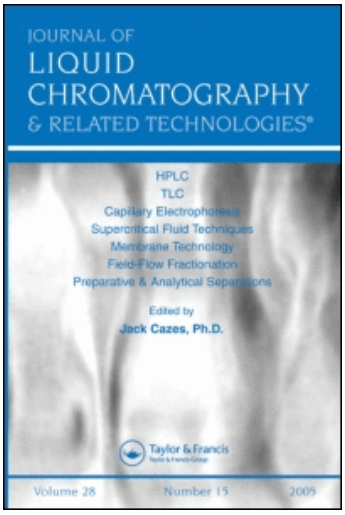

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Determination of Acetaminophen and Phenacetin in Porcine Microsomal Samples

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ABSTRACT

A simple, quick, and sensitive high performance liquid chromatography (HPLC) analysis of phenacetin and acetaminophen in porcine hepatic microsome samples is described. Chromatography was performed on a YMC-Pack ODS-AQ column using a gradient mobile phase consisting of 0.05% phosphoric acid (pH 3) : methanol. UV detection was measured at 254 nm. The procedure produced a linear curve for the concentration range 10–1500 ng/mL and had a limit of detection of 5 ng/mL for both compounds. This assay produced accurate and repeatable quantification of acetaminophen and phenacetin.

Key Words: Phenacetin; Acetaminophen; HPLC; Microsome.

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INTRODUCTION

The cytochrome P450 monooxygenase enzyme system is important in terms of its catalytic versatility and the sheer number of compounds it detoxifies or activates to reactive intermediates.[1,2] P450 enzymes are located in virtually all tissues however, the largest concentration is located in the liver endoplasmic reticulum (microsomes). These enzymes play a pivotal role in drug metabolism. They activate xenobiotics including drugs to toxic and/or tumorigenic metabolites, aid in determining intensity and duration of action of drugs, and detoxify xenobiotics. Therefore, it is essential to establish the activity and regulation of the P450 system for species selected for pharmacological and toxicological studies. In addition, impaired drug disposition in food-producing animals may lead to changes in residue levels of veterinary drugs and other xenobiotics in edible tissues, milk, or eggs. There is also concern about the increase in development of drug resistant bacterial strains in food animals. Much of the information known about P450s comes from studies conducted in rodents. While very little is known about P450s in animals used for food. The pig is becoming a viable alternative to traditional non-rodent species in pharmacological and toxicological testing;[3] however, information on the P450 system for this species is limited.

The hepatic P-450 enzymes in microsomes are mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P-450 mediated xenobiotic metabolism, individual forms of P450 have been found to catalyze specific reactions with certain substrates. These activities are designated as probes for specific P450 enzyme action. To date, at least one marker activity exists for the majority of human P450 enzymes.[4] Phenacetin-O-deethylation to acetaminophen (Fig. 1) is one method used to characterize cytochrome P450 enzyme CYP1A2 activity. Several high performance liquid chromatography (HPLC) methods have been developed to measure acetaminophen and phenacetin in biological fluids and microsomes.[5–14] Many microsome methods involve the use of liquid–liquid extractions and evaporation,[5,8–11] while one method[14] uses extraction cartridges. The majority of the methods also involve use of human or rat microsomes, but not porcine microsomes.

We describe an easy and efficient method for analysis of acetaminophen and phenacetin, which may facilitate characterization of P450 metabolism in the pig.

EXPERIMENTAL

Chemicals

Methanol (HPLC grade), phosphoric acid (reagent grade), magnesium chloride (enzyme grade), potassium phosphate (enzyme grade), and

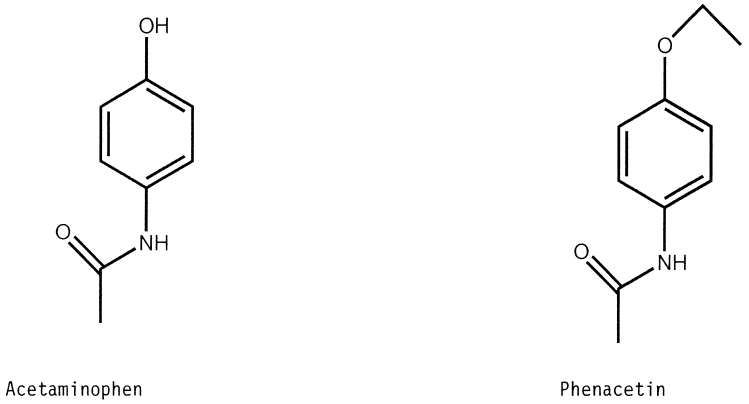

Figure 1. Structures of phenacetin and acetaminophen.

