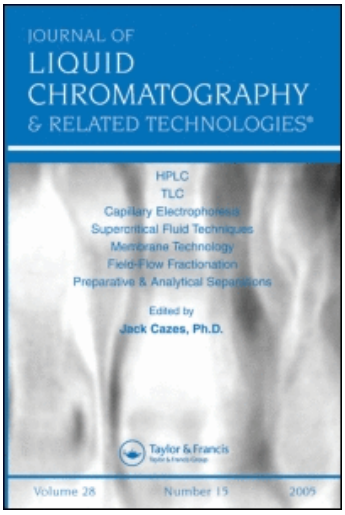

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Simultaneous Determination of Flunixin, Phenylbutazone, Oxyphenbutazone and γ-Hydroxyphenylbutazone in Equine Plasma by High-Performance Liquid Chromatography: With Application to Pharmacokinetics

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SIMULTANEOUS DETERMINATION OF FLUNIXIN, PHENYLBUTAZONE, OXYPHENBUTAZONE AND γ-HYDROXYPHENYLBUTAZONE IN EQUINE PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: WITH APPLICATION TO PHARMACOKINETICS

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ABSTRACT

A high performance liquid chromatographic method was developed for the simultaneous determination of flunixin, phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone in equine plasma. Samples of plasma or sera were deproteinated by addition of acetonitrile containing the internal standard naproxen. The concentration step consisted of taking an aliquot of deproteinated plasma, evaporating under nitrogen to dryness and redissolving in mobile phase. The extracts were chromatographed on a Spherisorb 5 μm ODS column using an isocratic mobile phase of methanol (30% v/v), acetonitrile (20% v/v) and pH 3.0 1% acetate buffer (50% v/v) at a flow rate of 1.2 ml/min using naproxen as the internal standard. The detection limit for flunixin, phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone was 50 ng/ml.

The developed chromatographic method was applied to the determination of equine nonsteroidal anti-inflammatory treatment. Plasma samples from clinically treated horses administered flunixin and phenylbutazone simultaneously are reported. Effect of different anticoagulants used in sampling is reported.

INTRODUCTION

Flunixin Meglumine (3-Pyridine carboxylic acid, 2 [[2-methyl-3 trifluromethyl] phenyl] amine) is an analgesic agent with anti-inflammatory and antiprostaglandin synthetase activities which has been approved for use in horses. Recently, flunixin has been used in the management of endotoxin-induced cardiovascular derangements associated with equine intestinal ischemia (1-3). Although physical improvement is seen immediately after and for the duration of flunixin therapy, little is done to correct the underlying cause of the condition. Consequently, the clinical signs upon which the veterinarian must rely to make the diagnosis are masked and referal of patients requiring emergency surgery is delayed. More recently, we have noticed the simultaneous use of phenylbutazone and flunixin further exacerbating the problems. A rapid, reliable method for determining extent of prior-to-clinic treatment with nonsteroidal anti-inflammatory agents may help in further cases or therapeutic management.

Recently, reports have been published on the simultaneous analysis of some anti-inflammatory agents in human serum and plasma (4, 5). These papers present a general method for the analysis of many agents but point out the necessity of developing a separation scheme which addresses the particular need of each assay. The concurrent use of phenylbutazone and flunixin appears to be increasing in veterinary practice and to determine pretreatment and proper therapy, an assay which determines therapeutic and subtherapeutic concentrations of phenylbutazone, oxyphenbutazone, and flunixin in icteric horse plasma is required. In this paper we describe the extraction, concentration and chromatographic analysis of these drugs. Results obtained in clinical cases of intestinal ischemia treated with nonsteroidial anti-inflammatories are also presented.

MATERIALS AND METHODS

Reagents

Acetonitrile and methanol were HPLC grade (Baker Chemical Co., Phillipsburg, N. J.). Oxyphenbutazone, γ-hydroxyphenylbutazone (γ-OH) and phenylbutazone reference standards were gifts from Ciba-Geigy (Ciba-Geigy Corp., Summit N. J.). Flunixin Meglumine reference standard was a gift from Schering Corporation (Schering Corp., Bloomfield, N. J.). Naproxen was a gift from Syntex Corporation (Syntex Corp., Palo Alto, CA.).

