pale	Brown	Grey	Grey	Pink, brown
Flunixin	0.14	—	Dark	Clear	—	—	Orange	Brown	Grey	Grey	Faint orange
Acetic acid derivatives											
Tolmetin	0.45	—	Dark	Clear	Red	Dark	Orange	—	—	Grey	—
Zomepirac	0.49	—	Dark	Clear	Pink	Dark	Orange	Tan	Grey	Faint grey	Faint purple
Sulindac	0.30	Yellow	Dark	Clear	White	Orange	Orange	Tan	Grey	Grey	Grey
Diclofenac	0.70	—	—	Clear	Brick red	Dark	—	Tan	Brown	Grey	Pink
Propionic acid derivatives											
Naproxen	0.66	—	—	Clear†	Violet	Dark	Faint pale	Brown	Grey	Grey	Grey
Ketoprofen	0.65	—	—	Clear	Tan	Deep yellow	Pale orange	Brown	Grey	Grey	Grey
Ibuprofen	0.71	—	—	Faint	White	Pale yellow	Faint pink	Brown	Grey	Faint grey	Faint pink
Fenoprofen	0.69	—	—	Faint	White	Pale yellow	—	—	Grey	Grey	Grey
Salicylates											
Salicylic acid	0.59	—	Pale blue	Clear	Blue-pink	Pale	Pale orange	NA	NA	NA	NA
Diflunisal	0.58	—	Green	Clear	Blue-grey	Green	Pale	Tan	Grey	Grey	Grey
Thiosalicylic acid	0.59	—	Blue	Diffuse	NA	NA	NA	—	Grey	Grey	—
Diuretics											
Furosemide	0.14	—	Blue	Clear	NA	NA	NA	—	—	—	Pink
Ethacrynic acid	0.24	—	—	Small	—	—	—	Tan	Bluish white	Grey	Grey
Bumetanide	0.25	—	Bright blue	Clear	Lavender	—	Faint pale	Yellow	Grey	Grey	Bright purple
Methylxanthines											
Caffeine	0.16	—	—	Clear	—	—	Pink	—	—	Yellow	Orange
Theobromine	0.07	—	—	Clear	NA	NA	NA	—	—	—	—
Theophylline	0.17	—	—	Clear	—	—	Pink‡	—	—	Grey	—

NA *Not applicable (not extracted in system)*
— *No reaction, no color*
* *Crescent in presence of phenylbutazone*
† *Triangle in presence of oxyphenbutazone*
‡ *Extracts poorly in System 1*

Theobromine	0.07	—	—	Clear/	NA	NA	NA	—
Theophylline	0.17	—	—	Clear	—	—	Pink‡	Grey

NA *Not applicable (not extracted in system)*
— *No reaction, no color*
• *Crescent in presence of phenylbutazone*
† *Triangle in presence of oxyphenbutazone*
‡ *Extracts poorly in System 1*

Figure 2 *Thin-layer chromatogram of phenylbutazone, oxyphenbutazone, and co-migrating acidic parent drugs. Dichloromethane solutions of the drugs were spotted directly onto the plate at approximately 50 μg drug per spot. The sample numbers correspond to: (1) phenylbutazone, (2) diclofenac, (3) fenoprofen, (4) ibuprofen, (5) oxyphenbutazone, (6) sulindac. The plate was visualized under SUV. On this plate, these* R_f *values were observed: phenylbutazone, 0.74; oxyphenbutazone, 0.33; diclofenac, 0.71; fenoprofen, 0.71; ibuprofen, 0.74; sulindac, 0.29.*



spot which reacted with Mandelin's reagent and appeared just below phenylbutazone.

Ketoprofen extracted well in both systems and yielded a dark spot under SUV (R_f=0.65) just below phenylbutazone (R_f=0.69). This drug was also distinguished from phenylbutazone by its color with Mandelin's reagent under LUV after heating.