ethylenediaminetetraacetic acid (EDTA) (enzyme grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phenacetin, acetaminophen, glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), β-nicotinamide adenine dinucleotide phosphate (NADP) and trimethoprim (TMP), the internal standard, were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Stock standard solutions of phenacetin (100, 5, and 1 μg/mL) and acetaminophen (100, 5, and 1 μg/mL) were prepared in methanol and stored at 4°C. These solutions were stable for six months. Working standards were prepared fresh daily by dilution of the stock standards. Stock standard solutions of TMP (50 and 25 μg/mL) were prepared in methanol and stored at 4°C. These solutions were also stable for six months. Phenacetin is light sensitive and all solution containers were wrapped in aluminum foil.

Apparatus and Chromatographic Conditions

The analytical system consisted of a 626 solvent delivery system, a model 717 WISP autosampler, a 996 scanning UV detector, and a computer equipped with Millennium software (Waters, Milford, MA, USA). The column was a YMC-Pack ODS-AQ (5 μm, 6 × 150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford, MA, USA).

The mobile phase consisted of a mixture of (A) 0.05% phosphoric acid pH 3.0 and (B) methanol. The mixture was pumped as a gradient, starting at 74% A and 26% B, and maintained for 4 min. Then, over a 5 min period the mixture changed to 66% A and 34% B, and was maintained for 9 min. Over

the next 4 min period, the system was returned to initial conditions and ready for the next injection, without further equilibration. The mobile phase was prepared fresh daily using double-distilled, deionized water, filtered (0.22 μM), and degassed before use. Flow rate was 1.5 mL/min. Column temperature was ambient and detection was measured at 254 nm.

Sample Treatment

Spiked samples were prepared by the addition of appropriate volumes of both phenacetin and acetaminophen. The internal standard, TMP (50 μL of 25 μg/mL) was added, and appropriate amounts of the solution used in microsomal preparation were added to produce a 0.5 mL final volume. Samples were vortex-mixed and a 190 μL sample injected onto the liquid chromatograph.

Microsomal samples were prepared using Lake's ultracentrifugation method.[15] Incubation mixtures contained 0.5 mg of microsomal protein, 100 mM phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mM EDTA, and an NADPH-generating system (1 mM NADP, 10 mM G-6-P, and 0.7 U of G-6-PDH) in a total volume of 0.5 mL. Incubation mixtures contained phenacetin and inhibitors, and the reactions were initiated by addition of the NADPH-generating system after a 5 min pre-incubation step at 37°C. Reactions were quenched with 0.1 mL of ice cold acetonitrile after 20 min in a 37°C shaking water bath and then placed on ice for 1 hour. Samples were centrifuged at 14,000 rpm for 15 min. The supernatant was removed and stored at −80°C until analysis could be performed. Reaction rates were linear with incubation time under these conditions. Frozen samples were thawed on ice and vortex-mixed before use. Trimethoprim (50 μL of 25 μg/mL) was added to a 0.5 mL microsome sample and vortex-mixed. Those samples containing particulates were centrifuged for 5 min at 14,000 rpm in an Eppendorf centrifuge (Brinkman Instruments, New York, NY). A 190 μL aliquot of the supernatant was injected onto the liquid chromatograph.

RESULTS

A blank chromatogram for a microsomal sample with no drug added is shown in Fig. 2(A) with a large peak at 2.85 min and smaller peaks at 3.62 and 3.88 min. These peaks are the result of the NADPH generating solution used in the microsome sample; however, they do not interfere with peaks of interest. The *x*-axis on chromatograms 2B and 2C start at 4.5 min in order to eliminate the large NADPH peak and to provide a better image of the peaks of

