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Short communication

Monoethylglycinexylidide and lidocaine determination in porcine microsomal preparations

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Abstract

A simple, accurate, and sensitive HPLC analysis of monoethylglycinexylidide (MEGX) and lidocaine in porcine microsome samples is described. Lidocaine and MEGX were measured by direct injection after the addition of the internal standard. Chromatography was performed on a μBondapak C_{18} column using an isocratic mobile phase of 0.03 M potassium dihydrogen phosphate:acetonitrile (87:13), pH 5.9. UV absorbance was measured at 205 nm. The procedure produced linear curves for the concentration range 50–1000 ng/mL with a limit of detection of 10 ng/mL. Recoveries for both compounds were greater than 90%. This assay produced accurate and repeatable results.

Keywords: HPLC; Lidocaine; Microsome

1. Introduction

Cytochrome P450 enzymes in the liver play a pivotal role in metabolism. They are important in terms of their catalytic versatility and sheer number of compounds detoxified or activated to reactive intermediates [1,2]. P450 enzymes are present in all species examined to date. They are classified into families, which in turn are divided into subfamilies. The subfamilies in turn consist of highly related individual forms. The number of enzymes does differ between species [1]. Enzyme levels can vary because of environmental as well as genetic factors. The largest concentration of P450 enzymes is located in the liver endoplasmic reticulum (microsomes), but they are located in virtually all tissues.

P450 enzymes activate xenobiotics including drugs to toxic and/or tumorigenic metabolites, detoxify xenobiotics, and aid in determining intensity and drug exposure. Drug or xenobiotic metabolism is a direct reflection of the multiple enzyme systems that characterize different animal species and is an important factor in the regulation of their concentrations. 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. Much of the information known about P450 comes from studies conducted in rodents. However, the pig is becoming a popular alternative to traditional non-rodent species in pharmacological and toxicological testing [3]. The information on the P450 enzyme system for this species is limited.

P-450 enzymes are mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P-450 enzyme mediated xenobiotic metabolism, individual forms of P450 have been found to catalyze specific reactions with certain substrates. Thus these specific activities or reactions can be utilized as probes for this particular kind of enzyme. To date, at least one marker activity exists for the majority of human P450 enzymes [4]. The conversion of lidocaine to MEGX (Fig. 1) by *N*-deethylation is one of the methods used

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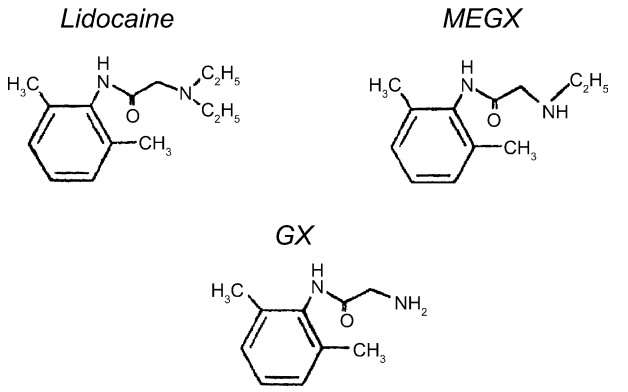


Fig. 1. Structures of lidocaine, MEGX and GX.

to characterize cytochrome P450 enzyme CYP3A4 activity. Several high performance liquid chromatography (HPLC) methods have been developed to measure MEGX and lidocaine in biological fluids and microsomes [5–13]. Many microsome methods involve the use of liquid–liquid extractions and evaporations [5–9,11,12]. The majority of methods also involve the use of human or rat microsomes, but not from swine.

This article describes a quick and efficient method for analysis of MEGX and lidocaine, which may facilitate characterization of P450 enzyme activity in the pig.

2. Materials and methods

2.1. Reagents and standards

Acetonitrile (HPLC grade), potassium dihydrogen phosphate (reagent grade), sodium hydroxide (reagent grade), magnesium chloride (enzyme grade), potassium phosphate (enzyme grade), and EDTA (enzyme grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Lidocaine was purchased from US Pharmacia (Rockville, MD, USA). MEGX and glycinexylidide (GX) were gifts from Astra laboratories (Westboro, MA, USA). Trimethoprim (TMP), glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), and β-nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma (St. Louis, MO, USA).

Stock standard solutions of lidocaine (100, 5 and 1 μg/mL) were prepared in methanol, while stock standard solutions of MEGX and GX (100, 5, and 1 μg/mL) were prepared in water. All solutions were stored at 4 °C. 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.

2.2. Apparatus and chromatography

The analytical system consisted of a 626 solvent delivery system, a model 717 WISP autosampler, a 486 UV detector and a computer equipped with Millennium software (Waters, Milford, MA, USA). The column was a μBondapak C_{18} (10 μm, 3.9 mm × 300 mm) equipped with a μBondapak C_{18} Guard-Pak precolumn insert (Waters, Milford, MA, USA).

The mobile phase consisted of a premixed isocratic mixture of 0.03 M potassium dihydrogen phosphate pH 5.9 (87%) and acetonitrile (13%). It was prepared fresh daily using double-distilled deionized water, filtered (0.22 μM) and degassed before use. The flow rate was 2.0 mL/min. Column temperature was ambient and UV absorbance was measured at 205 nm.