Instrumentation and Quantitation

An Altex model 110A HPLC pump with a 254 nm fixed wavelength absorbance detector (Altex model 153) was used with a strip chart recorder. Signals were read as peak heights above the base line. A pellicular ODS 30 μm, 5 cm guard column coupled with an analytical column of Spherisorb 5 μm, ODS I 25 cm (Universal Scientific Inc., Atlanta, GA) was eluted with a degassed mobile phase of 30% v/v acetonitrile, 20% v/v methanol and pH 3.0 acetate (1%) buffer at a flow rate of 1.2 ml/min.

Peak heights were measured by a recorder-integrator (Hewlett-Packard 3390A, Hewlett-Packard, Atlanta, GA.). Peaks were identified by comparison of retention times to samples spiked with authentic standards. Under the conditions described the elution times for γ-OH, oxyphenbutazone, naproxen, flunixin and phenylbutazone were 3.5, 5.8, 7.7, 9.0 and 14.2 min. respectively. Quantitation of each compound was achieved using the peak ratio method with respect to the internal standard, naproxen.

Sampling and Extraction Procedure

Blood was drawn into heparinized syringes and centrifuged at 1000 xg at 4C for 10 minutes. Plasma was pooled from healthy horse samples for preparation of the standard curves. Clinical samples were collected in the same way except that selected samples were

collected with sodium fluoride (NaF), citrate-phosphate-dextrose (CPD), ethylenediaminetetraacetic acid (Na_2EDTA) or no anti-coagulants. Plasma or serum samples were stored at -10C as 2.5 ml aliquots until use. All samples were thawed and two 1 ml aliquots taken for extraction.

To 1 ml of plasma (or serum) 4 ml of acetonitrile containing naproxen (250 ng/ml) was added to precipitate the plasma proteins (6). Samples were vortexed for 30 seconds and then centrifuged for 15 minutes at 1000 x g. From the supernatant a 4 ml aliquot was taken and evaporated to dryness under a stream of nitrogen at 37C. The residue was redissolved in 500 µl of the HPLC mobile phase and a portion was withdrawn to rinse and load the 50 µl loop for injection.

One normal, thoroughbred mare (454 kg) was fitted with a 12 gauge cannula inserted into the right jugular vein. Phenylbutazone (4.4 mg/kg) and flunixin (1.1 mg/kg) were mixed in a syringe and administered. Blood samples (10 ml) were withdrawn at 0 min. and 5, 10, 15, 30, 45, 60, min., 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8, 12, 16, 20 and 24 hr post administration. Blood samples were immediately transferred to the heparinized tubes, refrigerated, centrifuged, separated and frozen. Other samples were drawn from clinical cases at the time of admission.

RESULTS

Sensitivity, Linearity, Reproducibility and Recovery

Standard solutions containing 1 µg/ml, 10 µg/ml and 100 µg/ml of flunixin, phenylbutazone, γ-OH and oxyphenbutazone each in acetonitrile/methanol 50% were prepared. These three solutions were used to spike known amounts of drug into empty test tubes. To tubes containing .05, .10, .20, .25, .40, .50, 1.0, 2.0, 2.5, 5.0, 10.0 and 25.0 µg of each drug 1 ml of pooled normal plasma or 1 ml of mobile phase was added. Figure 1 shows the chromatograms obtained from injections of: 1) spiked mobile phase, 2) spiked

