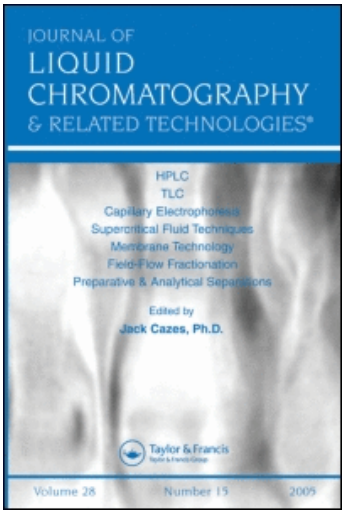

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

An Investigation of the Metabolism of Amitriptyline Using High Performance Liquid Chromatography

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To cite this Article Smith, Cynthia S. , Abramson, Ruth K. and Morgan, Stephen L.(1986) 'An Investigation of the Metabolism of Amitriptyline Using High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 9: 8, 1727 — 1745
To link to this Article: DOI: 10.1080/01483918608076714
URL: http://dx.doi.org/10.1080/01483918608076714

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(8), 1727-1745 (1986)

AN INVESTIGATION OF THE METABOLISM OF AMITRIPTYLINE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A systematic study of the metabolism of the antidepressant amitriptyline was conducted using an inbred strain of rats. Variables affecting rat liver metabolism in vitro that were examined include age of the rat, the substrate concentration, and pH. A liquid-liquid extraction with hexane-butanol and back extraction into phosphoric acid was developed to provide efficient extraction for the wide range of polarity exhibited by amitriptyline and seven metabolites (amitriptyline-N-oxide, cis and trans isomers of 10-hydroxy-amitriptyline, cis and trans isomers of 10-hydroxynortriptyline, nortriptyline, and desmethylnortriptyline). HPLC was performed with 5 μm C-8 reversed phase column and a methanol/sodium phosphate buffer/amine modifier mobile phase.

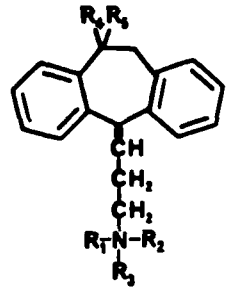
 0148-3919/86/0908-1727$3.50/0

INTRODUCTION

High performance liquid chromatography (HPLC) is widely employed for profiling drugs and their metabolites in physiological fluids and tissues. The chemical structures and functional groups that give molecules biological activity can also serve as the basis for liquid chromatographic separations. Reversed phase HPLC is particularly attractive for separating compounds which are predominantly hydrophobic in character with subtle differences in polar functional groups. The use of an aqueous/organic mobile phase and a bonded hydrocarbon stationary phase allows the chromatographer to exploit these functional group differences; in general, compounds elute in order of decreasing polarity.

Fundamental knowledge of drug metabolism and the factors that influence metabolic pathways is essential for the effective treatment of patients who may vary in their metabolic makeup. Changes in metabolism as a function of age have been shown to be substrate dependent (1,2). Metabolism and, therefore, the elimination of drugs have been observed to be dependent on concentration (3). Studies in which the pH optima for production of different metabolites of drugs help define the enzyme systems involved in specific metabolic pathways (4).

Amitriptyline (AMI) is a widely prescribed tricyclic antidepressant used in the clinical treatment of major depression. Although AMI has been the subject of many studies, there are aspects of its metabolism that are still not well understood (3). The major metabolites of AMI are shown in Figure 1. In human livers, AMI is rapidly demethylated to nortriptyline (NOR) and further demethylated to desmethylnortryptyline (DMN). Both AMI and NOR undergo hydroxylation to their cis- and trans- 10-hydroxy analogs (C or T 10-OH-AMI or NOR) (3). Amitriptyline N-oxide (AMI-N-O) has been



COMPOUND	R_1	R_2	R_3	R_4	R_5	PEAK NO.
AMI	CH_3	CH_3	-	H	H	9
NOR	CH_3	H	-	H	H	6
DMN	H	H	-	H	H	5
T-10-OH-AMI	CH_3	CH_3	-	H	OH	4
C-10-OH-AMI	CH_3	CH_3	-	OH	H	3
T-10-OH-NOR	CH_3	H	-	H	OH	2
C-10-OH-NOR	CH_3	H	-	OH	H	1
AMI-N-O	CH_3	CH_3	O	H	H	7

Figure 1. Amitriptyline and major metabolites

reported only in small amounts in human systems (5), but is an important metabolite in rats and dogs (6). All of these metabolites have shown pharmacological activity (5,7). Although a number of HPLC methods for the determination of AMI and some of its metabolites have been developed (7-10), no current methods include all seven major metabolites. Kiel *et al.* (11) developed a reversed phase method to quantitate AMI and six metabolites. In this study we present a

modification of that method which includes the trans-isomer of 10-OH-AMI and demonstrate the use of HPLC to profile differences in drug metabolism.

