ELSEVIER

Journal of Chromatography B, 681 (1996) 227–232

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Fast determination of low-level cytochrome P-450 1A1 activity by high-performance liquid chromatography with fluorescence or visible absorbance detection

I. Leclercq, J.P. Desager, C. Vandenplas, Y. Horsmans*

Departments of Gastroenterology and Pharmacotherapy, University Hospital St-Luc, Catholic University of Louvain, 1200 Brussels, Belgium

Received 15 September 1995; revised 24 November 1995; accepted 24 November 1995

Abstract

A method to determine the activity of the cytochrome P-450 1A1 enzyme, by measuring 7-ethoxyresorufin-O-deethylase (EROD) activity using high-performance liquid chromatography (HPLC) with fluorescence or with visible absorbance detection of resorufin, is described. The lowest quantifiable activity (0.2 pmol/mg min) is obtained by incubation of 0.3 mg of human duodenal microsomal proteins using HPLC fluorescence detection. Using HPLC with visible absorbance detection, sensivity was ten times lower. However, the equipment for this last method is available in most laboratories. The use of both HPLC assays allows determination of the low EROD activity level in samples of small size, such as two or three human duodenal biopsies obtained by routine endoscopy. These methods will be a useful tool to study the role of drug intestinal metabolism by cytochrome P-450 1A1.

Keywords: Cytochromes; Enzymes

1. Introduction

Cytochrome P-450 1A1 (CYP-450 1A1) is the major enzyme induced in response to polycyclic aromatic hydrocarbons. As a result of biotransformation reactions associated with this cytochrome, chemicals may be changed into cytotoxic or carcinogenic substances. 7-Ethoxyresorufin-O-deethylase (EROD) activity was found to be a highly specific measure of the activity of this cytochrome [1,2]. In the alimentary tract of untreated rats, EROD activity is only detected in the jejunum (20 pmol/mg min) [3,4]. After β-naphthoflavone (βNF) treatment, a potent inducer of the CYP-450 1A1, EROD activity is increased throughout the alimentary tract tissues [3]. Whereas liver does not express CYP-450 1A1 constantly in human subjects [4–6], this cytochrome is constitutively expressed in the duodenum [4] and is induced by omeprazole [7]. The digestive location of CYP-450 1A1, in addition to being the site of absorption, failed to provide a major role for this enzyme against environmental chemicals and drugs.

Most laboratories use an EROD assay that is adapted from the fluorometric method described by Burke and Mayer [8] and by Pohl and Fouts [9].

*Corresponding author. Address for correspondence: Laboratoire de Pharmacothérapie, Université Catholique de Louvain, FATH 53.49, Avenue E. Mounier, 53, B-1200 Bruxelles, Belgium.

0378-4347/96/$15.00
SSDI 0378-4347(95)00543-9

With these methods, the reaction product (resorufin) is quantified by a spectrofluorimeter. Recently, Kennedy et al. [10,11] described a new technique to determine EROD activity using a fluorescence plate reader. However, the detection level of these techniques does not allow the determination of low activities from very small sized samples.

The aim of our study was therefore to develop a more sensitive quantitative assay for EROD activity. Resorufin was quantified by HPLC using fluorescence detection with reference to an internal standard. Since this equipment is not available in most laboratories, we also developed a method using the same conditions but with a visible absorbance HPLC detector.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a solvent delivery pump (Model 307, Gilson, Middleton, WI, USA), a variable-wavelength fluorescence detector (Spectra SYSTEM FL 2000, Spectra-Physics, San Jose, CA, USA) or a variable-wavelength UV–Vis absorbance detector with a Z-shaped cell (Dynamax UV-1, Rainin Instrument, Emeryville, CA, USA). Signal output was monitored with a CR501 integrator (Chromatopac, Bio-Rad, Hercules, CA, USA).

