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Short Communication

Automated high-performance liquid chromatographic assay for the determination of 7-ethoxycoumarin and umbelliferone

Robert R. Evans and Mary V. Relling

Pharmaceutical Division, St. Jude Children's Research Hospital, 332 North Lauderdale, and Department of Clinical Pharmacy, University of Tennessee, Memphis, TN 38101 (USA)

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ABSTRACT

An improved high-performance liquid chromatographic assay is presented for the determination of 7-ethoxycoumarin O-deethylase activity. Following a 30-min microsomal incubation, 7-ethoxycoumarin, 4-methylumbelliferone (internal standard), and the metabolite umbelliferone were extracted with chloroform. Separation was achieved with an isocratic mobile phase using a μBondapak phenyl (300 mm × 3.9 mm I.D.) analytical column. The effluent was monitored by fluorescence detection with an excitation wavelength of 360 nm and an emission wavelength of 470 nm. The intra- and inter-assay coefficients of variation were 10 and 6%, respectively. A detection limit of 0.07 μg/ml was achieved, making this method suitable for characterizing P-450 activity of human livers.

INTRODUCTION

Cytochromes P-450 are a superfamily of isoenzymes comprising at least 24 different genes of the human genome [1]. Although some of these membrane-bound proteins have broad and overlapping substrate specificities, other isoenzymes are responsible for the metabolism of a limited and specific group of drugs. Furthermore, inter-individual variability in the activity of some of these enzymes occurs, which can result in either beneficial or toxic effects in prone individuals [2].

Correspondence to: Dr. R. R. Evans, Pharmaceutical Division, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38101, USA.

The activities of specific isoenzymes are often determined by incubating the drug of interest in the proper environment with human microsomes. Comparisons of the level of enzyme activity between different individuals are difficult to interpret without a non-specific substrate that could provide an index of overall liver condition and general oxidative activity. Many different agents have been proposed as probes to indicate overall P-450 activity; however, one of the most frequently used substrates is 7-ethoxycoumarin. 7-Ethoxycoumarin undergoes O-deethylation to umbelliferone by as many as eleven different isoenzymes [3]. The metabolism of 7-ethoxycoumarin by multiple isoenzymes may serve as an index of the general level of microsomal drug me-

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tabolizing activity. Several different methods for determining 7-ethoxycoumarin O-deethylase activity have been published [4–7]. Some of these assays are limited by insensitivity, lengthy extraction methods, the inability to be automated, and use of gradient mobile phases which can be cumbersome. This paper describes a simple, sensitive high-performance liquid chromatographic (HPLC) method for measuring umbelliferone that is suitable for automation.

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Shimadzu LC-6A pump (Columbia, MD, USA), a Perkin Elmer ISS100 autosampler (Norwalk, CT, USA), and a Spectroflow 980 fluorescence detector (Ramsey, NJ, USA). Compounds were separated on a μBondapak phenyl column (300 mm × 3.9 mm I.D.) with 10-μm packing (Waters, Bedford, MA, USA) and chromatograms were recorded on a Shimadzu CR501 integrator.

Reagents and standards

HPLC-grade methanol was obtained from Baxter (Muskegon, MI, USA), analytical-reagent grade trichloroacetic acid was supplied by J. T. Baker (Phillipsburg, NJ, USA) and glacial acetic acid and sodium acetate were obtained from Fisher Scientific (Houston, TX, USA). 7-Ethoxycoumarin, 4-methylumbelliferone, umbelliferone, isocitrate dehydrogenase, isocitrate and sodium NADP were purchased from Sigma (St. Louis, MO, USA).

A stock solution of 7-ethoxycoumarin was prepared by dissolving 10 mg of 7-ethoxycoumarin in 10 ml of methanol. An umbelliferone stock solution was prepared by dissolving 2 mg of umbelliferone in 10 ml of methanol. Standard curve calibrators were prepared exactly like the ethoxycoumarin incubations (see below) with NADPH except that enzyme activity was prevented by immediate addition of trichloroacetic acid and they were spiked with metabolite so that final concentrations of umbelliferone were 0.15, 0.3, 0.75, 1.2, and 1.5 μg/ml. The internal standard (I.S.), 4-methylumbelliferone, was dissolved in methanol to a final concentration of 0.025 mg/ml.