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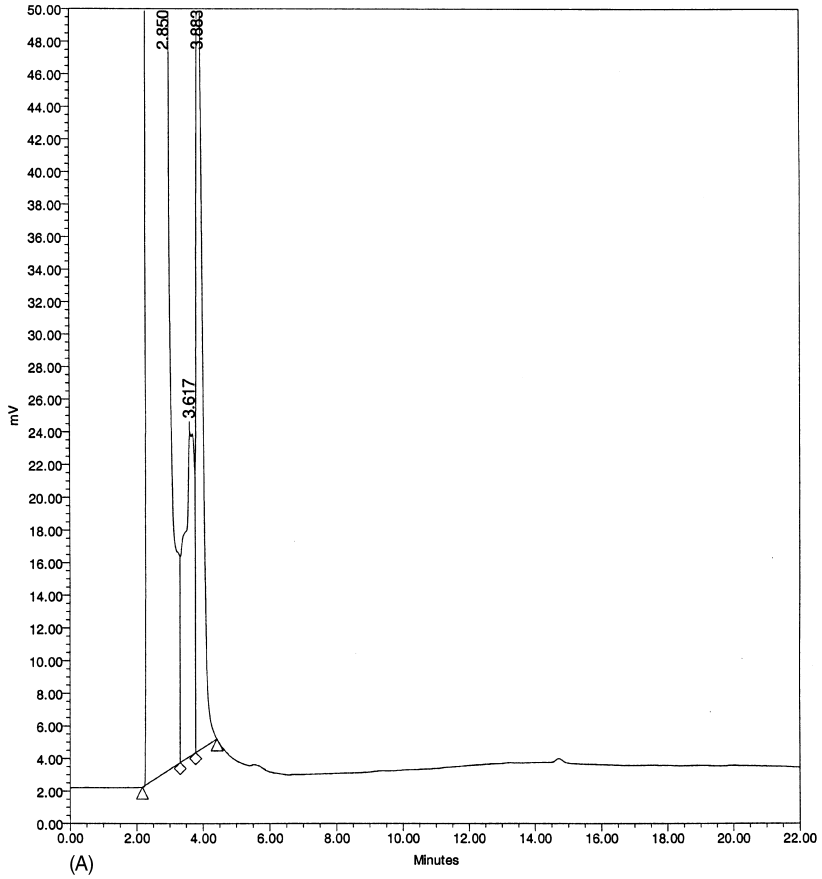

Figure 2. (A) Blank microsome chromatogram with no drug added. Peaks 2.85, 3.62, and 3.88 result from microsomal generating solution. (B) Chromatogram of a porcine microsomal sample after incubation with 10 μM of phenacetin. Peaks: acetaminophen (525 ng/mL); TMP (internal standard); Phen = phenacetin (687 ng/mL). (C) Chromatogram of a 500 ng/mL standard. Peaks: acetaminophen; TMP (internal standard); Phen = phenacetin.

(continued)

interest. The chromatogram in Fig. (2B) represents a porcine liver, microsome sample after incubation with 10 μM of phenacetin for 20 min. Retention times for acetaminophen, TMP, and phenacetin were 5.25, 8.08, and 19.48 min. The chromatogram in Fig. (2C) represents a 500 ng/mL standard with retention

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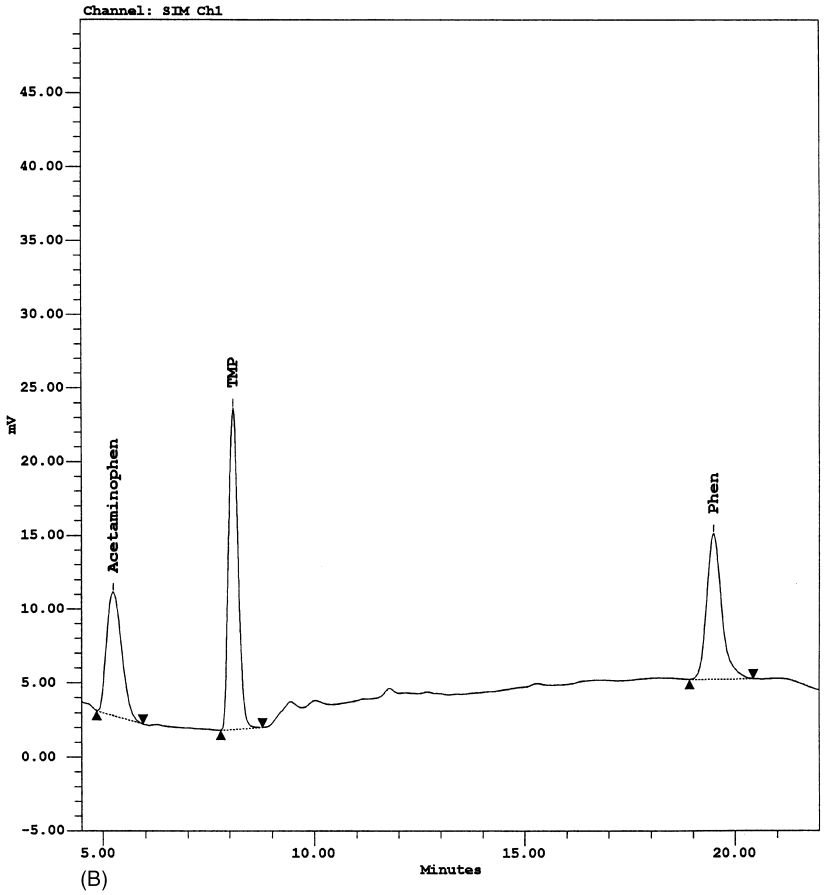

Figure 2. Continued.

times of 5.28, 8.05, and 19.50 min for acetaminophen, TMP, and phenacetin, respectively.