2.2. Reagents

Resorufin and 7-ethoxyresorufin were obtained from Sigma (St. Louis, MI, USA). Scoparone was from Aldrich (Milwaukee, WI, USA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH were from Boehringer (Mannheim, Germany). Methanol (from LabScan, Dublin, Ireland) was of HPLC grade. All other materials were obtained from the usual commercial suppliers.

2.3. Standards

The stock solution of resorufin (100 m*M*) was prepared in absolute ethanol; ethoxyresorufin (100 m*M*) in methanol and scoparone (0.8 m*M*) in 0.1 *M* Tris–HCl buffer, pH 7.8, and stored in aliquots at −20°C. The solutions used for the assay were diluted daily with 0.1 *M* Tris–HCl buffer, pH 7.8.

2.4. Sample preparation

Rat liver microsomes were obtained by differential centrifugation according to previously described procedures [12].

For the preparation of rat intestine, the first 20 cm of the small intestine, distal to the pylorus, was excised. It was flushed with ice-cold phosphate–KCl buffer. The mucosa was removed by scraping with the edge of a glass microscope slide and microsomes were extracted following the procedure used for the liver with some modification; the homogenate was sonicated on ice for 15 s using a sonifier B-12 (Branson Sonic Power, Danbury, CT, USA) before centrifugation.

Three human duodenal biopsies (30 mg) were homogenized in ice-cold phosphate buffer and microsomes were extracted as described for rat intestine.

Protein concentration was assayed with bicinchoninic acid (BCA) assay reagent (Pierce, Oud-Beijerland, Netherlands) using bovine serum albumin as the standard [13].

2.5. Assay of EROD activity

The EROD activity was determined by quantification of the resorufin production from deethylation of 7-ethoxyresorufin by microsomal proteins. The incubation mixture contained glucose-6-phosphate (0.8 mg), glucose-6-phosphate dehydrogenase (0.175 U), $MgCl_2$ (5 m*M*), bovine serum albumin (1.6 mg), varying concentrations of microsomal proteins and 7-ethoxyresorufin (1.5 m*M* in 0.1 *M* Tris–HCl buffer, pH 7.8) in a final volume of 1 ml. The tubes were warmed to a temperature of 37°C over 1 min. The reaction was initiated by adding 20 μl of 6.36 m*M* NADPH (the blank contained no NADPH) and was carried out at 37°C for 3 min. The reaction was stopped by adding 0.6 ml of 15% $ZnSO_4$. The internal standard solution (scoparone: 50 μl, 56 n*M* for fluorescence detection and 50 μl, 5.6 μ*M* for visible detection) was then added. Resorufin and scoparone were extracted, after addition of 50 μl of

20% trichloracetic acid (w/v), into 4 ml of diethyl ether, by manual shaking for 1 min. Following centrifugation at 3500 *g* for 5 min, the supernatant was evaporated to dryness under a stream of nitrogen. The residues were redissolved in 350 μl of mobile phase, filtered on a 0.22-μm filter and 200 μl aliquots were injected onto the HPLC column.

To determine the optimum conditions to be used, all measurements were performed with an incubation time and with amounts of microsomal proteins that would ensure linear reaction rates. The concentrations of microsomes used varied from 0.002 to 0.300 mg according to the origin and to the induction status of the microsomes.

2.6. Chromatography

Separation of the compounds was achieved at room temperature on a C_{18} reversed-phase column (150 × 4.6 mm I.D., 5 μm, cat no. 195-5028, Bio-Rad) equipped with a RP18 guard column (15 × 3.2 mm I.D., 5 μm, Applied Biosystems, Foster City, CA, USA). The mobile phase used was 25 m*M* phosphate buffer, pH 7–methanol (58:42, v/v), at a constant flow-rate of 0.8 ml/min (90 bar). Resorufin and scoparone were monitored using fluorescence detection at 530 nm (excitation), 580 nm (emission) and 326 nm (excitation), 416 nm (emission), respectively, and using visible detection at 570 nm from 0 to 5 min and after manual change, at 325 nm. The retention times for resorufin and the internal standard were 4.1 and 6.4 min, respectively. Resorufin was quantified by calibration curves obtained with authentic resorufin.