Mobile phase

The mobile phase consisted of methanol and 0.05 *M* acetate buffer (pH 4.7) (55:45, v/v). This mobile phase was filtered through a 0.45-μm Nylon filter (Rainin, Woburn, MA, USA) and degassed prior to use. The flow-rate was 1 ml/min and column temperature was ambient. Fluorescence detection of the peaks occurred with an excitation wavelength of 360 nm and an emission wavelength of 470 nm.

Assay of 7-ethoxycoumarin metabolism in liver microsomes

Microsomes were prepared from human livers as described by Meier *et al.* [8]. Protein concentrations of the microsomal preparations were determined according to the method of Bradford [9]. Microsomal incubations were performed in glass tubes by pre-incubation of 1 m*M* 7-ethoxycoumarin, 0.05–0.3 mg of microsomal protein in 0.1 *M* potassium phosphate buffer (pH 7.4) for 10 min at 23°C. The reactions were started by the addition of 50 μl of a NADPH-regenerating system (10 U/ml isocitrate dehydrogenase, 50 m*M* isocitrate, 10 m*M* sodium NADP, and 50 m*M* magnesium chloride) and incubated at 37°C for 30 min. The final volume of the reaction mixture was 250 μl. The reactions were stopped by the addition of 25 μl of 15% trichloroacetic acid. The internal standard was added and then the samples were extracted with 7 ml of chloroform, by mixture on an end-over-end rotary shaker at 40 rpm for 5 min, and centrifuged at 160 *g* for 5 min. The aqueous phase was frozen in an acetone dry ice bath, and the organic phase evaporated to dryness at room temperature under a stream of nitrogen. The residue was reconstituted in 200 μl of methanol–water (70:30, v/v) and 150 μl were injected onto the HPLC system. The system was automated with injections made every 15 min.

Calculations

Concentrations in unknowns were calculated by comparing peak-height ratios of umbellife-

rone to internal standard to those ratios obtained from spiked calibrators. The spiked calibrators were used to prepare a standard curve to assess linearity between the umbelliferone concentration and the peak-height ratios of umbelliferone to the internal standard. The extraction efficiency for umbelliferone was estimated by comparing the peak height of the extracted samples at both high and low concentrations with those of directly injected standard solutions dissolved in methanol–water (70:30, v/v).

RESULTS

Chromatography

As shown in Fig. 1, the retention times of umbelliferone, 4-methylumbelliferone (I.S.) and 7-ethoxycoumarin were 4.7, 5.8 and 11.6 min, respectively. No other peaks corresponding to these retention times were noted in either the blank standards or in the microsomal incubations performed in the absence of an NADPH-regenerating system. The standard curve was linear over the range of concentrations tested. The regression equation indicating the parameters (± their standard deviations) was as follows: concentration of umbelliferone = 1.92 (±0.04) · (peak-height ratio of umbelliferone to I.S.) + 0.016 (±0.02) with r^2 = 99.5%. The limit of detection, defined by a signal-to-noise ratio of 3, was 0.07 μg/ml or 17.5 ng. The extraction recovery of umbelliferone ranged from 91 to 98% over the concentrations in the standard curve. The intra-assay variability of umbelliferone was assessed by analyzing ten spiked samples at two different concentrations (1.5 and 0.3 μg/ml) on the same day. The inter-assay variability was determined at two concentrations (1.5 and 0.3 μg/ml), in duplicate on ten different occasions over a period of 30 days. Intra- and inter-assay variability is summarized in Table I. The intra-assay variability ranged between 5 and 10%. The inter-day mean variability was less than 6%.

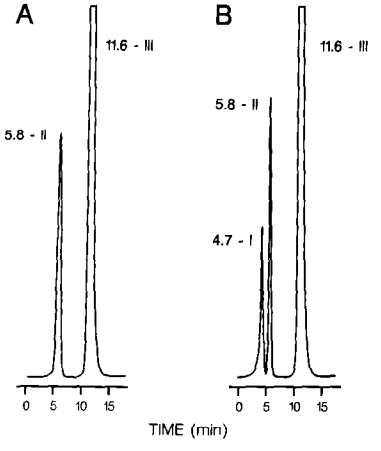

Fig. 1. Representive chromatograms of extracts. (A) Standard containing the I.S. 4-methylumbelliferone (II, retention time 5.8 min) and 7-ethoxycoumarin (III, retention time 11.6 min); (B) microsomal incubation indicating formation of the metabolite, umbelliferone (I, retention time 4.7 min).