2.3. Sample treatment

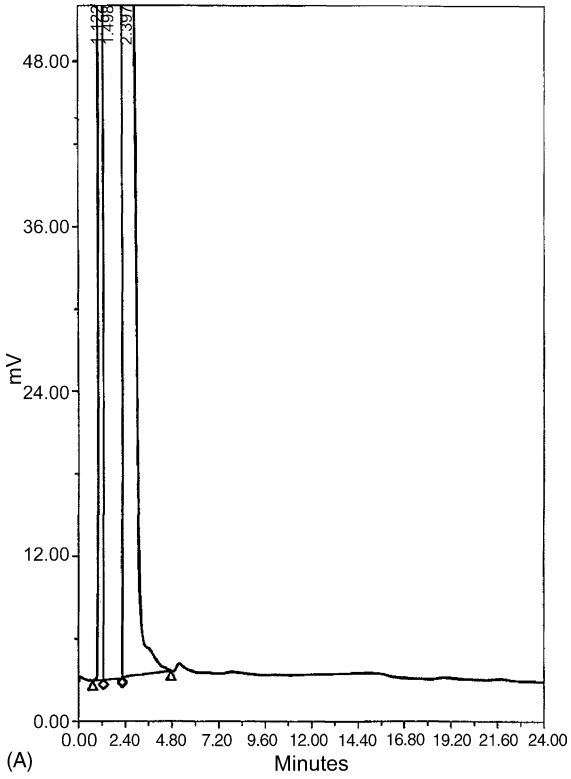
Spiked samples were prepared by the addition of appropriate volumes of GX, MEGX and lidocaine. The internal standard, TMP (25 μL of 25 μg/mL) was added and appropriate amounts of incubation solutions used in microsomal preparations were added to produce a 0.5 mL final volume. The composition of the spiked standards was identical to that of the samples. Samples were vortex-mixed and a 190 μL sample injected onto the liquid chromatograph.

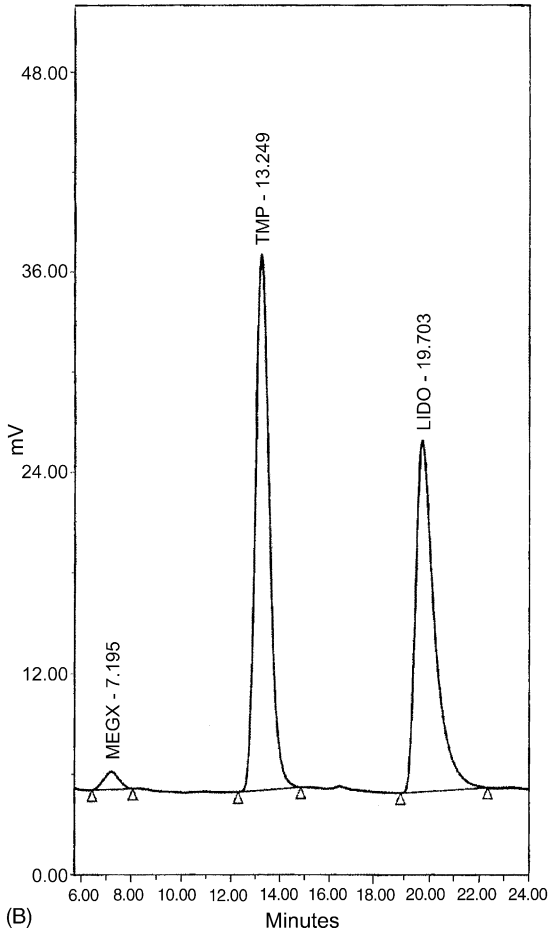
Previously frozen microsomal samples were prepared using Lake's ultracentrifugation method [14]. Incubation mixtures contained 0.5 mg of microsomal protein, 100 mmol/L phosphate buffer at pH 7.4 containing 6 mM magnesium chloride, 1 mmol/L EDTA, and a NADPH-generating system (1 mmol/L NADP, 10 mmol/L G-6-P and 0.7 U of G-6-PDH) in a total volume of 0.5 mL. Incubation mixtures contained lidocaine 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 0.05 mL of 1 M NaOH after 20 min in a 37 °C shaking water bath and then placed on ice for 1 h. Reaction rates were linear with incubation time under these conditions. Samples were centrifuged at 14,000 rpm for 15 min; the supernatant was removed and stored at −80 °C until analysis could be performed the next day. Samples were thawed only one time for analysis.

Frozen samples were thawed on ice and vortexed before use. TMP (25 μL of 25 μg/mL) was added to a 0.5 mL microsome sample and vortex-mixed. Samples were centrifuged for 5 min at 14,000 rpm in an Eppendorf centrifuge (Brinkman Instruments, New York, NY, USA). A 190 μL aliquot of the supernatant was injected onto the liquid chromatograph.