The method used produced a linear curve for the concentration range of 10–1500 ng/mL for phenacetin and acetaminophen, with correlation coefficients ranging from 0.998 to 0.999 for both compounds. Replicate analyses performed on the same day for microsomal samples, spiked with specific concentrations of phenacetin, produced coefficients of variation (CV) of 1.5% for 60 ng/mL, 1.3% for 750 ng/mL, and 0.1% for 1200 ng/mL. The metabolites' CV was 6.9%, 2.3%, and 1.0%, respectively, for the same concentrations (Table 1). Results for day-to-day variability for microsomal replicates

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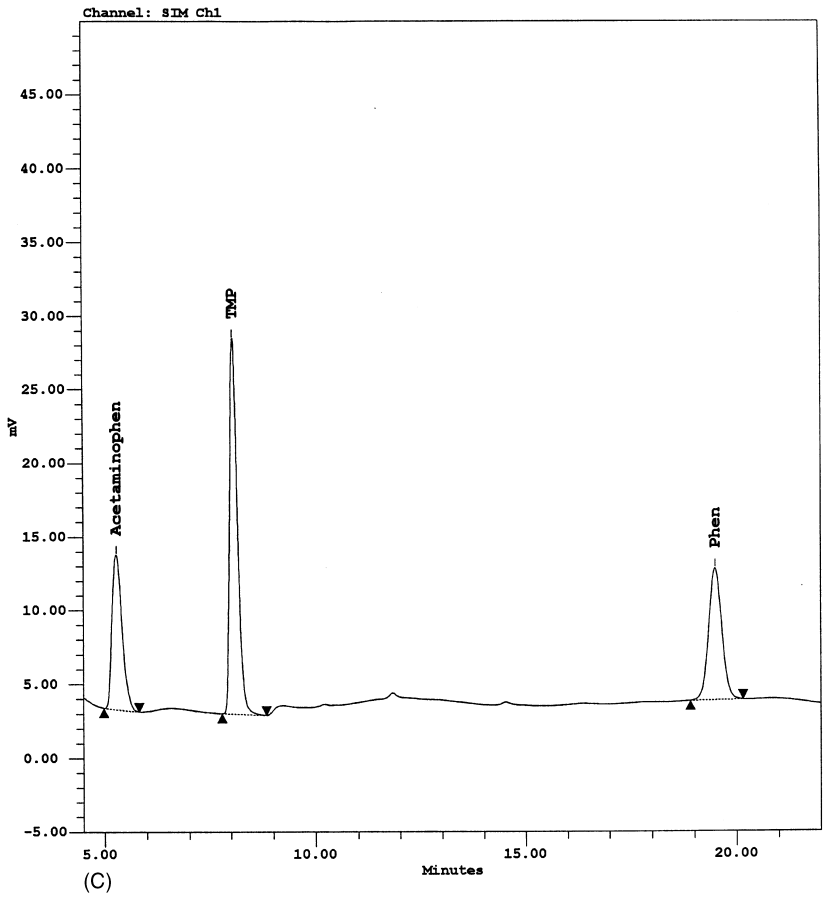

Figure 2. Continued.

appear in Table 2. Mean recoveries of phenacetin were 99%, 96%, 103%, 97%, 98%, 94%, 98%, 98%, and 98% for 10, 25, 50, 100, 250, 500, 800, 1000, and 1500 ng/mL. Mean recoveries of acetaminophen were 98%, 96%, 98%, 99%, 100%, 99%, 99%, 99%, and 96% for 10, 25, 50, 100, 250, 500, 800, 1000, and 1500 ng/mL. The detection limit for both compounds was 5 ng/mL. This represents a peak approximately three times baseline noise.

Numerous drugs and chemicals used in inhibition studies were tested for interference with the chromatographic procedure (Table 3). Furafylline was found to co-elute with phenacetin using the present gradient. This was

Table 1. Intra-assay precision for phenacetin and acetaminophen ($n = 4$).