MATERIALS

Glucose-6-phosphate (sodium salt), glucose-6-phosphate dehydrogenase, type XII, nicotinamide adenine dinucleotide phosphate (reduced form), and bis-tris propane were obtained from Sigma Chemical Co., (St. Louis, MO). Metabolite standards were generously supplied by Lundbeck and Co., (Copenhagen, Denmark). HPLC grade methanol and water were obtained from Burdick and Jackson, (Muskegon, MI). HPLC grade phosphoric acid, hydrochloric acid, butanol, hexane, sucrose, magnesium chloride, acetone, and sodium chloride came from Fisher Scientific (Atlanta, GA). Reagent grade sodium hydroxide, sodium phosphate dibasic, and methylamine (40 % in water) came from Aldrich Chemical Co., Inc. (Milwaukee, WI).

METHODS

Experimental Animals

Male Fischer 344 rats of different ages (6 and 30 months) were obtained from Charles River Breeding Labs (Wilmington, MA). Animals were housed in metal cages for at least one week prior to sacrifice. Animals had free access to water and solid pellet food. All animals used in these studies were healthy and tumor free. Rats were sacrificed by decapitation, livers excised, rinsed in cold 0.85 % saline and immediately placed on dry ice. Livers were stored at −80°C until used.

Sample Preparation

Rat livers were homogenized in 20 volumes (w/v) of 0.25 M sucrose, 0.05 M bis-tris buffer at the appropriate pH in glass-teflon homogenizers at 4°C. Each sample contained 0.2 mL homogenate and 0.8 mL reaction mixture for a total of 1 mL. The reaction mixture contained 0.5 µmole NADPH, 5 µmole glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase from torula yeast in 0.8 mL of a 0.05 M bis-tris and 3 µM $MgCl_2$ buffer. Samples and buffers were prepared just prior to use, and kept at 4°C prior to addition of substrate. Just before incubation, an aliquot of a 1 mg/mL solution of AMI in 0.85 % saline was added to each sample. Samples were incubated at 37°C for 0, 10, or 20 min. The reaction was stopped by addition of 1 mL cold acetone. Reaction mixtures were extracted immediately or stored at −80°C until extraction. Unless otherwise indicated the reaction was run at pH 7.5 using 10 µL amitriptyline solution.

To the quenched reaction mixture, 100 µL of a 4000 ng/mL imipramine solution was added as an internal standard. A 250 µL aliquot of a 25 % (w/v) solution of Na_2CO_3 and 5 mL 10 % (v/v) butanol in hexane were also added. The mixture was vortexed, then centrifuged 3 min at 500 x g to break the emulsion. The aqueous layer was aspirated and the mixture transferred to a 15 mL centrifuge tube. A 100 µL aliquot of 4.5 M H_3PO_4 was added, the mixture vortexed and centrifuged three additional min. The organic layer was then aspirated and the acid layer neutralized with 80 µL of a 4.5 M NaOH solution. After a final vortexing, 50 µL of the resulting mixture was sampled for injection into the HPLC.

Liquid Chromatography

All separations were performed on a Waters HPLC system (Waters Associates, Milford, MA) consisting of a Model 730 System

Controller, a Model 720 Data Module, a Model 710 autoinjector, two Model 6000A pumps, and a Model 441 detector. The detector monitored UV absorbance at 214 nM.

The isocratic mobile phase was composed of 55% methanol, 45% water, 275 mM methylamine, and 25 mM Na_2HPO_4 adjusted to pH 7.35 with H_3PO_4. A 4.6 x 250 mm analytical column packed with 5 μm C-8 bonded phase coupled to a 4.6 x 50 mm guard column packed with 40 μm C-8 bonded phase (both from Supelco, Bellefonte, PA) was employed. The column was heated to 40°C by a water bath (Haake, Inc., Saddle Brooke, NJ) and a flow rate of 2-mL/min was maintained.

