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High-performance liquid chromatographic assay for 4-nitrophenol hydroxylation, a putative cytochrome P-4502E1 activity, in human liver microsomes

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ABSTRACT

A high-performance liquid chromatographic method which measures formation of product 4-nitrocatechol (4NC) has been developed and applied to the study of human liver microsomal 4-nitrophenol (4NP) hydroxylation. Following diethyl ether extraction, 4NC and the assay internal standard (salicylamide) were separated by reversed-phase (C_{18}) liquid chromatography. Extraction efficiencies of 4NC and internal standard were both >90%. The assay, which has a limit of detection of 15 pmol injected (or an incubation 4NC concentration of 0.25 μM), is accurate, reproducible and straightforward. With a chromatographic time of 12 min, 40–50 samples may be analyzed per day. Rates of 4NC formation were linear with time and protein concentration to 50 min and 0.5 mg/ml, respectively. Preliminary studies of 4NP hydroxylation showed that this reaction followed single enzyme Michaelis–Menten kinetics in human liver microsomes.

INTRODUCTION

Cytochrome P-450 comprises a family of enzymes of major importance in the oxidation of drugs, environmental pollutants, dietary chemicals and endogenous compounds. The various cytochrome P-450 isoforms exhibit distinct but overlapping patterns of substrate specificities and are independently regulated. In recent years particular interest has focused on the ethanol-inducible cytochrome P-4502E1 (CYP2E1), which has been implicated in the activation of many low-molecular-mass toxins and carcinogens, including acrylonitrile, benzene, carbon tetrachloride, N-nitrosodimethylamine, styrene and vinyl chloride [1]. CYP2E1 also has the capacity to metabolize other endo- and xenobiotics such as ketone bodies, acetaminophen, chlorofluorohydrocarbons, chlorzoxazone and ethanol [2–5].

Given the importance of CYP2E1, a number of compounds have been investigated as substrate probes for this enzyme. One such compound is 4-nitrophenol (4NP); the hydroxylation of 4NP to form 4-nitrocatechol (4NC) has been used as a marker of CYP2E1 activity in a number of animal species [6–8]. Assays for 4NP hydroxylation typically utilize spectrophotometric quantitation at 546 nm. However, the spectrophotometric method generally requires a high microsomal protein concentration which may result in high background absorbance. In addition, sensitivity is limited and rapid loss of 4NC absorbance has been reported at 546 nm [5].

In the present paper we describe an isocratic

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high-performance liquid chromatographic (HPLC) method for the determination of 4NP hydroxylase activity in human liver microsomes. The method is sensitive, precise, and has been applied to a preliminary study of human liver microsomal 4NP hydroxylation kinetics.

EXPERIMENTAL

Materials

4NP, 4NC, salicylamide, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from the Sigma (St. Louis, MO, USA). All other reagents and solvents were of analytical grade.

HPLC conditions

The assay was developed using a Model 6000A solvent delivery system, a Model 481 variable-wavelength UV–VIS detector, a Model U6K injector (Millipore–Waters, Milford, MA, USA) and a Model SE120 BBC Goetz Metrawatt recorder (Brown-Boveri, Vienna, Austria). Absorbance was monitored at 250 nm. The instrument was fitted with a μBondapak C_{18} column (30 cm × 3.9 mm I.D.; 10 μm particle size; Millipore–Waters) and operated at ambient temperature. The mobile phase, delivered at a flow-rate of 1.5 ml/min, comprised acetonitrile–glacial acetic acid–water (22:1:77) containing 30 m*M* triethylamine; the pH of this mixture was adjusted to 3.0 with phosphoric acid.

Microsomal incubations and sample preparation

Microsomes were prepared from human livers of renal transplant donors as previously described [9] and protein concentration was determined according to the method of Lowry *et al.* [10] with bovine serum albumin as standard. Reaction mixtures contained microsomal protein (0.2 mg), ascorbic acid (1 m*M*), 4NP (2.5–200 μ*M*) and NADPH-generating system (consisting of 1 m*M* $NADP^+$, 10 m*M* glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase and 5 m*M* $MgCl_2$) in a final volume of 0.5 ml of 0.1 *M* potassium phosphate buffer pH 6.8. Aqueous 4NP stock solutions were prepared freshly each day and stored in the dark at 0°C (ice bath). Incubations were started after 5 min of pre-incubation (37°C) by addition of the NADPH-generating system. After 30 min, reactions were terminated by the addition of 0.25 ml of 0.6 *M* perchloric acid. The assay internal standard, salicylamide (50 μl of a 8 μg/ml solution), was added to the mixture which was then saturated with ammonium sulfate (0.5 g) and extracted with 4 ml of diethyl ether (vortex mixer for 1 min). The organic layer was separated by centrifugation (3000 *g* for 15 min) and evaporated to dryness under nitrogen. Residues were redissolved in 120 μl of the mobile phase and a 15-μl aliquot was injected onto the HPLC system. Standard curves were constructed in the 4NC concentration range 0.5–20.0 μ*M* and unknown concentrations were determined by comparison of metabolite/internal standard peak-height ratio with those of the calibration curve.

RESULTS

Under the chromatographic conditions employed, retention times for salicylamide, 4NC, and 4NP were 5.6, 7.2, and 10.8 min, respectively, allowing sequential analysis of multiple samples at 12-min intervals (Fig. 1). The detector wavelength of 250 nm maximized sensitivity and specificity. At the mobile phase pH (3.0), 4NC exhibits near maximal absorbance at 250 nm, whereas substrate absorbance is minimal at this wavelength (Fig. 2). The detection limit of the assay, defined as a signal-to-noise ratio of 5:1, was 15 pmol of 4NC injected (or an incubation 4NC concentration of 0.25 μ*M*). Standard curves were linear over the concentration range 0.5–20.0 μ*M* and passed through the origin.

