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Short communication

High-performance liquid chromatographic method for measurement of cytochrome P450-mediated metabolism of 7-ethoxy-4-trifluoromethylcoumarin

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Abstract

An HPLC method for analysis of deethylation of 7-ethoxy-4-trifluoromethylcoumarin (ETFMC), a substrate of various enzymes of the cytochrome P450 superfamily, was developed. ETFMC was incubated at 37°C with human hepatic microsomes or microsomes prepared from a lymphoblastoid cell line that expresses human CYP2B6. Under these conditions, the highly fluorescent metabolite 7-hydroxy-4-trifluoromethylcoumarin (HTFMC) is formed. The metabolite was analyzed by reversed-phase HPLC with fluorescence detection. The limits of detection of the metabolite were 5.0 fmol per injection, a sensitivity at least one order of magnitude greater than the standard method, which does not involve HPLC. This method will be of great utility when quantities of microsomal protein from cell lines expressing human CYP enzymes are limited. © 1998 Elsevier Science B.V.

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1. Introduction

7-Ethoxy-4-trifluoromethylcoumarin (ETFMC) is a useful substrate for the determination of certain cytochrome P450 (CYP) enzyme activities, such as CYP2B6 [1–4]. CYP2B6 and other CYP enzymes convert ETFMC (Fig. 1) to 7-hydroxy-4-trifluoromethylcoumarin (HTFMC), a metabolite with intense fluorescence. The standard method of determining deethylation of ETFMC involves the use of a static fluorimeter [2]. While this assay is quite easy to perform and reasonably sensitive, there is a need for a method allowing even greater sensitivity. Such an enhancement in sensitivity would allow the use of sparing quantities of microsomes prepared from cell lines designed to stably express CYP2B6. Therefore, our goal in this study was to develop a highly sensitive, quantitative HPLC method to assay ETFMC deethylase activity. This method is rapid and highly specific, and requires no extraction.

2. Experimental

2.1. Instrumentation

The HPLC system used for analysis consisted of an Alcott Model 738 autosampler (Norcross, GA,

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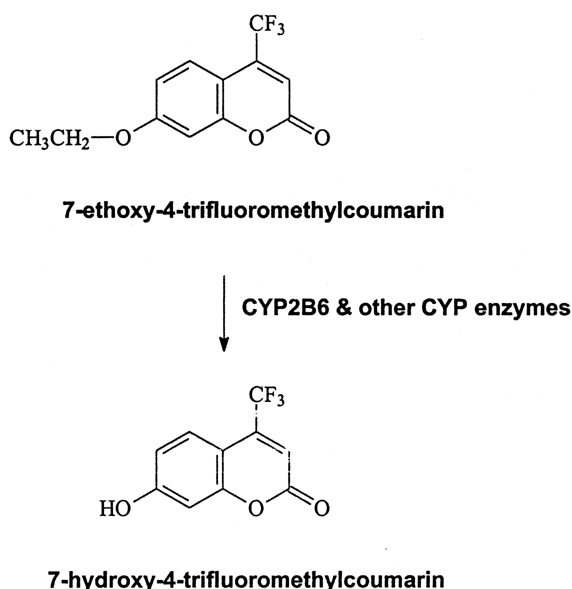

Fig. 1. Formation of HTFMC by cytochrome P450 enzymes.

USA) or a Rheodyne Model 7125 manual injection valve (Cotati, CA, USA), an ESA Model 580 solvent delivery module (ESA, Bedford, MA, USA), an Eppendorf CH-430 column heater (Westbury, NY, USA), a Waters 470 programmable fluorescence detector (Milford, MA, USA), and a ChromJet Model 4400 integrator (Spectra-Physics, San Jose, CA, USA).

2.2. Chemicals

ETFMC, HTFMC, glucose-6-phosphate, NADP, $MgCl_2$, EDTA, glucose-6-phosphate dehydrogenase, Tris–HCl, and sodium phosphate were obtained from Sigma (St. Louis, MO, USA). HPLC-grade phosphoric acid and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid was obtained from Jenneile Enterprises (Cincinnati, OH, USA). Bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce (Rockford, IL, USA). All other chemicals were of reagent grade or better.