Experimental Design and Data Handling

Samples were run in triplicate and experiments were performed at zero reaction time to evaluate the baseline. Multipoint linear calibration was performed using duplicate extracted standards at three concentration levels (usually 20, 200, and 2000 ng/mL). A straight line was fitted to peak height ratio of sample to internal standard as a function of concentration using a BASIC program on a Commodore 64 microcomputer (Wayne, PA). All calibrations were linear over the concentration range examined, with intercepts not significantly different from zero at the 95% level of confidence. The slopes were used to calculate response factors in the calibration table of the Waters 720 Data Module. Sample responses were automatically generated in the report immediately following each chromatographic run.

The experimental design to investigate the pH dependence of drug metabolism involved triplicate experiments at 7 different levels of reaction mixture pH (5.5, 6.5, 7.5, 8.0, 8.5, 9.0, and 9.5). After calculation of parent drug and metabolite concentrations these data were not processed further. Plots of the raw concentration data for each drug metabolite were produced as a function of pH; the mean of each set

of triplicate measurements was calculated and lines connecting these means were superimposed on the plot.

A factorial design was conducted to investigate the effects of three experimental factors on drug metabolism: (1) the initial concentration of the parent drug, AMI (at 4 levels); (2) the reaction time (at 3 levels); and (3) the age of the rat (at 2 levels, 6 months and 30 months). The results of this factorial design were concisely summarized by fitting the parent drug and metabolite concentrations to full second order models as a function of the two factors, concentration and reaction time, for each compound and each age of rat:

$$y = \beta_0 + \beta_1 * x_1 + \beta_{11} * x_1^2 + \beta_2 * x_2 + \beta_{22} * x_2^2 + \beta_{12} * x_1 * x_2 \quad (1)$$

where y represents the concentration of parent drug or metabolite, the β's represent parameters of the linear model, x_1 represents concentration of substrate, and x_2 represents reaction time. A matrix least squares program, written in BASIC on a Model 85A computer (Hewlett-Packard, Palo Alto, CA) was used to estimate the parameters. All models fit the data adequately with coefficients of determinaton (R^2) typically higher than 0.95 and with lack of fit not significant at the 95% level of confidence (12). Pseudo-three-dimensional plots of concentration versus time and level of metabolite were produced on the HP-85A interfaced to a Model 7225B plotter (Hewlett-Packard, Palo Alto, CA).

RESULTS AND DISCUSSION

Quantitative Aspects of the Analytical Method

Recovery and extraction time for all components were similar to that reported previously (11). The inclusion of an additional metabolite

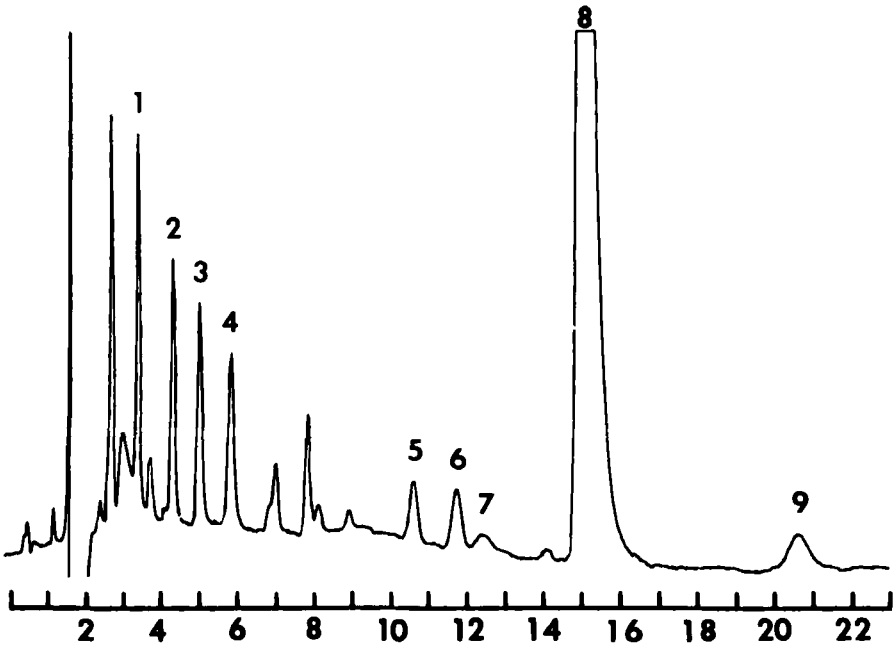


Figure 2. A chromatogram of a 20 ng extracted standard (see text for conditions).
See Figure 1 for peak identities.

and the exclusion of interference peaks from rat liver homogenate required an increase of 6-8 min in the chromatographic run time compared to our previous method. The internal standard, imipramine, elutes well away from the other peaks of interest.

