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Journal of Chromatography B, 749 (2000) 119–125

JOURNAL OF CHROMATOGRAPHY B

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# Sensitive and specific high-performance liquid chromatographic assay for 4′-hydroxyflurbiprofen and flurbiprofen in human urine and plasma

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Received 24 March 2000; received in revised form 22 June 2000; accepted 7 July 2000

**Abstract**

A high-performance liquid chromatographic assay has been developed for the simultaneous quantitation of flurbiprofen and its major metabolite, 4′-hydroxyflurbiprofen, in urine and plasma. No extraction procedure was necessary for analysis of these compounds, which reduced time involved in sample preparation. The analytes were separated on a Brownlee Spheri-5® $C_{18}$ column with a mobile phase of acetonitrile–20 m*M* dibasic potassium phosphate pH 3 buffer (40:60, v/v). Fluorescence detection was utilized with an excitation wavelength of 260 nm and an emission wavelength of 320 nm, providing excellent sensitivity. The limit of quantitation for 4′-hydroxyflurbiprofen and flurbiprofen was 0.25 μg/ml in urine and 0.05 μg/ml and 0.25 μg/ml, respectively, in plasma. All components were eluted within 16 min. Intra-day, inter-day, freeze–thaw, and in process stability were tested for both compounds and the coefficient of variation was less than 14% in all cases. This method provides a sensitive and specific assay for the detection of flurbiprofen and 4′-hydoxyflurbiprofen in urine and plasma and is suitable for use in in vivo studies evaluating the regulation of CYP2C9 activity. 

*Keywords:* Flurbiprofen; 4′-hydroxyflurbiprofen

## 1. Introduction

Flurbiprofen is an arylpropionic non-steroidal anti-inflammatory drug used in the treatment of pain and inflammation. In humans, flurbiprofen is eliminated primarily by oxidation to form 4′-hydroxyflurbiprofen (Fig. 1), which accounts for about 86% of the oxidative metabolites [1]. The oxidative metabolites and parent compound are also subject to glucuronidation or sulfation [2,3]. It has been shown that the formation of 4′-hydroxyflurbiprofen is mediated almost exclusively by cytochrome P450 2C9 in a non-stereoselective manner [4,5]. As a result, flurbip-

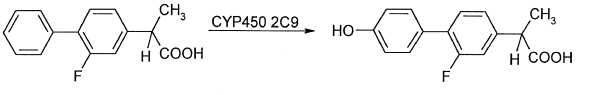


Fig. 1. Structures for flurbiprofen and its primary metabolite, 4′-hydroxyflurbiprofen.

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0378-4347/00/$ – see front matter 
PII: S0378-4347(00)00393-5

rofen may be used as an in vitro probe for CYP2C9 activity, without the need for measuring the enantiomers. More recently, it has been shown that flurbiprofen may be used as an in vivo probe to estimate the activity of CYP2C9 in human subjects [6].

Other HPLC methods have been described for the detection of racemic flurbiprofen and its metabolites in urine and plasma [3,7–9], in which the analytes must be extracted from their respective biological matrices. This paper describes a sensitive and specific procedure for the detection of flurbiprofen and its 4′-hydroxy metabolite in which no extraction step is utilized, thus reducing the amount of time and error involved in quantitation. The method involves a reversed-phase liquid chromatography column and fluorescence detection, and is sufficiently sensitive to quantitate flurbiprofen and 4′-hydroxyflurbiprofen in amounts as low as 0.25 and 0.05 μg/ml, respectively. This assay was used to quantitate concentrations of flurbiprofen and 4′-hydroxyflurbiprofen in urine and plasma from human subjects to evaluate the use of flurbiprofen as an in vivo probe for cytochrome P450 2C9 activity.