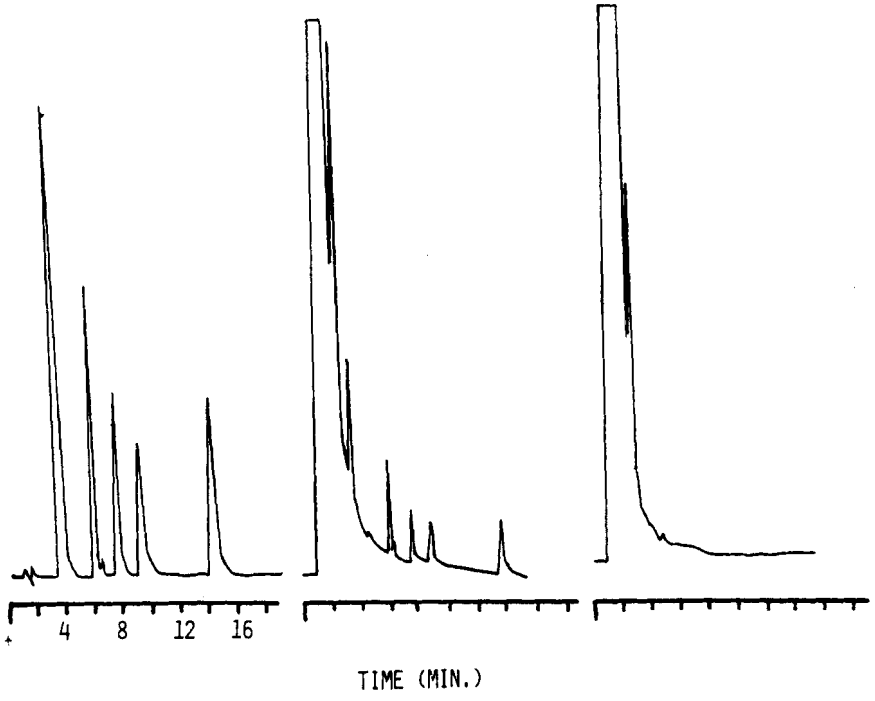

Figure 1. Chromatograph of; a) mobile phase spiked with 10 µg/ml each of γ-hydroxyphenylbutazone, oxyphenbutazone, naproxen, flunixin and phenylbutazone (.08 AUFS), b) extracted normal plasma spiked with 1 µg/ml of each drug (.02 AUFS), c) extracted normal plasma blank (.02 AUFS).

plasma carried through the extraction procedure, and 3) blank plasma carried through the extraction procedure. The peak eluting at 6.4 minutes was identified as the lactone form of γ-hydroxyphenylbutazone which exists in equilibrium with the γ-OH form. This lactone form, which accounts for approximately 4% of the total γ-OH present, appears in both spiked and experimental samples but was not quantitated in this study. The chromatograph of the blank plasma shows the absence of interferring peaks for γ-OH, naproxen, flunixin and phenylbutazone but shows a component of horse plasma which elutes before oxyphenbutazone. This peak fused with the

oxyphenbutazone peak at lower concentrations but the peak of interest was still able to be quantitated.

It was necessary to calculate recoveries by comparing spiked plasma to spiked mobile phase because: 1) recoveries from distilled-deionized water or 0.1 M phosphate buffer (pH 7.4) gave lower absolute and relative (to naproxen) recoveries than that obtained from plasma, 2) injection of the extract containing 4 volumes acetonitrile gave excessive broadening of the peaks and subsequent decreases in peak heights and 3) solubilization of the drugs by pure methanol, water or acetonitrile was incomplete. Absolute recoveries calculated by comparing spiked plasma to spiked mobile phase yielded: 99±2% for oxyphenbutazone, 96±3% for naproxen, 97±4% for flunixin, 96±3% for phenylbutazone and 98±2% for the γ-OH. Relative to the internal standard, naproxen, the recoveries were: 100±2% for oxyphenbutazone, 99±2% for flunixin, 97±2% for phenylbutazone and 100±2% for γ-OH.

Figure 2 shows the standard curves prepared from spiked plasma samples. Each point represents the average of three to six separation determinations. Table 1 lists the linear regression parameters for this peak height versus added drug concentration data.

The overall precision and accuracy of the method for each compound are represented in Table 2. Presented are the mean concentrations calculated, standard deviations and number of samples. The precision and accuracy were tested in three ways: 1) six different plasma samples were spiked with 5 µg of each compound followd by extraction and analysis the same day, 2) eight different aliquots of an actual sample from a dosed horse were analyzed the same day and 3) a spiked sample (5 µg each compound) was analyzed once a week for 5 weeks.

For this extraction, concentration and chromatographic procedure the minimum detectable limit, twice the signal to noise ratio, was 50 ng/ml for each compound.