A chromatogram of an extracted 20 ng/mL standard containing the parent drug, AMI, and all seven metabolites is shown in Figure 2. The detection limits for the early eluting compounds (the hydroxylated metabolites) are much less than 10 ng/mL. Later eluting compounds can not be detected as readily, with AMI-N-O having the highest limit of detection.

Replicate analyses of parent drug and metabolite concentrations typically produced relative standard deviations of 20% or less. For example, a set of triplicate determinations for a 30 month rat at pH 7.5, 10 μL AMI substrate, reacted for 20 min gave the following relative standard deviations: C-10-OH-NOR 9.5 %, T-10-OH-NOR 3.7 %, C-10-OH-AMI 14.7 %, T-10-OH-AMI 17.6 %, DMN 6.7 %, NOR 26.8 %, AMI-N-O 14.1 %, and AMI 25.2 %. Because this analysis requires the measurement of very large peaks in the same chromatogram as very small ones, integration is a problem. Variability in peak height for replicate runs is usually higher with the larger peaks. Also, wherever experimental conditions favored rapid metabolism, extra variability was introduced in the lag time between the quenching of replicate reactions.

Effect of pH on Drug Metabolism

The pH of the reaction mixture was varied from 5.5 to 9.5 to determine the pH at which the maximum amount of each metabolite was produced. Tissue from a 6 month old rat was used, a fixed amount of parent drug was employed, and the reaction time was 10 min. Figure 3 presents four representative chromatograms from these experiments. The cis- and trans-10-OH-NOR exhibited an optimum at 7.5 (physiological pH), while cis- and trans-10-OH-AMI exhibited an optimum at pH 6.5 (see Figures 4 and 5). These two different pH optima suggest that separate sets of enzymes may be responsible for the hydroxylation of these two compounds which differ only by a methyl group. The pH dependence of the concentration of demethylated metabolites indicate that these pathways were most productive at higher pH ranges. The optimum pH for the production of DMN was 7.5, while that for NOR was 8.0. AMI-N-O production was highest at pH 9.0. Separate enzyme systems may also control demethylation of AMI and NOR as well as N-oxidation of AMI.