## 2. Experimental

### 2.1. Reagents and chemicals

Acetonitrile, dibasic potassium phosphate buffer, hydrochloric acid and phosphoric acid were obtained from Fisher Scientific Co. (Pittsburgh, PA, USA). *rac*-Flurbiprofen was purchased from Sigma Chemical (St. Louis, MO, USA). 4′- Hydroxyflurbiprofen and 2-fluoro-4-biphenylacetic acid (internal standard (IS)) were gifts from Pharmacia (Kalamazoo, MI, USA). All other chemicals were obtained from commercial sources and were of the highest purity available. Drug-free human plasma was obtained from the blood bank and drug-free human urine was obtained from laboratory personnel.

### 2.2. Instrumentation

The HPLC system consisted of a Waters 501® HPLC pump, a Waters 717® autosampler and a Waters 470® scanning fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm (Waters Assoc., Milford, MA, USA). The mobile phase for both urine and plasma consisted of acetonitrile–20 m*M* $K_2HPO_4$, pH 3.0 (40:60, v/v) pumped at 1 ml/min through a Brownlee Spheri-5 ® $C_{18}$ 4.6×100 mm reversed-phase column, with a typical pressure of 67 bar.

### 2.3. Preparation of stock solutions

Flurbiprofen and 4′-hydroxyflurbiprofen stock solutions were prepared by dissolving each in 50:50 acetonitrile:$H_20$ to make a 1 mg/ml solution. From this stock solution, dilutions were made to prepare 50, 10, 5, and 0.5 μg/ml solutions for preparation of the standards. 2-fluoro-4-biphenylacetic acid (IS) was dissolved in acetonitrile to prepare a 1 mg/ml stock solution. From this stock solution a 1000 ng/ml solution was prepared by adding 0.5 ml of the 1 mg/ml stock to 500 ml acetonitrile. A 500 ng/ml internal standard solution was prepared by adding 0.25 ml of the 1 mg/ml stock to 500 ml acetonitrile. All stock solutions were stored at 4°C.

### 2.4. Flurbiprofen and 4′-hydroxyflurbiprofen in urine

Urine samples (50 μl) were prepared by adding 400 μl of purified water and 100 μl 6 *M* HCl, followed by vortexing and incubation at 90°C for 30 min to facilitate glucuronide cleavage via acid-hydrolysis. Following incubation, 500 μl acetonitrile containing IS (1000 ng/ml of 2-fluoro-4-biphenylacetic acid) was added and the samples vortexed. The samples were then centrifuged at 14 000 *g* for 5 min, and 150 μl of sample transferred to autosampler vials and 3–12 μl injected onto the HPLC system for analysis. Standard amounts for flurbiprofen and 4′-hydroxyflurbiprofen ranged from 0.5 to 40 μg/ml (0, 0.5, 1.0, 2.0, 5, 10, 20, and 40 μg/ml). Appropriate amounts of 4′-hydroxyflurbiprofen and flurbiprofen were added to 50 μl blank urine to prepare standards and then brought to final volume of 450 μl by addition of purified water and treated as subject samples.

### 2.5. Flurbiprofen and 4′-hydroxyflurbiprofen in plasma

Plasma samples (100 μl) were prepared by adding 100 μl acetonitrile, 200 μl IS (500 ng/ml of 2-fluoro-4-biphenylacetic acid in acetonitrile), 40 μl of half-strength $H_3PO_4$ (50:50 $H_3PO_4$: $H_20$), followed by vortexing. The samples were then centrifuged at 14 000 g for 10 min, and 150 μl placed into autosampler vials and 20–75 μl injected onto the HPLC. The standards were prepared to contain concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, and 3.0 μg/ml for 4′-hydroxyflurbiprofen and 0, 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 15 μg/ml for flurbiprofen. Appropriate amounts of 4′-hydroxyflurbiprofen and flurbiprofen were added to 100 μl blank plasma and acetonitrile was then added to bring the final volume to 200 μl, followed by treatment as with the subject samples.