Ibuprofen and fenoprofen extracted poorly in both systems. When added to urine (40 μg/ml) only faint SUV spots (ibuprofen: R_f=0.71; fenoprofen: R_f=0.69) which closely migrated with phenylbutazone (R_f=0.69) were observed [Figure 2]. Both drugs reacted with Mandelin's reagent to give pale lavender spots undetectable when phenylbutazone was present. After fenoprofen was treated with Mandelin's reagent and heated it gave a pale, luminescent spot under LUV just above phenylbutazone. Ibuprofen, after Mandelin's reagent and heat, appeared as a faintly visible white spot just above phenylbutazone. No ibuprofen or fenoprofen reaction was distinctive enough to allow identification with phenylbutazone present.

Salicylates The salicylates examined in this study were: salicylic acid; diflunisal; thiosalicylic acid. All three salicylates had identical R_f values (R_f=0.59) and were visible in the window between phenylbutazone and oxyphenbutazone. Therefore the drugs were not masked by phenylbutazone or oxyphenbutazone.

Salicylic acid is, however, an endogenous component of urine samples from both racehorses and nonracing research horses, as it is found in grass and hay. Therefore, when urine samples were spiked with diflunisal or thiosalicylic acid these drugs were masked by endogenous salicylate (but this is a separate issue).

Diuretics The diuretics examined were: furosemide; ethacrynic acid; bumetanide. Furosemide did not extract in System 1 but extracted well in System 2. Furosemide was best distinguished from phenylbutazone and oxyphenbutazone by its R_f and blue LUV spot.

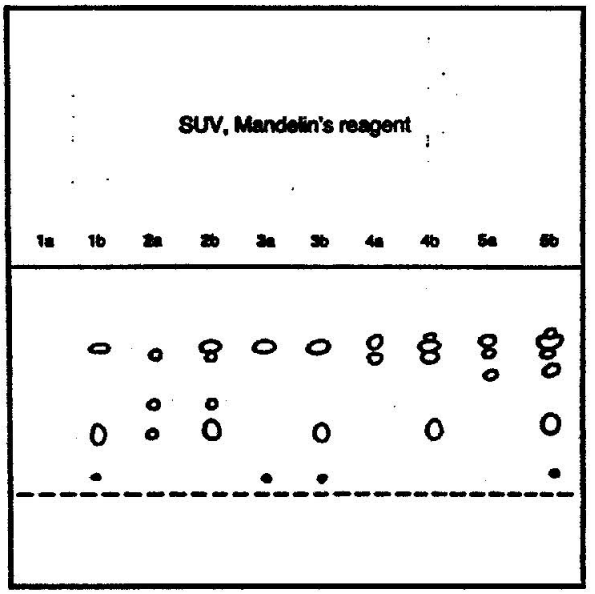
Ethacrynic acid (10 μg/ml) extracted well in both systems yielding a small spot (R_f=0.24) below the major oxyphenbutazone spot (R_f=0.30). Ethacrynic acid gave a characteristic reaction with Folin–Denis reagent when the plate was heated to 425°C.

Bumetanide (10 μg/ml) extracted well in both systems with a bright blue LUV spot (R_f=0.25) below the major oxyphenbutazone spot. Bumetanide was best distinguished from phenylbutazone and oxyphenbutazone by the LUV spot and its reactions with Mandelin's and Ehrlich's reagents.

Methylxanthines The methylxanthines included in this study were: caffeine; theobromine; theophylline. Caffeine extracted well in both systems, yielding a spot at R_f=0.16. Caffeine was best distinguished from phenylbutazone and oxyphenbutazone by its R_f and its reactions with Lady Tenger's reagent and Ehrlich's reagent.

Theobromine and theophylline did not extract in System 1 but extracted better in System 2 (theobromine: R_f=0.07; theophylline: R_f=0.17). However, neither drug reacted well with any of the visualization reagents.