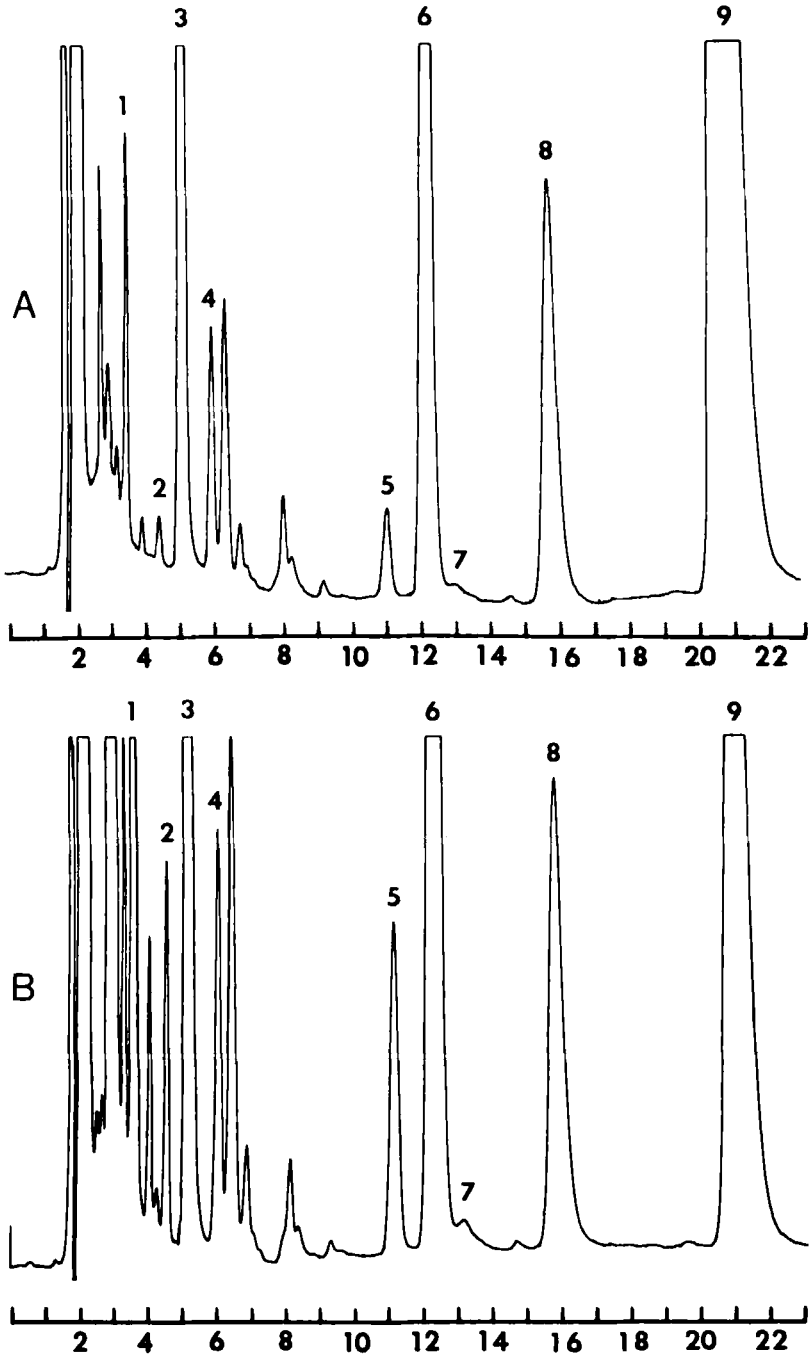


Figure 3. Variations in metabolism with reaction pH. See Figure 1 for peak identities.