### 2.6. Assay validation procedure

Urine assay validation consisted of replicates of a low concentration (1 μg/ml), a middle concentration (12 μg/ml), and a high concentration (30 μg/ml) quality control (QC) sample for flurbiprofen and 4′-hydroxyflurbiprofen. Plasma assay validation QC concentrations were 1 μg/ml, 12 μg/ml, and 30 μg/ml for flurbiprofen, and 0.1 μg/ml, 0.5 μg/ml, and 1.5 μg/ml for 4′-hydroxyflurbiprofen. Intra-day variability was tested with 12 replicates of each quality control concentration. Inter-day variability was conducted over 3 days with 6 replicates of each QC concentration. Furthermore, low and high QC concentrations (in triplicate) were subjected to three freeze–thaw cycles and analyzed. Finally, processed stability after pretreatment was tested by injecting low and high QC samples every 2 h for 24 h. Means, standard deviations and coefficients of variation were calculated by standard methods.

## 3. Results

Representative HPLC chromatograms illustrating the analysis of 4′-hydroxyflurbiprofen and flurbiprofen in urine and plasma, as well as a blank for each, are shown in Fig. 2 A–D. 4′-Hydroxyflurbiprofen, IS, and flurbiprofen were separated within 16 min, with retention times of approximately 3.6, 9.0, and 14.0 min, respectively. The standard curves for 4′-hydroxyflurbiprofen and flurbiprofen in urine were linear through 40 μg/ml, while the curves for 4′-hydroxyflurbiprofen and flurbiprofen in plasma were linear through 3 μg/ml and 15 μg/ml, respectively. Limits of quantitation for 4′-hydroxyflurbiprofen and flurbiprofen in urine and plasma were 0.5 μg/ml in urine and 0.05 μg/ml and 0.25 μg/ml, respectively, in plasma.. A representative subject log plasma concentration versus time curve in Fig. 3 shows the ability of this assay to sensitively detect flurbiprofen and low 4′-hydroxyflurbiprofen concentrations in plasma. In addition, in 12 subjects receiving flurbiprofen orally, the average urinary recovery of total drug after acid cleavage of glucuronide metabolite was 21 mg out of a 50 mg dose, or about 42% (data not shown).

### 3.1. Calibration and linearity

Calibration curves were constructed using seven standard concentrations in urine or plasma. Curves were obtained daily for 4 days by plotting the peak-area ratios of flurbiprofen and 4′-hydroxyflurbiprofen to the IS against the corresponding nominal concentration. Linear calibration curves were generated by weighted ($1/y$) linear regression analysis. The mean±SD coefficients of determination and slopes for 4′-hydroxyflurbiprofen and flurbiprofen in urine ($n=4$) were $0.996\pm0.005$ ($slope=6.84\times10^{-4}\pm3.99\times10^{-5}$) and $0.994\pm0.003$ ($slope=1.92\times10^{-3}\pm1.40\times10^{-4}$), respectively, while in plasma ($n=4$) they were $0.995\pm0.004$ ($slope=4.49\times10^{-3}\pm1.78\times10^{-4}$) and $0.996\pm0.001$ ($slope=1.42\times10^{-2}\pm2.45\times10^{-4}$), respectively. The lower limit of quantitation (LOQ) for flurbiprofen and 4′-hydroxyflurbiprofen in each matrix was selected as the lowest concentration of the standard curve (representative chromatogram for plasma is presented in Fig. 2.C).

### 3.2. Precision and accuracy

The precision and accuracy of the urine and plasma assays were determined by 3 days of quality