TABLE I

INTRA- AND INTER-ASSAY VARIATION FOR UMBELLIFERONE (n = 10)

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Intra-assay		
1.5	1.48 ± 0.07	5.2
0.3	0.3 ± 0.03	10.0
Inter-assay		
1.5	1.48 ± 0.04	2.7
0.3	0.30 ± 0.02	5.5

Accuracy for the umbelliferone assay was tested by a blinded analyst measuring three spiked samples of umbelliferone in triplicate (Table II).

TABLE II

ACCURACY OF THE ASSAY FOR UMBELLIFERONE (n = 3)

Concentration introduced (μg/ml)	Concentration found (μg/ml)	Accuracy[a] (%)
0.18	0.17	95
0.75	0.76	101
1.35	1.23	91

[a] Accuracy = (found/added) × 100%.

TABLE III

PRECISION OF INCUBATION PLUS EXTRACTION METHOD

7-Ethoxycoumarin concentration (n = 5) (mM)	Umbelliferone formed (mean ± S.D.) (nmol/mg/h)	Coefficient of variation (%)
1.0	4.7 ± 0.34	7.3
0.1	2.88 ± 0.18	6.3
0.05	2.25 ± 0.17	7.8
0.025	1.59 ± 0.07	4.7
0.01	0.96 ± 0.07	7.3

The concentrations of the unknown samples were found to be within 91–101% of the theoretical values. The overall precision of the assay, including variability inherent in the microsomal incubations and extraction process, was determined by measuring the amount of umbelliferone formed at five different 7-ethoxycoumarin concentrations (Table III). All incubations were conducted with microsomes from the same liver at a protein concentration of 0.4 mg/ml. The coefficient of variation ranged from 4.7 to 7.8%.

Fig. 2 depicts the rate of formation for umbelliferone production *versus* substrate concentration from one human liver. A derivation of the Michaelis–Menten equation that incorporates inhibition of metabolite formation at high substrate concentration ($v = V_{max} \cdot [S]/(K_M + [S] + K_I[S]^2)$) was used to calculate the kinetic parameters. The K_M (Michaelis constant) and V_{max} (maximum velocity) values in these liver microsomes, 0.1 μM and 7.1 nmol/mg/h, were similar to those in other published reports [10]. The K_I constant, or the amount of inhibition of metabolite formation at high substrate concentrations, was 0.32 μM. The formation of umbelliferone by human microsomes was linear with respect to time for 45 min at varying substrate concentrations and linear with respect to microsomal protein concentration up to 2 mg/ml.

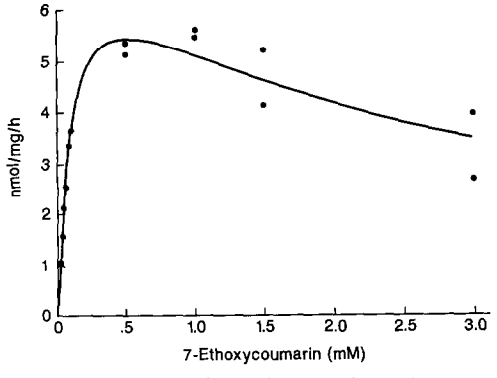

Fig. 2. Kinetic plot of umbelliferone formation *versus* 7-ethoxycoumarin concentration.

DISCUSSION

The present HPLC assay provides a reliable and rapid method for measuring umbelliferone. Unlike other assays [7], this method uses an isocratic rather than gradient mobile phase which is more convenient and yet produces chromatograms with symmetrically shaped peaks and short retention times. In addition, the extraction procedure is simple and results in a high level of precision and extraction efficiency. Moreover, the level of detection for this assay is well below the range of maximal umbelliferone production from human liver microsomal samples, using small concentrations of microsomal protein. The automation of the system allows results of several experiments to be quickly analyzed in an overnight run.

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