A) Reaction pH 5.5 B) Reaction pH 6.5

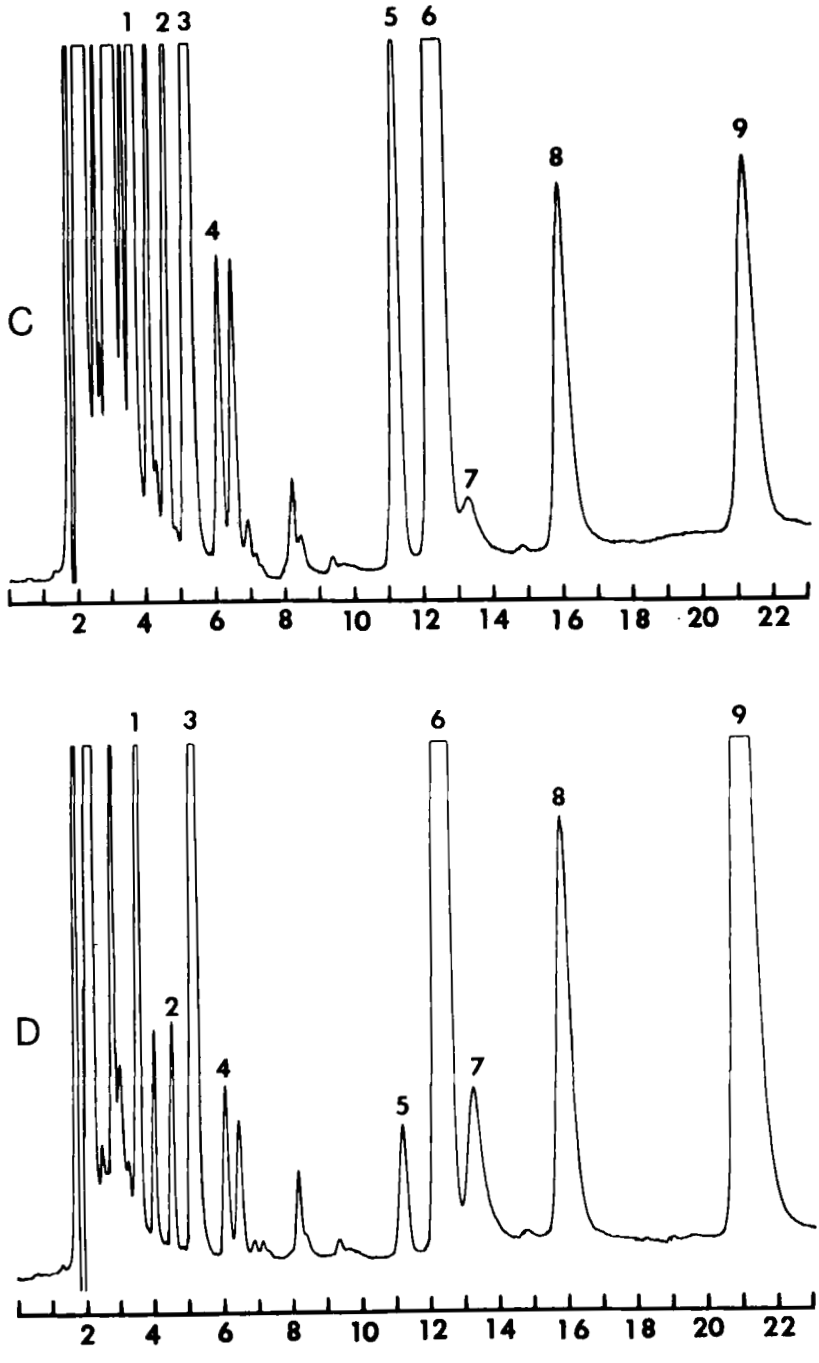


Figure 3. C) Reaction pH 7.5 D) Reaction pH 8.5

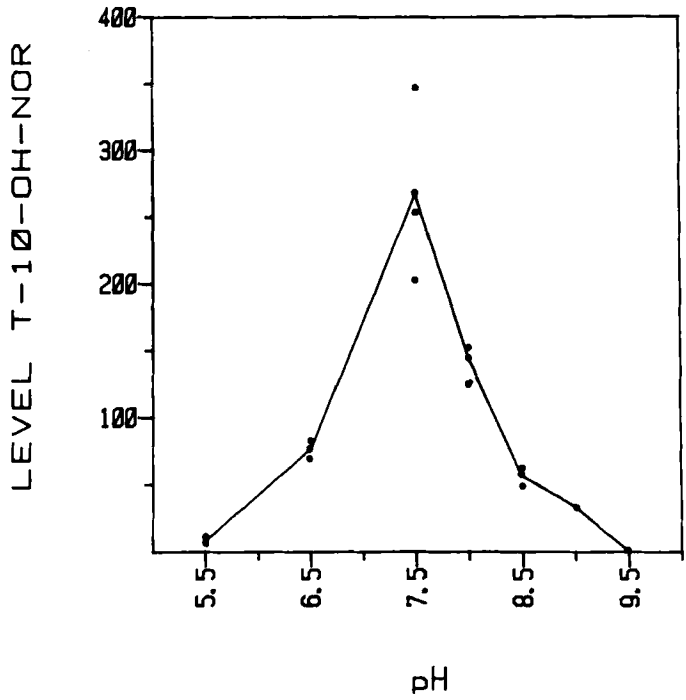


Figure 4. Profile for T-10-OH-NOR over pH range

Effect of Substrate Concentration on Drug Metabolism

The effect on the metabolite profile of increasing the substrate concentration from 5 to 50 µg AMI per sample was examined in 6 and 30 month old rats at physiological pH. Generally, increased substrate concentration produced an increase in metabolite concentration, as seen in the response surfaces plotted in Figures 6 and 7.

Major differences exist, however, in the appearance of the plots for the production of NOR (Figure 6), C-10-OH-AMI (Figure 7), and C-10-OH-NOR (Figure 8) as a function of substrate concentration (in this case, AMI). Mellstrom and Bahr (3) previously reported that increased