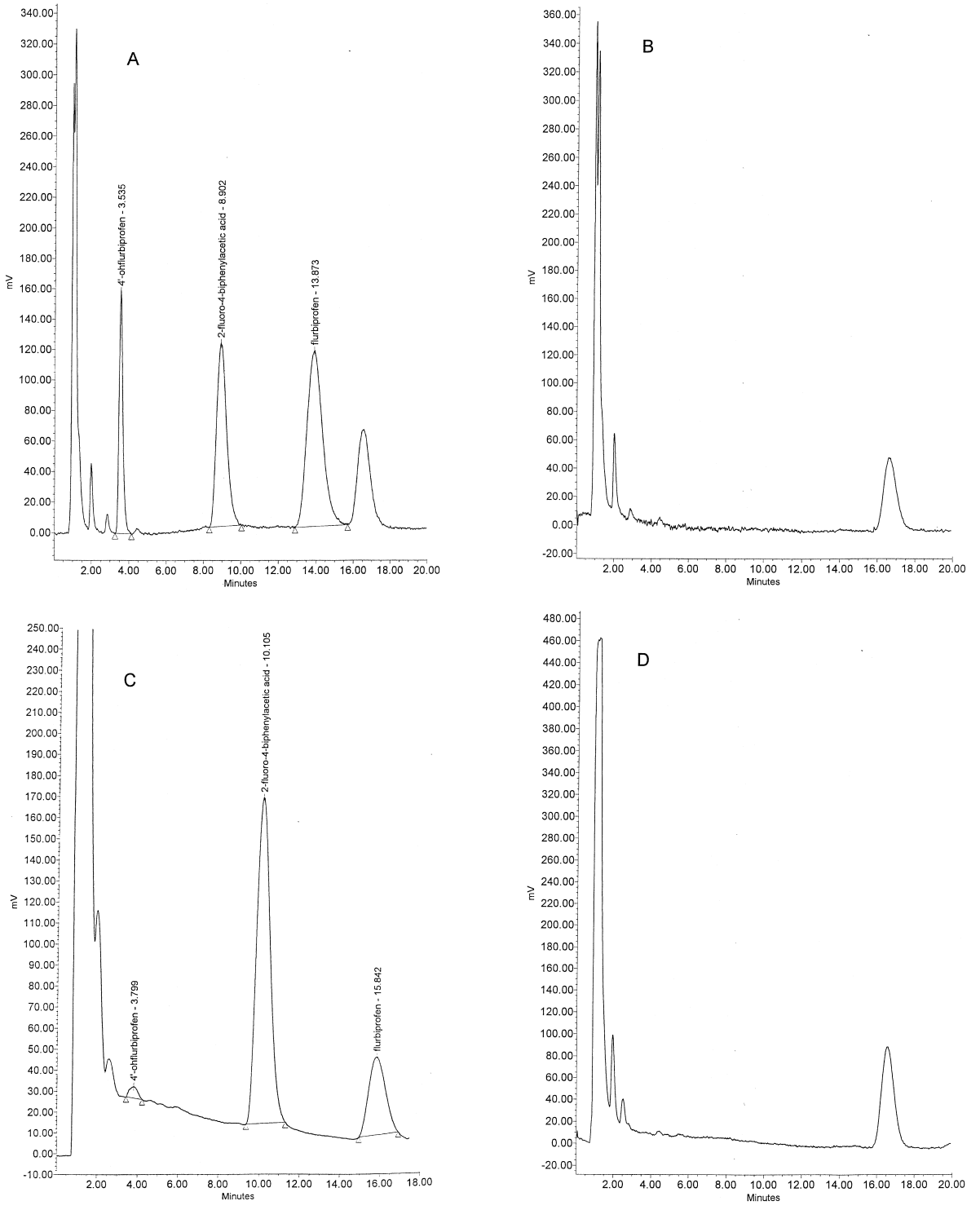


Fig. 2. A., B., C., and D. Representative HPLC chromatograms illustrating the detection of 4′-hydroxyflurbiprofen (5 μg/ml), internal standard, and flurbiprofen (5 μg/ml) in urine [Fig. 2.A.], blank urine [Fig. 2.B.], plasma at the LOQ of 4′-hydroxyflurbiprofen (0.05 μg/ml) and flurbiprofen (0.25 μg/ml) [Fig. 2.C.] and blank plasma [Fig. 2.D.].