The data were analyzed by linear regression and the results were expressed in pmol of resorufin formed per mg of protein, per minute.

2.7. Calibration curves

Calibration curves were prepared as described above for eight different concentrations of resorufin (0.1 to 40 pmol resorufin added to 1 ml of 0.1 *M* Tris–HCl, pH 7.8, containing 0.08 mg of microsomal proteins). Standards were run before and after each set of assays.

2.8. Limits of detection

The limit of detection was defined as the lowest quantity for which the intra-day coefficient of variation is lower than 5%. This corresponded to 0.1 pmol in the assay (0.057 pmol on the column) with fluorescence detection and 2.5 pmol (1.43 pmol on the column) with visible detection.

2.9. Precision and accuracy

Precision and accuracy were determined for both methods by analysis of the enzymatic activity of microsomal proteins from rat liver and human duodenal samples. Three samples were analyzed ten times for the intra-day coefficient of variation and three times daily for 6 days for inter-day variation.

2.10. Recovery

Recovery of resorufin, after extraction, was determined by comparing the peak-height ratio of four different quantities (10, 20, 30 and 40 pmol, in triplicate) of authentic resorufin in mobile phase (volume of 350 μl) to the same quantities of resorufin extracted by diethyl ether (the internal standard solution was added after the evaporation step).

2.11. Specificity

Visible absorbance spectra of resorufin and scoparone dissolved in the elution solvent after extraction were found to be similar to those of the pure compounds. The optimal wavelenghts with fluorescence detection were 530 nm (excitation) and 580 nm (emission) for resorufin and 326 nm (excitation) and 416 nm (emission) for scoparone, whereas with the visible detector, they were 570 nm for resorufin and 325 nm for scoparone. At the wavelengths used, unchanged 7-ethoxyresorufin was not detected.

3. Results and discussion

Resorufin was accurately detected by reversed-phase HPLC using fluorescence or visible detection

(Fig. 1). Fig. 2 shows typical chromatograms obtained after incubation of microsomal proteins from human duodenum as described before, with fluorescence detection. The resorufin was eluted in 4.1 min and scoparone, in 6.4 min. An automatic change of the wavelength (excitation and emission) was made at 5 min. Unchanged 7-ethoxyresorufin did not disturb the analysis.

A blank (sample without NADPH and treated as described in Section 2) was incubated for each sample. The resorufin present in the blank is derived from the acid hydrolysis of the substrate. To calculate the activity, the peak-height ratio of the blank was subtracted from the peak-height ratio of its sample.

A linear relationship between peak-height ratio and concentration of resorufin was found with fluorescence detection from 0.1 to 40 pmol ($y = 6.12x - 0.1166$, $r^2=0.998$) and with visible detection from 5 to 40 pmol ($y = 28.96x - 0.7522$, $r^2=0.997$). Recovery of resorufin after extraction was 96.5% (coefficient of variation = 5.5%). The limit of detection for resorufin solutions was 0.1 pmol under the analytical fluorescence HPLC conditions and 2.5 pmol under visible detection conditions. The lowest quantifiable activity was 0.2 pmol resorufin/mg