Figure 3 *Thin-layer chromatogram of phenylbutazone and oxyphenbutazone, co-migrating drugs and their metabolites. The plate was spotted with dichloromethane solutions of extracts (System 1) of horse urine 8 hours post-dose (a) unspiked and (b) spiked with phenylbutazone and oxyphenbutazone. The sample numbers correspond to: (1) blank urine, (2) sulindac and metabolites, (3) diclofenac and metabolite, (4) fenoprofen and metabolite, (5) ibuprofen and metabolites. The plate was visualized under SUV and with Mandelin's reagent.*

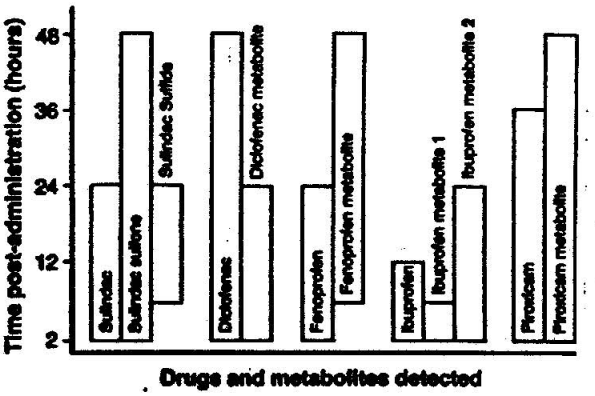


Detection as metabolites

The drugs sulindac, diclofenac, fenoprofen, and ibuprofen co-migrated with phenylbutazone or oxyphenbutazone, so these drugs were administered to horses and the R_f values of their major metabolites 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 how long these drugs and their metabolites were detectable by HPTLC.

Sulindac Sulindac and two of its metabolites extracted well in both systems yielding bright yellow spots appearing as dark spots under LUV and SUV. The parent drug (R_f = 0.30) co-migrated with the major oxyphenbutazone spot (R_f = 0.30) and the sulfide metabolite spot (R_f = 0.67) appeared just below the phenylbutazone spot (R_f = 0.69). Oxyphenbutazone

Figure 4 *Detection periods of acidic drugs and their metabolites by HPTLC. Horses were dosed orally with 2 g of the parent drug. Urine samples were taken after 2, 4, 8, 12, 24, 36, 48, and 72 hours. The urine samples were extracted and the drugs and metabolites were detected by HPTLC.*



completely obscured the parent sulindac spot during reactions with visualization reagents but the phenylbutazone and sulindac sulfide spots were always separate. The sulfone metabolite spot (R_f = 0.42) was located between phenylbutazone and oxyphenbutazone and was easily observable [Figure 3]. The yellow sulindac parent and sulfide spots were observed in urine up to 24 hours post-dose; the sulfone spot was visible up to 48 hours [Figure 4].

Diclofenac Diclofenac (R_f = 0.70) extracted well in both systems but its small metabolite spot was seen only in System 1. This diclofenac metabolite (R_f = 0.09) was often obscured by the minor oxyphenbutazone spot (R_f = 0.10) [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 phenylbutazone occurred more slowly. The immediate Mandelin's reaction of diclofenac allowed the parent drug to be detected up to 48 hours [Figure 4] even with co-migrating phenylbutazone (R_f = 0.69) [Figure 3].

Fenoprofen Fenoprofen (R_f = 0.69) was not readily detectable by the visualization techniques employed. Reaction with Mandelin's reagent resulted in a faintly visible white spot which was pale under LUV. This spot was small, appeared just above the phenylbutazone spot (R_f = 0.69) [Figure 3], and could be seen up to 24 hours.

A fenoprofen metabolite (R_f = 0.64) appeared be-

low phenylbutazone as an easily visible coral-pink spot after Mandelin's reagent and heat [Figure 3]. This spot first appeared at 8 hours post-dose and was easily distinguishable (with or without phenylbutazone present) for up to 48 hours.