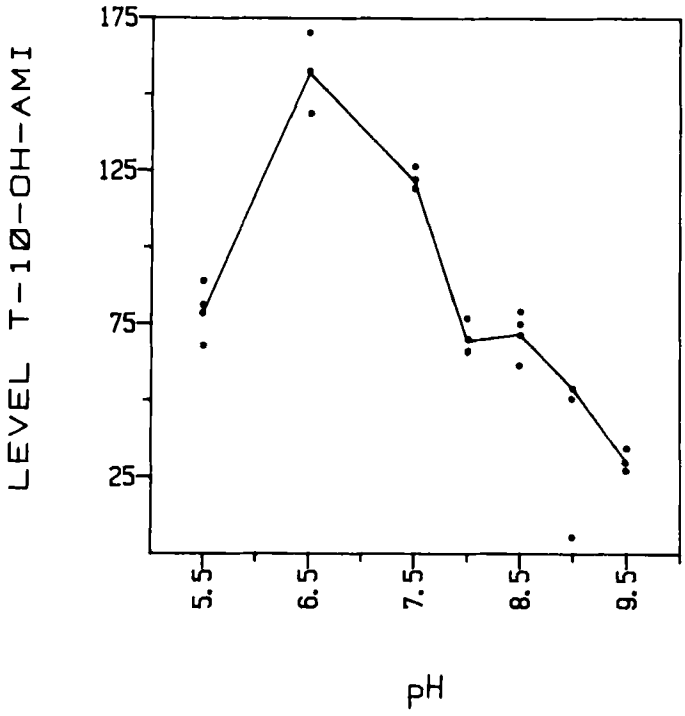


Figure 5. Profile for T-10-OH-AMI over pH range

concentration of NOR substrate inhibited the hydroxylation of AMI to C-10-OH-AMI. Figure 7 shows that AMI also inhibits the production of its hydroxylated metabolite, C-10-OH-AMI; this is seen in the curvature and slight leveling off of the response surface with respect to AMI at higher levels. The response surface for C-10-OH-NOR shown in Figure 8, however, exhibits a dramatically different appearance than Figures 6 and 7: the concentration of C-10-OH-NOR increases with increasing reaction times for all substrate concentrations, but a consistent negative effect is associated with increasing AMI substrate concentration. Since NOR is produced as a metabolite of AMI, this result is not surprising in view of the previously reported inhibition of the hydroxylation of AMI by NOR.

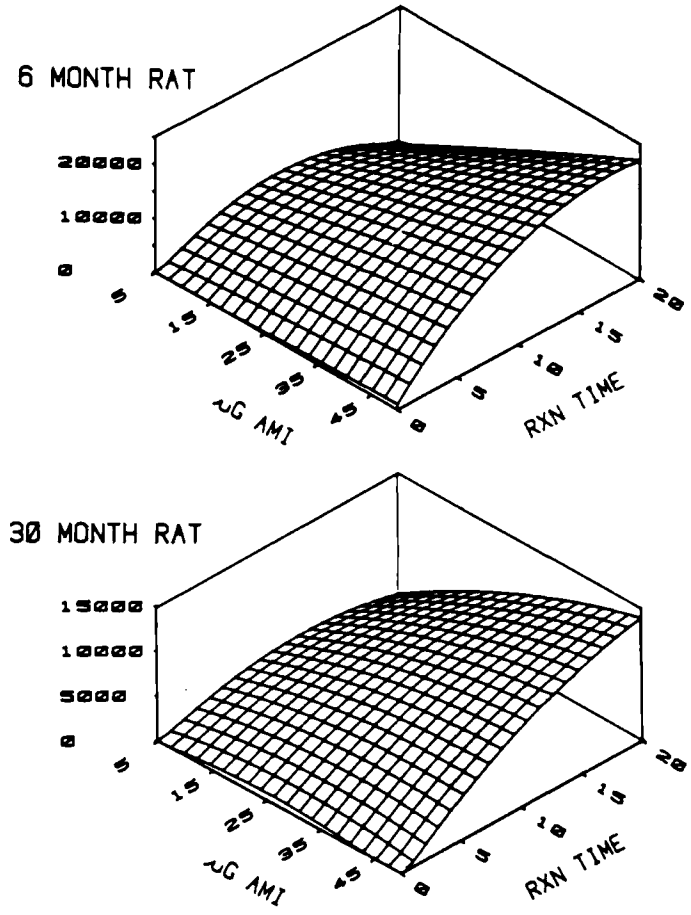


Figure 6. Nortriptyline level (ng/mL) produced as a function of concentration and time for 6 and 30 month rats