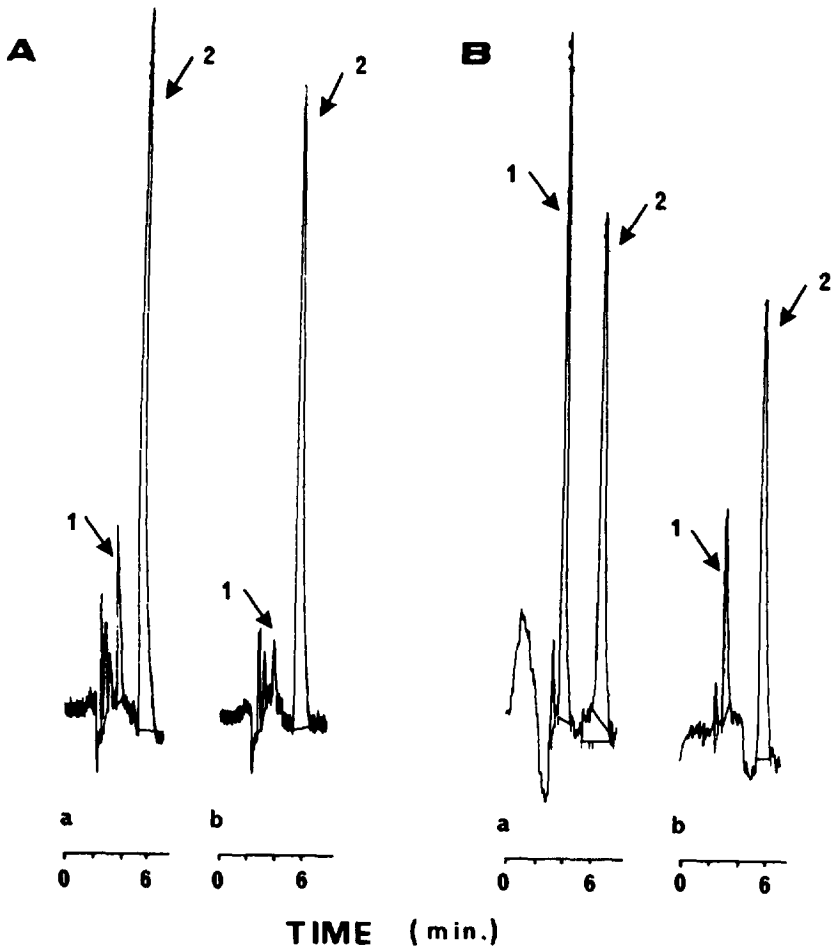

Fig. 1. Chromatograms obtained with 5 pmol of resorufin (a), and with the blank (b). A: Visible absorbance detection at 570 nm for resorufin and UV absorbance detection at 325 nm for the internal standard (scoparone). B: Fluorescence detection at 530 nm (excitation) and 580 nm (emission) for resorufin and at 326 nm (excitation) and 416 nm (emission) for the internal standard. Peaks: 1 = resorufin (4.1 min) and 2 = scoparone (6.5 min).