Ibuprofen The reactions of ibuprofen (R_f = 0.71) were similar to fenoprofen [Table 4]. A small ibuprofen spot appeared just above phenylbutazone (R_f = 0.69) causing the phenylbutazone spot to form a crescent around the ibuprofen spot [Figure 3]. This spot was apparent only up to 12 hours.

Ibuprofen had two urine metabolites appearing as distinctive light yellow spots under LUV after treatment with Mandelin's reagent. Metabolite 1 (R_f = 0.50) appeared well below phenylbutazone but could be seen only up to 8 hours [Figure 4]. Metabolite 2 (R_f = 0.65) appeared just below phenylbutazone and was visible up to 24 hours.

Other drugs Piroxicam (R_f = 0.42) was clearly visible as a green LUV spot for up to 36 hours; a piroxicam metabolite (R_f = 0.13) appeared in System 2 as a very distinctive dark grey spot up to 48 hours [Figure 4].

Both meclofenamic acid (R_f = 0.71) and naproxen (R_f = 0.66) were found unchanged post-race in urine samples. Meclofenamic acid appeared as a distinctive dark crescent above phenylbutazone when treated with Mandelin's reagent. Naproxen was seen as a triangular spot just below phenylbutazone with a lavender reaction to Mandelin's reagent. A possible meclofenamic acid metabolite was occasionally seen (R_f = 0.45). No distinctive naproxen metabolites were observed.

Discussion

Of the 20 acidic drugs in this study, 16 were located on the plate far enough removed from phenylbutazone and oxyphenbutazone so that their SUV spots were distinct from those of phenylbutazone and oxyphenbutazone. Most also had color reactions with certain visualization reagents that further distinguished the spots. Drugs which (because of their R_f values and color reactions) were easily detectable in urine samples spiked with at least 10 μg/ml of the drug and high levels of phenylbutazone (25 μg/ml) and oxyphenbutazone (50 μg/ml) were: piroxicam; tolmetin; zomepirac; naproxen; flunixin; furosemide; meclofenamic acid; bumetanide; ethacrynic acid; ketoprofen; caffeine. The salicylates were not masked by phenylbutazone or oxyphenbutazone but by endogenous salicylate. Theobromine and theophylline were not detected well by the acidic extraction procedures.

The four drugs which (because they co-migrated with phenylbutazone or oxyphenbutazone) could potentially be masked were: diclofenac; sulindac; ibuprofen; fenoprofen. Diclofenac (added to urine at 10 μg/ml), although co-migrating exactly with phenylbutazone [Figure 2], posed no detection problems—even with as much as 50 μg/ml phenylbutazone added to the urine. The distinctive quality of diclofenac is that it reacts immediately with Mandelin's reagent to give a characteristic brick-red whereas phenylbutazone is much slower to react, giving a brown color. In early post-administration samples (2–12 h) the brick-red dominated over the brown of added phenylbutazone indefinitely. However in later samples (24–48 h) the brown phenylbutazone color became predominant after a few minutes. Thus the detectability of diclofenac with phenylbutazone present depends on the relative concentration of the two drugs in the urine sample (determined by the administration levels of the drugs and time of the sampling). After an oral dose of diclofenac (2 g) the drug can be detected for up to 48 hours with high concentrations (25 μg/ml) of phenylbutazone present.

Young *et al.*[14] reported that parent diclofenac was readily detected by TLC in equine urine (at more than 1 μg/ml) 6 hours after a therapeutic dose (400 mg orally) and that a urinary metabolite of diclofenac (which also reacted with Mandelin's reagent) was present. We also observed a small metabolite spot but it was often obscured by an oxyphenbutazone breakdown product (the oxyphenbutazone minor spot).