The amounts of extraneous components carried through the extraction procedure are very important when concentrating the sample

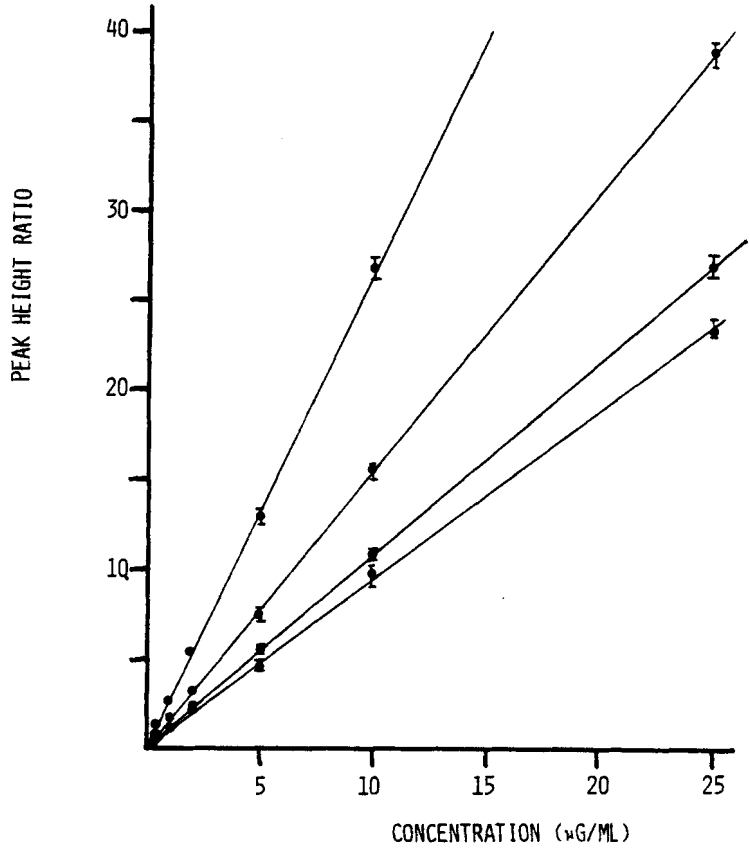

Figure 2. Calibration curves constructed for each compound in plasma. Lower concentrations not shown but included in regression functions included in Table 1.

TABLE 1

Linear Regression Parameters for the Drug Calibration Curves Presented in Figure 2.

Compound	Slope	Intercept	Correlation Coefficient
γ-OH	2.631	-.041	.9995
Oxyphenbutazone	1.541	.024	.9994
Flunixin	1.162	.035	.9992
Phenylbutazone	.9359	-.030	.9997

TABLE 2

Precision and Accuracy of Method for Different Samples

Sample		γ-OH	Oxyphen-butazone	Fluni-xin	Phenyl-butazone
1) Spiked Plasma (5 μg)	mean concentration	4.94	4.96	4.84	4.85
	std. dev.	.13	.10	.26	.16
	n	6	6	6	6
2) Plasma Sample (60 minute)	mean concentration	5.25	.42	5.31	12.23
	std. dev.	.10	.01	.14	.57
	n	8	8	8	8
3) Spiked Plasma (5 μg repeated)	mean concentration	4.99	4.84	4.81	4.90
	std. dev.	.11	.23	.17	.13
	n	10	10	10	10

to obtain maximum sensitivity. In the dosed horse, 30 and 60 minutes post administration, samples were collected in different anticoagulants. Standard blood collection tubes containing: 1) no anticoagulants, 2) NaHeparin, 3) Na_2EDTA, 4) NaF and 5) CPD were used for collection of samples. All samples were separated and stored in the same way except the samples in the tubes containing no anticoagulants were allowed to clot at room temperature before separation and storage.

All anticoagulants produced the same absolute and relative extraction ratios. However, the sample which was allowed to clot produced higher relative extractions due to lower absolute amounts of naproxen recovered. Each of the samples gave acceptable, peak free, blanks for the analysis of naproxen, flunixin and phenylbutazone except CPD which produced excessive solvent front tailing. Samples taken in the EDTA tubes proved to be the cleanest and this became especially important when measuring low levels of γ-OH and

oxyphenbutazone where heparin samples gave peaks which fused with the compounds of interest.

The volume of acetonitrile used for deproteinization was found to be critical; volume ratios of less than 4:1 gave much dirtier extractions which made quantitation of lower concentrations difficult. Also, methanol and acetone were used to precipitate the proteins but failed to give samples as clean as the acetonitrile treatment.