2.3. Preparation of stock solutions

ETFMC stock solutions in the millimolar range were initially prepared in DMSO and then diluted to the micromolar range in methanol. HTFMC stock solutions in the millimolar range were also prepared in DMSO and then diluted to the micromolar range in methanol. For the injection of standards to serve as a basis of comparison for spiked sample matrices, HTFMC solutions were further diluted to the nanomolar range using HPLC mobile phase (described below).

2.4. Preparation of microsomes

Freshly excised tissues or frozen tissues were placed into normal saline on ice and then homogenized in four volumes of 0.05 *M* Tris–HCl, 1.15% KCl, pH 7.4. The homogenates were centrifuged at 9000 *g* for 30 min at 0°C. After discarding the pellet, the supernatants were centrifuged at 105 000 *g* at 0°C for 90 min. The 105 000 *g* supernatant was removed and the pellet was resuspended in 0.25 *M* sucrose. The resuspended pellets were centrifuged again at 105 000 *g* for 60 min. The microsomal pellets were re-suspended in 0.25 *M* sucrose and stored at −80°C until use. Protein concentrations were determined using the Pierce BCA kit following the manufacturer's instructions.

2.5. Incubation of microsomes

ETFMC stock solutions were initially prepared in DMSO and then diluted in methanol. Microsomes (100 μg CYP2B6, 1.0 mg mouse or human liver protein) were mixed with ETFMC (300 μ*M* final concentration), 50 m*M* Tris–HCl (pH 7.4), 2.5 m*M* NADP, 12.5 m*M* glucose-6-phosphate, 7.5 m*M* $MgCl_2$, and 2.5 m*M* EDTA. Mixtures were preincubated for 3 min at 37°C and then 5 U/ml of glucose-6-phosphate dehydrogenase were added. The final volume was 250 μl. Samples were incubated at 37°C for 10 min. The reaction was terminated with 50 μl of 60% trichloroacetic acid. Samples were then centrifuged at 7800 *g* for 10 min at 4°C. Samples were filtered through 0.45-μm Gelman acrodiscs, diluted 1:5 in HPLC buffer, and 10-μl aliquots were analyzed by reversed-phase HPLC.

2.6. HPLC detection of 7-hydroxy-4-trifluoromethylcoumarin

A 5-μm Burdick and Jackson 15×0.46 cm I.D. octadecyl column was eluted isocratically with 20

m*M* sodium phosphate, pH 7.5–40% methanol at a flow-rate of 1.0 ml/min. The temperature of the injection valve and the column was maintained at 30°C. The eluate was monitored at an excitation wavelength of 410 nm and an emission wavelength of 510 nm for a period of 15 min after each injection.

2.7. Generation of standard curves

Standard curves were prepared daily by plotting the integrated peak area of samples spiked with HTFMC versus the known concentration. Linear least-squares regression was performed without using the origin as a data point to determine the slope, y-intercept, and correlation coefficient.

3. Results

Fig. 2 shows chromatograms for the system we have developed. The upper trace is of an injection of microsomes (incubated without ETFMC) spiked with an HTFMC standard, the middle trace is of a 50-μg CYP2B6 incubation with 300 μ*M* ETFMC, and the lower trace is of an incubation with all system components (except microsomal protein) and 300 μ*M* ETFMC. The first peak is not merely a system peak, since injections of solvent only or standards only do not result in a significant peak. The peak eluting at 7.8 min in both the upper and middle traces corresponds to HTFMC.

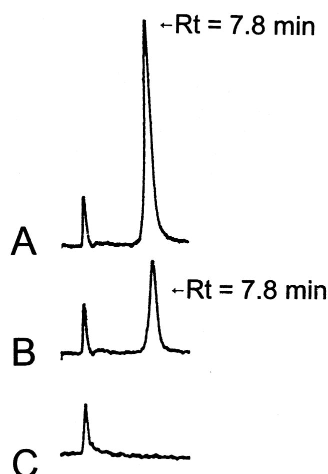

Fig. 2. Representative chromatograms. (A) Injection of a microsomal incubation performed without ETFMC spiked with an HTFMC standard. (B) Injection of a CYP2B6 incubation performed with 300 μ*M* ETFMC. (C) Injection of an incubation with all system components (except microsomal protein) plus 300 μ*M* ETFMC.