Sulindac (added to urine at 20 μg/ml as the parent drug) showed as a bright yellow spot in visible light. Some urine samples, however, give a faint yellow streak on the HPTLC plate due to unknown endogenous materials, and low levels of sulindac might not be readily observable against such a "dirty" background. Sulindac co-migrated with oxyphenbutazone [Figure 2] and was completely obscured in all visualization techniques by oxyphenbutazone (added to urine at 50 μg/ml). Thus low levels of sulindac parent drug would most probably be masked by oxyphenbutazone. However, the potential for oxyphenbutazone to mask sulindac is greatly reduced by its metabolites: two urinary metabolites have been reported—the sulfone and

Table 4 *HPLC visualization of acidic drugs and metabolites*

Drug	R_f	Visible	LUV	SUV	System 1			System 2			
					Mandelin + heat	LUV post-Mandelin	Ludy Tenger	H_2O_2 + heat	Folin–Denis	Ammonia	Ehrlich + heat
Phenylbutazone	0.69	—	—	Clear	Brown	Dark	Brown, dark	Brown	Grey	Grey	Pink, purple
Oxyphenbutazone	0.30	—	—	Clear	Gold	Dark	Red	Brown	Grey	Grey	Pink
Sulindac	0.30	Yellow	Dark	Clear	White	Orange	Orange	Tan	Grey	Grey	Grey
Sulindac sulfide	0.67	Yellow	Dark	Clear	Yellow	—	Orange	Yellow	Greyish yellow	Dark yellow	Yellow
Sulindac sulfone	0.42	Yellow	Dark	Clear	Yellow	Dark	Orange	Yellow	Greyish yellow	Dark yellow	Yellow
Diclofenac	0.70	—	—	Clear	Brick red	Dark	—	Tan	Brown	Grey	Pink
Diclofenac metabolite	0.09	—	—	—	Grey	—	—	—	—	—	—
Fenoprofen	0.69	—	—	Faint	White	Pale yellow	—	—	Grey	Grey	Grey
Fenoprofen metabolite	0.64	—	—	—	Coral	—	Grey	—	—	—	—
Ibuprofen	0.71	—	—	Faint	White	Pale yellow	Faint pink	Brown	Grey	Faint grey	Faint pink
Ibuprofen metabolite 1	0.50	—	—	—	—	Pale yellow	Faint pink	—	—	—	—
Ibuprofen metabolite 2	0.65	—	—	—	—	Pale yellow	—	—	—	—	—
Piroxicam	0.42	Yellow	Green	Clear	White	Green	White	—	—	Faint grey	Faint grey
Piroxicam metabolite	0.13	Yellow	—	Clear	—	—	—	Brown	Grey	Deep grey	Grey

— *No reaction, no color*

the sulfide—in rats, dogs, monkeys,[15] guinea pigs, and rabbits.[16]

Sulindac was detectable as the highly colored (yellow) sulfone metabolite for up to 48 hours even when the parent drug was totally obscured by oxyphenbutazone. Up to 24 hours, the parent sulindac was visible (yellow) and distinctive with oxyphenbutazone present, before the plate was sprayed. The visible sulfide metabolite seen from 8 to 24 hours post-dose may be extra confirmation for the detection of sulindac in the urine.

Fenoprofen and ibuprofen (of all the 20 drugs examined) appeared the most likely to be masked by phenylbutazone. Both closely migrated with phenylbutazone [Figure 2] and were difficult to detect even without phenylbutazone present. Although these drugs reacted with Mandelin's reagent to give a visible bluish-white color fairly well distinguishable when they were added alone to urine (at 20 μg/ml), phenylbutazone completely masked them when they were added up to 40 μg/ml. When the spots treated with Mandelin's reagent were heated both drugs responded with weakly visible reactions: fenoprofen palely luminescent under LUV; ibuprofen faintly white. Both spots appeared just above phenylbutazone when extracted with phenylbutazone from spiked urine. These reactions were not very distinctive and could easily be obscured by phenylbutazone at low drug concentrations.

Fenoprofen has been reported to have one major urinary metabolite in man: 4-hydroxyfenoprofen.[17] When fenoprofen calcium was administered orally in man approximately 45 per cent appeared in the urine as fenoprofen, 55 per cent as 4-hydroxyfenoprofen.