Blood Levels and Pharmacokinetic Data

The log plasma concentration versus time course of flunixin and phenylbutazone obtained in a single horse after simultaneous IV administration of flunixin (1.1 mg/kg) and phenylbutazone (4.4 mg/kg) is shown in Figure 3. These doses are recommended by the manufacturer and commonly used in clinical practice. The plasma concentration versus time curves for γ-OH and oxyphenbutazone are shown in Figure 4.

Clearly, this data suggests that a two compartment model best fits the data derived from this horse for both flunixin and phenylbutazone. While the distribution component of the phenylbutazone disposition is shallow, frequent sampling at the early times allows for its delineation. Flunixin shows a more pronounced distribution phase which requires six to eight hours for equilibration. Applying two compartment model fitting to this data the pharmacokinetic parameters, presented in Table 3, are calculated. Data for the first 15 minutes are omitted from these calculations as this appears to be the time required for complete mixing of the drug and blood after I. V. administration (see Figure 3).

DISCUSSION

The procedures described allow for the rapid and sensitive assay of flunixin, phenylbutazone, oxyphenbutazone, γ-hydroxyphenylbutazone and naproxen in plasma samples. In developing the assay it became apparent that not only the choice of mobile phase

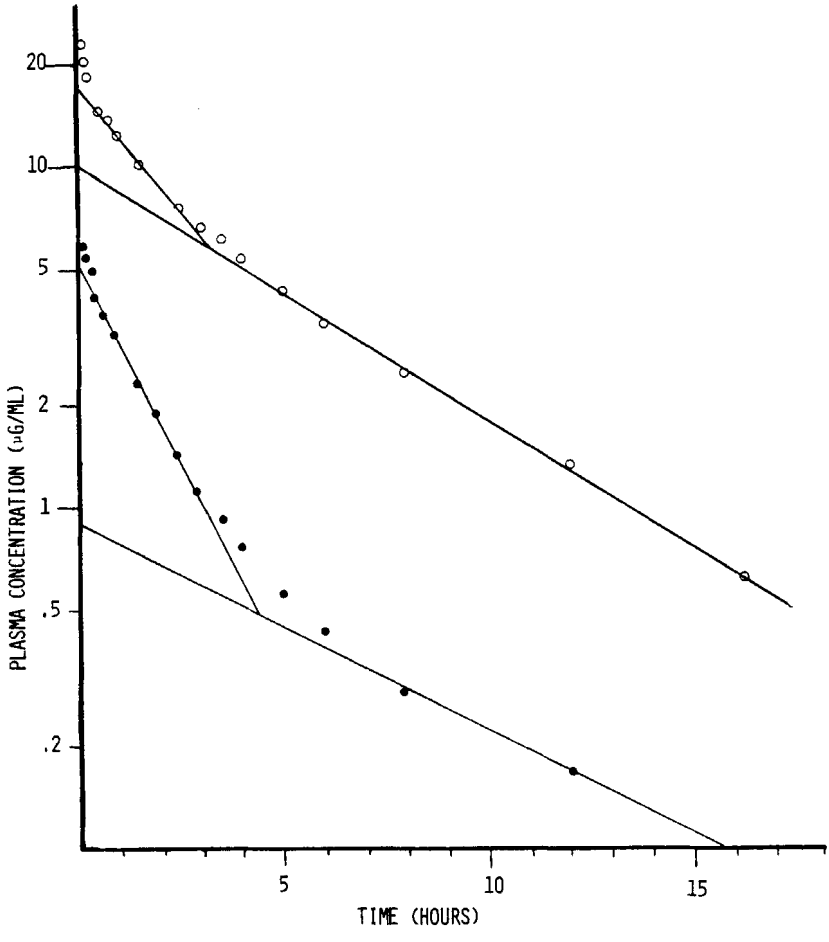

Figure 3. Semilog plot of plasma concentrations; (○) - phenylbutazone, (●) - flunixin. Lines drawn represent best fit of data to a two compartment pharmacokinetic model, 10 and 20 hr samples used for computations not shown.

composition but the sampling procedure and protein precipitation procedure are all extremely important when seeking the maximum sensitivity. The sensitivity obtained with this assay provides a useful tool in studying the pharmacokinetics of flunixin.