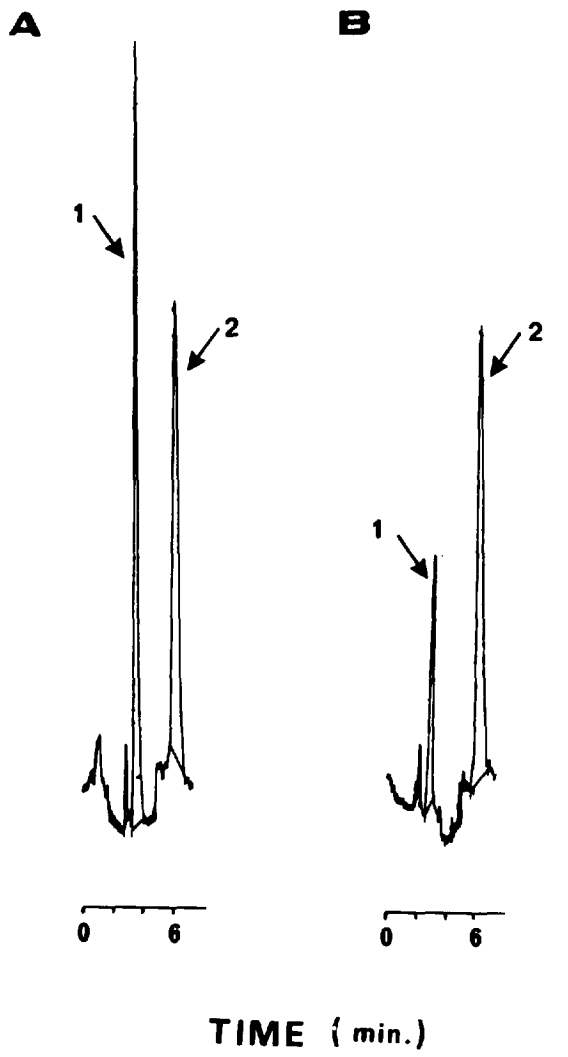

Fig. 2. Typical chromatograms obtained as described in Section 2.4 and Section 2.5 for (A) human duodenal microsomes (0.2 mg protein, incubation time, 3 min) and (B) the blank. Fluorescence detection at 530 nm (excitation) and 580 nm (emission) for resorufin and at 326 nm (excitation) and 416 nm (emission) for the internal standard (scoparone). Peaks: 1 = resorufin (4.1 min) and 2 = scoparone (6.5 min).

protein min with fluorescence and 2 pmol with visible detection (incubation of 0.3 mg of human duodenal microsomes for 3 min). Measurements were performed with an incubation time and with amounts of microsomal proteins that would ensure linear reaction rates. Production of resorufin was linear with incubation times from 1.5 to 8 min. Optimal concentrations of microsomal proteins were determined for liver microsomes of 3-methylcholanthrene (3-MC)-induced and untreated rats and for microsomal proteins from human duodenum (Fig. 3). The coefficient of variation of replicate enzymatic reactions with rat liver microsomes using the described procedure, was 3.9% for within-run assays and 6.1% for between-run assays (Table 1).

A very sensitive quantitative assay for EROD activity using HPLC with fluorescence detection is

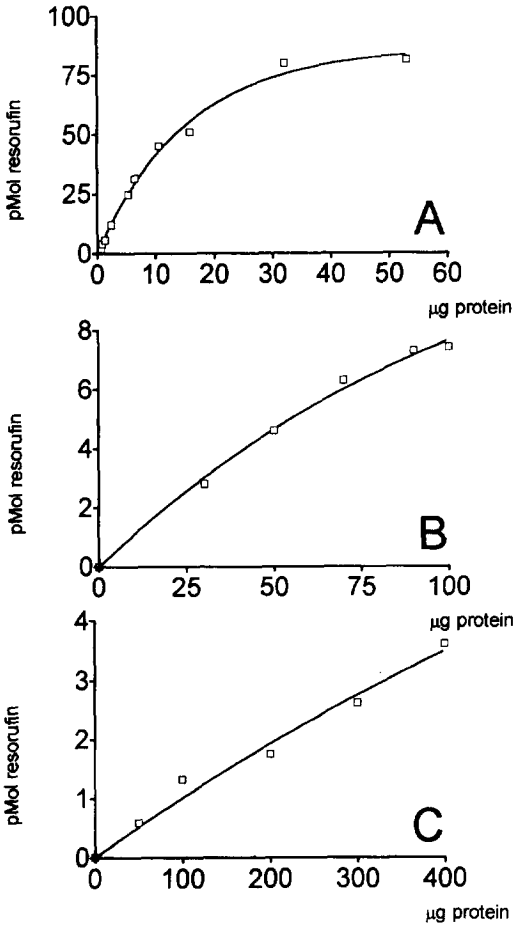

Fig. 3. Determination of optimal protein concentrations for 7-ethoxyresorufin-O-deethylase activity with A 3-MC-induced rat liver microsomes, B untreated rat liver microsomes and C human duodenal microsomes. Values are the means of duplicates. Activities were obtained with protein concentrations that would ensure linear reaction rates and were as follows: A = 4663 ± 86.1 pmol/mg min (C.V. = 4.5%), B = 29.4 ± 0.9 pmol/mg min (C.V. = 6.1%), C = 2.85 ± 0.05 pmol/mg min (C.V. = 4%).