Fenoprofen was most easily detected by the metabolite which occurred below and separate from the phenylbutazone spot. Though the parent fenoprofen spot was detectable soon after administration (2–8 h) this spot was poorly visible after 8 hours and barely visible with phenylbutazone present at 24 hours. The fenoprofen metabolite, however, was readily apparent as a colored (coral pink) spot from 8 to 48 hours and visualization of this spot was not diminished by the phenylbutazone spot above it.

Ibuprofen has been reported to have two major metabolites in horse urine detectable by TLC.[18–19] Only 3 to 6 per cent of administered ibuprofen was found in the urine as the parent drug with the rest 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.[20–22]

Ibuprofen detection was also aided by metabolites, although not as much as fenoprofen was. For up to 12 hours the added phenylbutazone appeared as a crescent curled under the small, indistinctive ibuprofen parent drug. Of the two ibuprofen metabolites observed, metabolite 2 was very useful in detecting ibuprofen. This metabolite was observable for up to 24 hours and was located in the window between phenylbutazone and oxyphenbutazone. Ibuprofen remains a somewhat difficult drug to detect by these visualization methods but its detection does not seem to be hampered by phenylbutazone.

The diuretics bumetamide and ethacrynic acid (added to urine at 10 μg/ml) were readily detectable. The R_f values for both drugs (approx. 0.25) were sufficiently less than the major oxyphenbutazone spot's R_f to prevent any masking problem. These drugs may occur in track urine samples at levels as low as 50 ng/ml urine which could make them difficult to detect. However, it is unlikely that phenylbutazone or oxyphenbutazone would affect their detectability whatever their concentrations, and low levels of these drugs might be difficult to detect regardless of phenylbutazone or oxyphenbutazone use.

The methylxanthines—except for caffeine—were not readily extractable in either extraction system although theophylline was seen occasionally in System 1. These drugs require re-extraction of the final aqueous fraction by a neutral extraction method.

The three salicylates (salicylic acid; thiosalicylic acid; diflunisal) have identical R_f values (0.59) which fall in the window between phenylbutazone and oxyphenbutazone. Thus there is no masking problem with phenylbutazone or oxyphenbutazone. However, a substantial background level of salicylate was found with track horses and pastured research horses which made detection of administered doses of any salicylate impossible.

Even when parent drugs are not masked by phenylbutazone, phenylbutazone could still interfere with detection if they occur predominately as metabolites in the urine. For instance, piroxicam has been reported to have one metabolite (5-hydroxypiroxicam) in the horse which occurs in urine at approximately 100-fold the concentration of the parent drug after oral dosing.[23]

For this reason, urines from horses dosed with piroxicam—or known to have been administered on a racetrack with meclofenamic acid or naproxen—were examined using our HPTLC acidic extraction method. Piroxicam parent was found to be easily detected in urines for up to 36 hours after an oral dose (2 g). A piroxicam metabolite was also readily evident up to 48 hours. Phenylbutazone and oxyphenbutazone did not interfere with the parent or its metabolite. Meclofenamic acid and naproxen were both easily seen as the parent drugs in urine samples taken from Kentucky track horses post-race and found to contain those drugs by the Kentucky Equine Drug Testing Laboratory. Although these two drugs have urine metabolites, in our experience they are more often seen as the parent.

For the drugs administered to horses (2 g orally) it was found that the metabolites play an important role in detection. It was also found that piroxicam, naproxen, and meclofenamic acid—although they have urine metabolites—occur in urine samples as the parent drug (whose detection is not affected by phenylbutazone or oxyphenbutazone) in high enough concentrations to be detected post-race after pre-race dosing. Diclofenac, which co-migrated with phenylbutazone, had no easily detectable metabolite to aid detection. However, after diclofenac sodium was administered (2 g orally) the parent drug could be seen for up to 48 hours if the observer was careful to examine the plate immediately after treatment with the visualization reagent. Therefore, detecting this drug when phenylbutazone is present—as indeed all forensic analysis—depends on the chemist's skill and experience.