Concentration added (ng/mL)	Phenacetin concentration measured (ng/mL) (mean ± SD)	Coefficient of variation (%)	Acetaminophen concentration measured (ng/mL) (mean ± SD)	Coefficient of variation (%)
60	62 ± 1	1.5	58 ± 4	6.9
750	720 ± 9	1.3	722 ± 17	2.3
1200	1206 ± 1	0.1	1186 ± 12	1.0

Note: SD, standard deviation.

corrected by changing the gradient. The first 10 min remains the same as the original gradient. The gradient changes from 66% A, 34% B to 76% A, 24% B from 10 min to 18 min. This is maintained for 8 min, then, over 1 min the gradient is returned to original conditions (74% A, 26% B).

DISCUSSION

Enzyme metabolism studies require a method that is simple, quick, accurate, sensitive, and reproducible. Such an HPLC assay, utilizing UV detection, has been developed to investigate the conversion of phenacetin to

Table 2. Inter-assay precision for phenacetin and acetaminophen ($n = 4$).

Concentration added (ng/mL)	Phenacetin concentration measured (ng/mL)	Coefficient of variation (%)	Acetaminophen concentration measured (ng/mL)	Coefficient of variation (%)
10	9.9	5.0	9.8	3.2
25	24	3.7	24	4.2
50	51	2.8	49	6.1
100	97	3.1	99	6.1
250	246	3.5	249	3.8
500	467	1.2	498	3.6
800	789	1.0	791	1.6
1000	978	1.0	987	3.3
1500	1481	1.2	1440	1.3

Table 3. Chemicals tested for assay interference.

Quinidine
Furafylline
7,8-Benzoflavone
Diethyldithiocarbamate
Ketoconazole
Ciprofloxacin
Bufuralol
Lidocaine
Itraconazole
Chlorzoxazone
NADP
Potassium phosphate
Magnesium chloride
Glucose-6-phosphate
Glucose-6-phosphate dehydrogenase

acetaminophen by microsomal fractions of porcine liver. The assay is sensitive, specific, and reproducible, with a high recovery of the metabolite.

Because we did not use an extraction for our samples, the NADPH generating solution produced a very large peak initially. In order to prevent any interference with acetaminophen, the mobile phase was initially adjusted to 74% A and 26% B. We did try to optimize the chromatography in order to reduce the time between TMP and phenacetin; however, we found that quinidine, which is used in microsomal studies, would interfere with TMP when the % methanol in the mobile phase was higher than 34%. A higher percentage of methanol also caused a large shift in the baseline, which was not acceptable. However, if the quantitation of phenacetin is not necessary or important for the experiment, the analysis time can be reduced.

Most of the procedures in the literature do not list validation parameters, such as limit of detection or recoveries for acetaminophen and phenacetin. However, we feel that our limit of detection and recovery for both compounds are more than adequate for microsomal studies. In cases where validation parameters are listed, ours are equal to or better than existing methods. Use of TMP as the internal standard corrects for intra- and inter-assay variability in the method.

High performance liquid chromatography procedures involved in the determination of acetaminophen and phenacetin by McKillop et al.[5] used a propanol : ethyl acetate extraction, flash freezing of the aqueous phase, and evaporation, while Zhang et al.[9] used an ethyl acetate extraction. Borm et al.[8]

used 10 mL of dichloromethane for 20 min, followed by hydrochloric acid and, then, a back extraction into diethyl ether for 20 min, which must be evaporated. Eagling et al.[13] used a similar procedure with 10 mL dichloromethane and 10 mL ethyl acetate, followed by evaporation, with inter and intra assay variabilities of 8.7% and 6.5%. Nakajima et al.,[10] Lillibridge et al.,[11] and Kobayashi et al.[12] used 2 mL of acetonitrile, vortexed for 10 min, then centrifugation and evaporation of the organic phase with a dri-block or vacuum evaporator. Kudo et al.[14] used extraction cartridges in their procedure. Our procedure eliminates use of time consuming liquid–liquid extractions involving toxic and expensive organic solvents, and does not require the use of dry ice, nitrogen, vacuum evaporation, or extraction cartridges. It is a rugged procedure with the column still in use after over 2300 injections, and the guard column replaced roughly every 300 injections.

This method was developed in order to facilitate microsome studies involving phenacetin, and has been applied to metabolism studies conducted in porcine microsomes in this laboratory. In conclusion, a simple, quick, and sensitive HPLC procedure has been developed for analysis of acetaminophen and phenacetin in hepatic microsome samples.

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