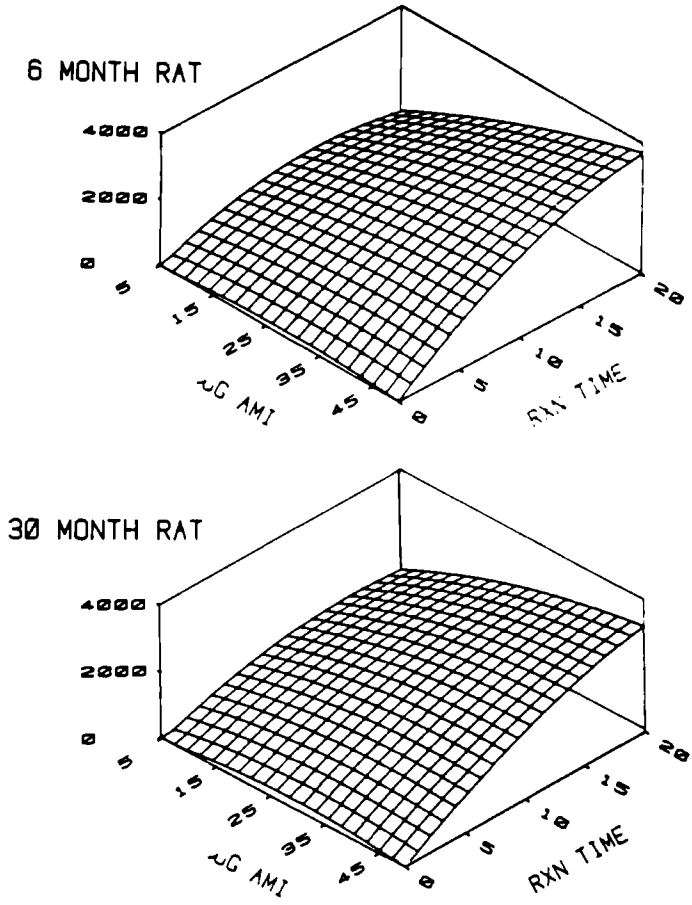


Figure 7. Level of C-10-OH-AMI (ng/mL) produced as a function of concentration and time for 6 and 30 month rat

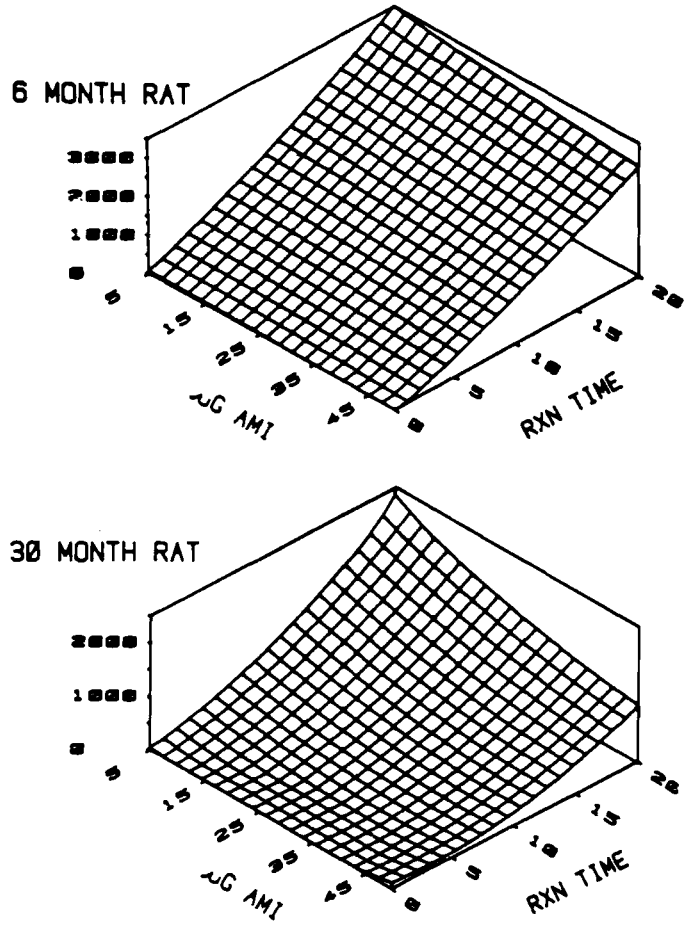


Figure 8. Level of C-10-OH-NOR (ng/mL) produced as a function of concentration and time for 6 and 30 month rat

Effect of Age on Drug Metabolism

The data obtained in this study and summarized by Figures 6-8 indicate that the rate of metabolism for all metabolites is slightly greater in the 6 month rat than in the 30 month rat. Furthermore, inhibition of hydroxylation of both AMI and NOR by AMI appeared more severe in 30 month old rats (Figures 6-8) Since the hydroxylated metabolite C-10-OH-NOR is a major vehicle for elimination of the drug (13), this observation implies that the drug would be eliminated more slowly in a 30 month than a 6 month old rat. If this result can be extrapolated to humans, it might in part explain the slower clearance of AMI in the elderly.

CONCLUSION

Reversed phase HPLC is an excellent technique for the rapid profiling of drugs and their metabolic products. We have further demonstrated the utility of HPLC in combination with a systematic experimental design to investigate the dependence of drug metabolism on experimental factors.

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