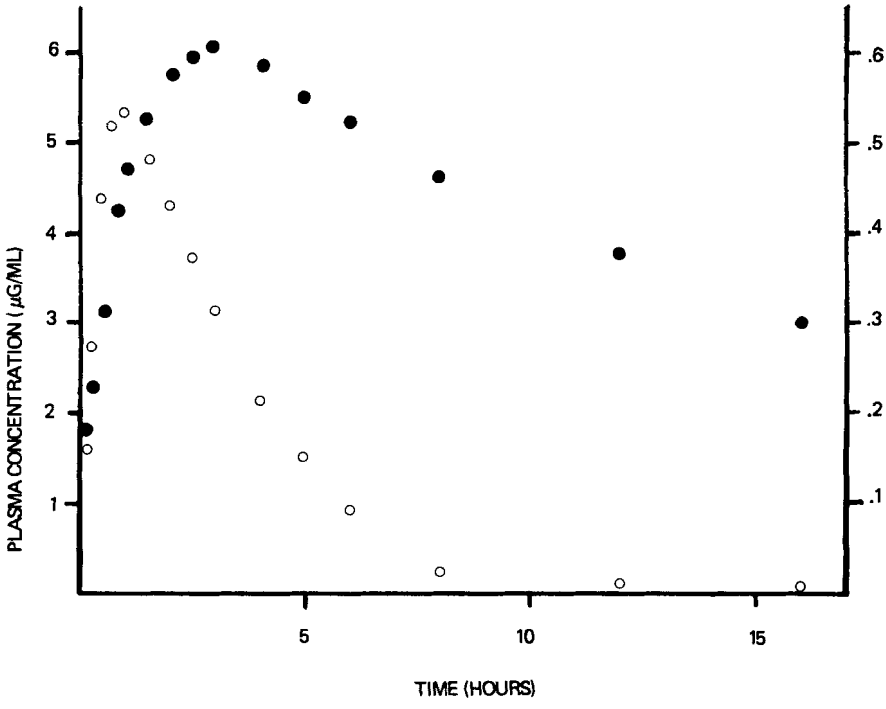

Figure 4. Linear plot of the major metabolites appearing in plasma from phenylbutazone; (○) - γ-hydroxyphenylbutazone (scale on right hand axis 0-6 µg/ml), (●) - oxyphenbutazone (scale on left 0-.6 µg/ml).

Previous studies on the pharmacokinetics of flunixin in horses report a single compartment model with an elimination half-life of 1.6 hours (2). These data were derived using an assay with a sensitivity of 2 µg/ml (7). Although the data derived here is from a single horse it agrees quite well with previous studies if data greater than 1 µg/ml are considered, i.e., one compartment model with an elimination half-life of 1.4 hours. The increased assay sensitivity allows delineation of the second compartment for flunixin. The importance of this second compartment in light of multiple dosing and minimum effective concentration is currently being studied in our laboratories.

TABLE 3

Pharmacokinetic Parameters Derived from the Two Compartment Treatment of the Experimental Data

Parameter	Flunixin	Phenylbutazone
Dose, mg/kg	1.1	4.4
β , hr^{-1}	.145	.172
$t_{\frac{1}{2}}$ elim., hr	4.8	4.0
α, hr^{-1}	.636	.624
A, µg/ml	4.61	6.05
B, µg/ml	.94	10.16
Vd, ℓ/kg	.552	.372
Vc, ℓ/kg	.198	.271
k_{21}, hr^{-1}	.221	.455
k_{10}, hr^{-1}	.417	.236
k_{12}, hr^{-1}	.143	.105

In a random sampling of clinic cases admitted to the University Veterinary Teaching Hospital for colic treatment, two of eight horses showed signs of pretreatment with flunixin. Plasma concentrations were measured to be 0.10 µg/ml and 0.50 µg/ml at the time of admission. Still another horse admitted for observation and subsequently diagnosed as a case of phenylbutazone toxicity presented no measureable levels of phenylbutazone upon admission. Oxyphenbutazone (650 ng/ml) and γ-hydroxyphenylbutazone (50 ng/ml) were measured in this sample; both decreased to undetectable levels (<50 ng/ml) within twenty four hours. The clinical consequences of prior-to-clinic treatment with nonsteroidal anti-inflammatory agents will be discussed elsewhere.

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