3. Results

A blank chromatogram of a microsomal sample with no drug added is shown in Fig. 2A with large peaks at 1.12, 1.49 and 2.39 min, which are the result of the NADPH-generating solution used in the microsome sample. However, none of these peaks interfere with the peaks of interest (MEGX, TMP, lidocaine). The *x*-axis on chromatogram 2B starts at 5 min in





order to eliminate the large NADPH peaks and provide a better image of the peaks of interest. The chromatogram in Fig. 2B represents the results of a porcine liver microsome sample after incubation with 10 μM of lidocaine for 20 min. Retention times for MEGX, TMP and lidocaine were 7.18, 13.25 and 19.70 min. GX, which is another metabolite of lidocaine, was not detected in any of the sample chromatograms.

The method used in the microsome study produced a linear line for the concentration range used (50–1000 ng/mL) for lidocaine and its metabolite, with correlation coefficients ranging from 0.998 to 0.999 for both compounds. Four replicate analyses performed on the same day for microsomal samples spiked with specific concentrations of lidocaine produced coefficients of variation (CV) of 5.9% for 100 ng/mL, 1% for 500 ng/mL and 2.2% for 800 ng/mL. The metabolites' CV was 3.2%, 1.7%, and 3.6% for the same concentrations (Table 1). Day-to-day variability from 4 days for microsomal replicates appears in Table 2. Mean recoveries of MEGX were 93%, 98%, 99%, 95%, 98%, and 99% for 50, 100, 250, 500, 800, and 1000 ng/mL. Mean recoveries of lidocaine were 103%, 94%, 98%, 94%, 96%, and 90% for 50, 100, 250, 500, 800, and 1000 ng/mL. The detection limit for both compounds was 10 ng/mL. This represents a peak approximately three times baseline noise. No interference from numerous drugs and chemicals used in inhibition studies was observed with the chromatographic procedure (Table 3).

4. Discussion

To be useful in enzyme metabolism studies requires a method to be simple, accurate, sensitive and reproducible. Such an HPLC assay, utilizing UV detection, has been developed to investigate the *N*-deethylation of lidocaine to MEGX by microsomal fractions of porcine liver. The assay is easy, specific, reproducible, and has adequate sensitivity for in vitro studies with a high recovery of MEGX.

We did not use an extraction for our samples, which resulted in very large peaks produced by the NADPH-generating solution at the front of the chromatogram. We did optimize the conditions considering this problem and found that an isocratic mixture of 87% phosphate buffer (pH 5.9) and 13% acetonitrile premixed would prevent interference from the generating solution peaks.

Most of the procedures in the literature do not list validation parameters such as limit of detection or recoveries for MEGX and lidocaine. 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

Fig. 2. (A) Blank microsome chromatogram with no drug added. Peaks 1.12, 1.49 and 2.39 result from microsomal generating solution. (B) Chromatogram of a porcine microsomal sample after incubation with 10 μM of lidocaine. Peaks: MEGX (50 ng/mL); TMP (internal standard); LIDO, lidocaine (2910 ng/mL).

Table 1
Intra-assay precision for MEGX and lidocaine ($n = 4$)

Concentration added (ng/mL)	MEGX concentration measured (ng/mL)	Coefficient of variation (%)	Lidocaine concentration measured (ng/mL)	Coefficient of variation (%)
100	99	3.2	95	5.9
500	492	1.7	503	1.0
800	809	3.6	809	2.2

n, number of replicates per day.

Table 2
Inter-assay precision for MEGX and lidocaine ($n = 4$)

Concentration added (ng/mL)	MEGX concentration measured (ng/mL)	Coefficient of variation (%)	Lidocaine concentration measured (ng/mL)	Coefficient of variation (%)
50	46	6.6	52	9.6
100	98	6.3	94	4.3
250	246	5.3	242	10.2
500	477	1.9	470	6.6
800	786	5.3	774	3.0
1000	990	5.0	897	1.0

n, number of days.

methods. The use of TMP as an internal standard corrects for intra- and inter-assay variability in the method.

In the determination of MEGX and lidocaine, Wang et al. [5,6] used a methyl tert-butyl ether extraction. Imaoka et al. [7], Tanaka et al. [8], Ohishi et al. [9] Nakamoto et al. [10] and Leclercq et al. [11] used varying amounts of ethyl acetate which are then evaporated with vacuum or nitrogen. Kawai et al. [12] used 10 mL of ethyl acetate followed by back extraction into sulfuric acid and neutralization with sodium hydroxide, which is evaporated under reduced pressure. Our procedure eliminates the use of time consuming liquid–liquid extractions involving toxic and expensive organic solvents and does not require the use of nitrogen or vacuum evaporation. The method did include validation of GX and could be used for its analysis. However, GX was not detected in any of the porcine samples; therefore, its validation parameters were not included in this manuscript. It is a rugged procedure with the column still in use after over 1000 injections and the guard column replaced roughly every 300 injections.

Table 3
Chemicals tested for assay interference

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

The chromatographic method described, was developed in order to determine the metabolism of lidocaine in porcine microsome samples. The procedure has been applied by our group to metabolism studies of lidocaine in porcine microsomes. In conclusion, a simple, sensitive and useful HPLC procedure has been developed for analysis of MEGX and lidocaine in microsome samples.

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