3.1. Limits of detection, quantitation and recovery

The limit of detection (LOD) was estimated as the quantity of injected HTFMC at a signal-to-noise ratio of 3. In this system, the LOD is 5 fmol. The limit of quantitation (LOQ) was estimated as the quantity of injected HTFMC at a signal-to-noise ratio of 10. In our system, the LOQ is 16.5 fmol. The recovery, calculated by comparison of peak areas of HTFMC-spiked microsomal samples with peak areas of injected standards, was 85.8%.

3.2. System linearity

Linearity was examined over a range of 50–10 000 fmol of HTFMC. The response was clearly linear (regression equation: $y=0.0023x-0.2383$; $r=0.9995$) over this 200-fold range.

Table 1
Precision and accuracy of HTFMC concentration determination

	Concentration of HTFMC (μ*M*)		C.V. (%)
	Actual	Determined	
Analyst 1			
Day 1	50	44.20±0.70	1.58
	500	427.7±3.9	0.92
Day 2	50	49.06±0.90	1.84
	500	464.5±5.3	1.14
Day 3	50	44.67±0.68	1.52
	500	421.1±11.3	2.68
Analyst 1			
Days 1–3	50	45.98±2.42	5.26
	500	437.8±21.3	4.87
Analyst 2			
Days 1–3	50	41.07±0.48	1.17
	500	435.6±20.7	4.75
Analysts 1 and 2			
	50	42.63±1.80	4.22
	500	431.7±14.0	3.25

Table 2
Metabolism of ETFMC by human liver microsomes and CYP2B6

Sample	Source	Production of HTFMC (pmol/min/mg microsomal protein)
HL-1	72-year-old caucasian female	93.8
HL-2	49-year-old caucasian female	89.2
HL-3	50-year-old caucasian male	381.8
HL-4	60-year-old caucasian male	340.5
HL-5	8-month-old black male	44.6
HL-6	45-year-old caucasian male	176.1
HL-7	63-year-old caucasian female	145.4
HL-8	32-year-old hispanic female	117.7
HL-9	38-year-old black male	274.8
HL-10	68-year-old caucasian female	59.3
CYP2B6	Lymphoblastoid cell line transfected with cDNA for human CYP2B6	106±16

3.3. Accuracy and precision

The accuracy and precision of the system is illustrated in Table 1. On each day, an analyst measured the concentrations of samples spiked with HTFMC in triplicate. In general, the calculated concentrations of samples were within 15% of the actual target value, with acceptably low variability. Daily coefficients of variation for analyst 1 ranged from 0.92 to 2.68%. The aggregate coefficients of variation for analyst 1 over a 3-day period were 5.26 and 4.87% for 50 and 500 μM concentrations, respectively. Combining the data for analysts 1 and 2, coefficients of variation were 4.22 and 3.25%, indicating good reproducibility.

3.4. Practical use of the assay

Table 2 presents data on ETFMC 7-deethylation for a panel of 10 human liver microsomal preparations and for microsomes derived from a lymphoblastoid cell line that expresses human CYP2B6. Enzymatic velocities in human liver microsomes ranged from 44.6 to 381.8 pmol/min/mg protein under the incubation conditions that were used. This indicates that other CYP enzymes besides CYP2B6 metabolize ETFMC, since CYP2B6 microsomes alone have a lower enzymatic velocity than all of the human liver microsomal samples that were analyzed.

4. Discussion

The present HPLC assay has a limit of detection of approximately 5 fmol per injection, at least a 10-fold improvement in sensitivity. Besides the simple fact that one is measuring the fluorescence of a much smaller volume of sample in the HPLC assay, there is also the matter of increased background in a non-HPLC assay due to interference by other components. As was seen in Fig. 2, the fluorescence due to system components is readily separated from the metabolic product HTFMC. This work provides the first HPLC-based assay of ETFMC deethylation. Given its much greater sensitivity than traditional methods, this HPLC assay will be useful in determination of the ETFMC deethylation activity of small quantities of microsomal preparations.

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