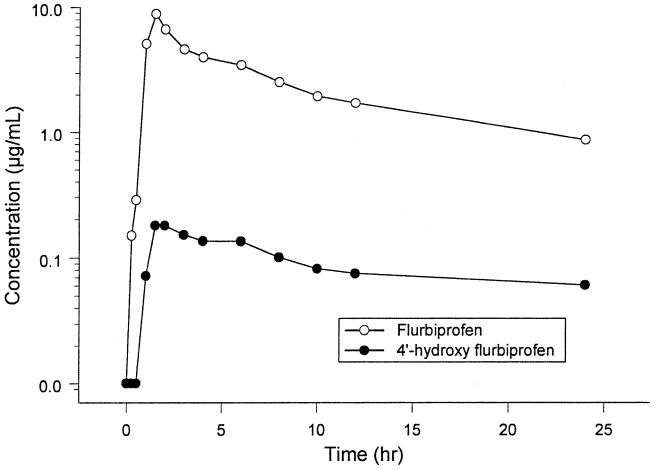


Fig. 3. Representative log plasma concentration-time profile for flurbiprofen and 4′-hydroxyflurbiprofen from a healthy subject who received a 50 mg dose of racemic flurbiprofen.

Table 1
Intra- and Inter-day precision and accuracy for flurbiprofen and 4′-hydroxyflurbiprofen in urine

| Compound | Concentration (μg/ml) | | | |
|---|---|---|---|---|
| | Added | Found | C.V. (%) | % Deviation |
| Intra-assay reproducibility[a] | | Mean± SD | | |
| Flurbiprofen | 1.0 | 0.90±0.09 | 9.5 | −10.0 |
| | 12.0 | 10.29±0.40 | 3.9 | −14.2 |
| | 30 | 28.23±2.58 | 9.1 | −6.0 |
| 4′-hydroxyflurbiprofen | 1.0 | 1.12±0.06 | 5.8 | 10.0 |
| | 12.0 | 11.46±0.47 | 4.1 | −4.2 |
| | 30 | 29.96±2.77 | 9.3 | −0.3 |
| Inter-assay reproducibility[b] | | Mean±S.E. | | |
| Flurbiprofen | 1.0 | 0.88±0.02 | 2.7 | −10.0 |
| | 12.0 | 10.39±0.34 | 3.6 | −13.3 |
| | 30 | 29.34±1.10 | 3.8 | −2.3 |
| 4′-hydroxyflurbiprofen | 1.0 | 1.09±0.03 | 3.0 | 10.0 |
| | 12.0 | 11.30±0.39 | 3.5 | −5.8 |
| | 30 | 30.54±1.00 | 3.4 | 1.7 |
| Freeze-thaw stability[c] | | Mean±SD | | |
| Flurbiprofen | 1.0 | 0.85±0.07 | 8.2 | −14.5 |
| | 30 | 29.64±1.56 | 5.3 | −1.2 |
| 4′-hydroxyflurbiprofen | 1.0 | 1.01±0.04 | 4.0 | 1.0 |
| | 30 | 30.65±1.39 | 4.5 | 2.2 |
| Processing stability[d] | | Mean±SD | | |
| Flurbiprofen | 1.0 | 0.92±0.03 | 3.3 | −7.7 |
| | 30 | 31.14±0.76 | 2.4 | 3.8 |
| 4′-hydroxyflurbiprofen | 1.0 | 1.01±0.02 | 2.0 | 0.8 |
| | 30 | 31.99±0.63 | 2.0 | 6.6 |

[a,d] Twelve samples per concentration.
[b] Six samples per day per concentration for 3 days.
[c] Three samples per concentration.

Table 2
Intra- and Inter-day precision and accuracy for flurbiprofen and 4′-hydroxyflurbiprofen in plasma