Acknowledgments

Sources of the drug standards included: Sigma Chemical Co., St. Louis, MO (furosemide, naproxen, ketoprofen, ibuprofen, theobromine, thiosalicylic acid, caffeine, salicylic acid, piroxicam, theophylline, ethacrynic acid); Ciba-Geigy, Summit, NJ (phenylbutazone, oxyphenbutazone, diclofenac); Hoffmann-LaRoche, Nutley, NJ (bumetanide); Eli Lilly, Indianapolis, IN (fenoprofen); McNeil Pharmaceutical, Fort Washington, PA (tolmetin, zomepirac); Merck, Sharp & Dohme, Rahway, NJ (diflunisal, sulindac, sulindac sulfide, sulindac sulfone); Schering, Bloomfield, NJ (flunixin); Warner-Lambert, Ann Arbor, MI (meclofenamic acid).

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Discussion

STEVENSON I have a question that goes back to your sample preparation. I'm a little concerned that you're hydrolysing right away prior to extraction. How long do you leave your urines with the sodium hydroxide?

BLAKE We add one millilitre two-tenths molar sodium hydroxide to one millilitre of urine. This is left for ten minutes prior to the addition of phosphate buffer and initial extraction with petroleum ether–dichloromethane.

STEVENSON Are all your acidic urine extracts treated in this way—you don't have any straight extracts? You're probably well aware of the degradation of barbiturates in sodium hydroxide after exposure to it for a very short time and, although barbiturates are not very common in doping these days, there's still a possibility of a sedative doping there.

BLAKE Yes, you're right. I would think that we'd pick up some of the barbiturates in our basic extract (and I'd have to go through that with you in some detail). Also, the blood sample corresponding to the urine is extracted at pH 5, which would recover the barbiturates, if present.

STEVENSON Oh, so you've got a neutral stage of extraction that you think might cope with it?

BLAKE Yes, there are many more extractions that we do. In fact, although I'm showing only two extracts here, we actually do a third extraction with this particular one mil of urine and that's a neutral extraction with ethyl acetate.

STEVENSON Prior to, or after, the hydrolysis step?

BLAKE This is after. This is the final extraction, but I think many of the barbiturates probably would appear in our neutral–base extract (and I'd have to go through that with you).

STEVENSON Yes, I'd be interested to know if this kind of system—this kind of extraction—would actually be sufficient to degrade them, or if they still would be detected. The parent compound can be detected in horse urine for several of the barbiturates at least for a few hours after administration.

BLAKE Yes, that's correct.

STEVENSON Thank you.

JOHNSTON I just want to ask whether your technical paper which will appear in the *Proceedings* will give the formulations of your locating reagents.

BLAKE Yes, the paper does list them, Gerry. I think all the sprays which I've cited here are listed in the paper.

JOHNSTON Thank you.

DELBEKE Jerry, is it really necessary to do alkaline hydrolysis for the NSAIDs?

BLAKE It is very important, particularly when you're starting with one millilitre of urine. The majority of these non-steroidal anti-inflammatories, particularly those containing carboxylic acid groups, apparently are conjugated. We're not absolutely certain whether all are conjugated with glucuronic acid, but an appreciable number are conjugated with glucuronic acid as an ester conjugate and it is necessary to do the hydrolysis prior to extraction. Otherwise the detectability is greatly reduced.

DELBEKE Yes, and the second question is how do you confirm a piroxicam 'positive'?

BLAKE Piroxicam?

DELBEKE Yes.

BLAKE The metabolite or the parent drug?

DELBEKE The parent drug.

BLAKE By GC/mass spec, although I don't recall whether it's necessary to derivatize this or not. I'd have to look at the data. We've looked at both parent and metabolite by mass spectrometry and I know that we had to derivatize the metabolite, but I don't recall the conditions for the parent.

DELBEKE Thank you.