Table 1
Within-run and between-run precision

	n	Activity (mean) (pmol/mg protein min)	S.D.	Coefficient of variation (%)
Within-run				
Liver microsomes from 3-MC-induced rats	10	1360	102	7.6
Liver microsomes from untreated rats	10	34.2	1.3	3.9
Duodenal microsomes from humans	6	2.7	0.2	7
Between-run				
Liver microsomes from untreated rats	6[a]	31.8	2	6.1

[a]Sample in triplicate.

described. The sensitivity of detection is 0.1 pmol of resorufin and allows activity as low as 0.2 pmol/mg protein min, with small amounts of microsomal proteins (0.3 mg), to be determined.

It has been found that the EROD activity is highly specific for the rat CYP-450 1A1 and for the human intestinal CYP-450 1A1 [1,2]. With the previous, less sensitive, methodologies, large biopsy capsules [14] or multiple endoscopic biopsy specimens [7] were needed to measure EROD activity from human small intestine. High sensitivity is thus needed to determine CYP 450 1A1 activity from small sized human samples. With the fluorescence HPLC assay described here, EROD activity of the intestinal tract is accurately measured using only 0.3 mg of microsomal proteins from 30 mg of duodenal tissue. The fluorescence HPLC technique is ten times more sensitive than previous methods [1,9–11], thus allowing quantification of EROD activity from small amounts of duodenal proteins. Moreover, this technique works in an isocratic mode and, as shown, can be used with visible detection with only a small loss of sensivity. This assay is of particular interest for studying the effects of drugs or diet on the regulation of the digestive CYP-450 1A1, especially in human beings.

Acknowledgments

This work is supported by a research grant from Glaxo, Brussels, Belgium.

References

[1] M.D. Burke, S. Thompson, C.R. Elcombe, J. Halpert, T. Haaparanta and R.T. Mayer, Biochem. Pharmacol., 34 (1985) 3337.
[2] M.D. Burke, S. Thompson, R.J. Weaver, C.R. Wolf and R.T. Mayer, Biochem. Pharmacol., 5 (1994) 923.
[3] P.G. Traber, W.M. McDonnell, W. Wang and R. Florence, Biochem. Biophys. Acta, 1171 (1992) 167.
[4] I. De Waziers, P.H. Cugnenc, C.S. Yang, J.P. Leroux and P.H. Beaune, J. Pharmacol. Exper. Ther., 253 (1990) 387.
[5] H. Schweikl, J.A. Taylor, S. Kitareewan, P. Linko, D. Nagorney and J.A. Goldstein, Pharmacogenetics, 3 (1993) 239.
[6] R.A. McKinnon, P. Hall, L.C. Quattrochi, R.H. Tukey and M.E. McManus, Hepatology, 14 (1991) 848.
[7] M. McDonnell, J.M. Scheiman and P.G. Traber, Gastroenterology, 103 (1992) 1509.
[8] D. Burke and R. Mayer, Drug Metabol. Dispos., 3 (1975) 245.
[9] R.J. Pohl and J.R. Fouts, Anal. Biochem., 107 (1980) 150.
[10] S.W. Kennedy, A. Lorenzen, C.A. James and B.T. Collins, Anal. Biochem., 211 (1993) 102.
[11] S.W. Kennedy and S.P. Jones, Anal. Biochem., 222 (1994) 217.
[12] F.P. Guengerich, Biochem. Pharmacol., 26 (1977) 1909.
[13] P.K. Smith, R.I. Krohn and G.Y. Hermanson, Anal. Biochem., 150 (1985) 76.
[14] M.R. Stahlberg, E. Hietanen and M. Mäki, Gut, 29 (1988) 1058.