| Compound | Concentration (μg/ml) | | | |
|---|---|---|---|---|
| | Added | Found | C.V. (%) | %Deviation |
| Intra-assay reproducibility[a] | | Mean± SD | | |
| Flurbiprofen | 0.5 | 0.48±0.03 | 6.3 | −4.0 |
| | 6.0 | 5.49±0.23 | 4.2 | −8.3 |
| | 15 | 13.93±0.69 | 4.9 | −7.3 |
| 4′−hydroxyflurbiprofen | 0.1 | 0.12±0.02 | 13.7 | 16.7 |
| | 0.5 | 0.46±0.03 | 6.9 | −8.0 |
| | 1.5 | 1.39±0.07 | 4.9 | −6.7 |
| Inter-assay reproducibility[b] | | Mean±SE | | |
| Flurbiprofen | 0.5 | 0.46±0.02 | 4.4 | −8.0 |
| | 6.0 | 5.41±0.18 | 3.3 | −10.0 |
| | 15 | 13.97±0.04 | 0.3 | −6.7 |
| 4′-hydroxyflurbiprofen | 0.1 | 0.10±0.02 | 14.3 | 1.0 |
| | 0.5 | 0.45±0.01 | 2.7 | −10.0 |
| | 1.5 | 1.41±0.02 | 1.2 | −6.7 |
| Freeze–thaw stability[c] | | Mean±SD | | |
| Flurbiprofen | 0.5 | 0.49±0.01 | 2.0 | −2.9 |
| | 15 | 14.51±1.41 | 9.7 | −3.2 |
| 4′-hydroxyflurbiprofen | 0.1 | 0.09±0.01 | 11.1 | −6.9 |
| | 1.5 | 1.45±0.16 | 11.0 | −3.3 |
| Processing stability[d] | | Mean±SD | | |
| Flurbiprofen | 0.5 | 0.52±0.02 | 3.8 | 4.2 |
| | 15 | 14.11±0.55 | 3.9 | −5.9 |
| 4′-hydroxyflurbiprofen | 0.1 | 0.09±0.01 | 11.1 | −8.6 |
| | 1.5 | 1.45±0.04 | 2.8 | −3.1 |

[a,d] Twelve samples per concentration.
[b] Six samples per day per concentration for 3 days.
[c] Three samples per concentration.

control analysis. Intra-day ($n$=12) and inter-day ($n$= 3 days; $n$=24 samples) variability is shown in Table 1 and Table 2, respectively. Analyte stability was demonstrated in both urine and plasma by subjecting the samples to three freeze–thaw cycles, and injecting the processed quality control samples every two h over a 24 h period. Three freeze–thaw cycles had no effect on the stability of the analytes in both urine and plasma, as the accuracy of all low and high QC concentrations ($n$=3) for flurbiprofen and 4′-hydroxyflurbiprofen was within 15% of expected values (Tables 1 and 2). In addition, the coefficients of variation were below 11% for all LC and HC amounts (data not shown). Likewise, processed stability after processing was not a factor, as the accuracy of all low and high QC concentrations ($n$=12) was within 10% of expected values, and all coefficients of variations were within 8.5% (Tables 1 and 2).

## 4. Discussion

This study describes a rapid and sensitive HPLC method for the detection and quantitation of flurbiprofen and 4′-hydroxyflurbiprofen in urine and plasma. The method uses simple protein precipitation with acetonitrile followed by direct sample injection resulting in short sample preparation times. Quality control analysis indicates that the assay is precise (C.V.<14%) and accurate (within 15%). The urinary recovery data and the representative plasma concentration-time curve suggest that this method is sufficient for attaining accurate clinical phar-

macokinetic data following single dose administration of flurbiprofen. This assay has been used to validate use of the flurbiprofen 8 h urinary recovery ratio ($FLRR = \frac{4'OHF}{4'OHF + F}$) as an in vivo probe of cytochrome P450 2C9 activity in human subjects ([6], manuscript in preparation). Applications of this methodology will allow determination of relative CYP2C9 metabolizing capacity in individuals, phenotypic identification of individuals possessing mutant CYP2C9 alleles (e.g., *2 and *3 [10,11]) which result in altered metabolizing capacity and determination of the effects of disease states on CYP2C9 activity.

## Acknowledgements

This work was supported in part by a contract from the Food and Drug Administration (≠223-97-33005) and NIH/NCRR/GCRC grant ≠ 5M01 RR00056. J.M.H. was supported in part by a fellowship from the American Foundation for Pharmaceutical Education.

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