A number of extraction solvents were investigated which included chloroform, diethyl ether and ethyl acetate. Recovery was unsatisfactorily low with chloroform, and although ethyl acetate extraction resulted in quantitative recovery of 4NC, use of this solvent gave rise to a number of chromatographic interferences (data not shown). Thus diethyl ether, which produced no potentially interfering peaks, was chosen as the extraction

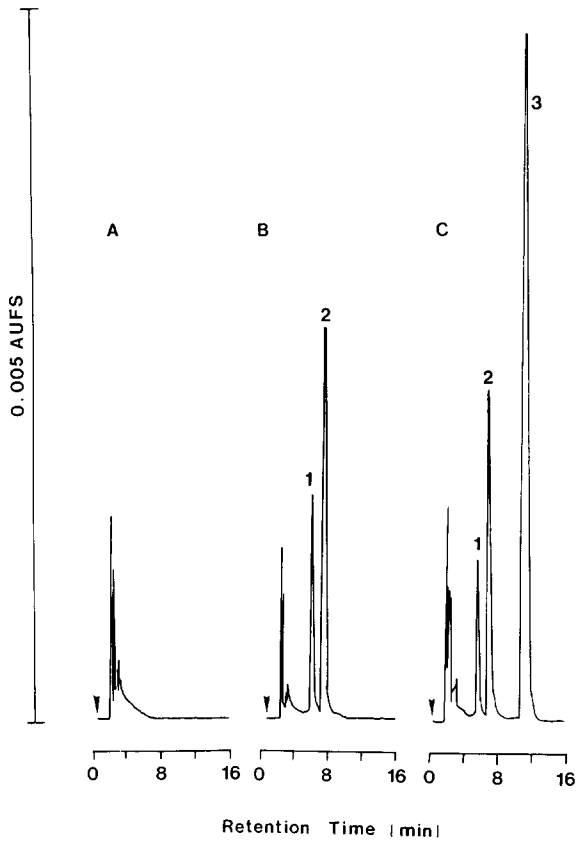

Fig. 1. Chromatograms of extracts. (A) Microsomal incubation without substrate; (B) aqueous standard containing 4NC (5 μM) and internal standard; (C) incubation of human liver microsomes in the presence of 4NP (25 μM). Arrow heads indicate time of injection. Peaks: 1 = internal standard; 2 = 4NC; 3 = 4NP.

solvent. The extraction procedure adopted was reproducible, both within and between concentrations. Mean (± S.D.) extraction efficiencies for spiked incubation 4NC concentrations of 0.5, 2.5 and 10 μM (n = 4 at each concentration) were 94.3 ± 5.7, 100.2 ± 1.5 and 98.8 ± 4.2%, respectively. The mean extraction efficiency for the internal standard (0.4 μg) was 91.9 ± 3.9% (n = 6).

Within-day coefficients of variation for spiked aqueous quality controls containing 0.8 and 7.5 μM 4NC (n = 8 at each concentration) were 8.3 and 5.5% respectively. Accuracy was 106.3 and 103.9% at 0.8 and 7.5 μM 4NC, respectively. Between-day coefficients of variation for the 0.8 and

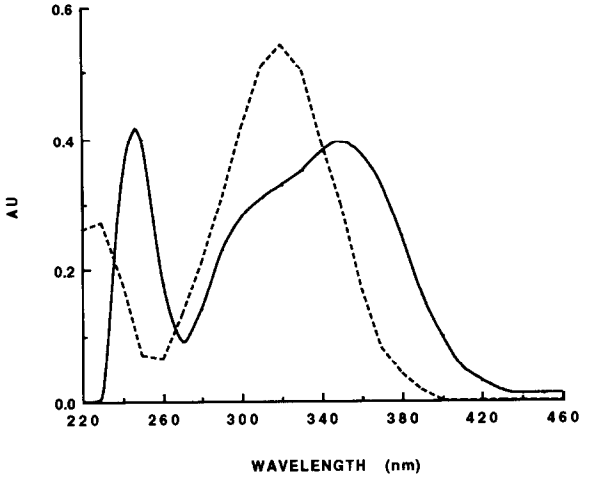

Fig. 2. Absorbance spectra of 4NP (broken line) and 4NC (solid line) dissolved in the mobile phase (concentration of both 50 μM).

7.5 μM spiked aqueous quality controls (n = 8 at each concentration) were 9.8 and 6.0%, respectively. The overall assay reproducibility was assessed by investigating formation of 4NC at an added 4NP concentration of 100 μM using the same batch of microsomes. The within-day and between-day (n = 6 estimations of each) coefficients of variation were 2.5 and 11.4%, respectively.

As demonstrated with rabbit liver microsomal incubations [7], the 4NP hydroxylase activity of human liver microsomes was maximal at pH 6.8 (data not shown). At this pH, 4NC formation was 1.3-fold higher than at pH 7.4. Thus an incubation pH of 6.8 was utilized.

The effect of incubation time and protein concentration is shown in Fig. 3. 4NC formation was linear for incubation times to 50 min and for microsomal protein concentrations to 0.5 mg/ml. 4NC formation by liver microsomes of a single donor over the 4NP concentration range 2.5–200 μM followed single enzyme Michaelis–Menten kinetics (Fig. 4). The apparent K_m and V_{max} values, estimated from the Eadie-Hofstee plot [11], were 26.3 μM and 1.10 nmol 4NC per min per mg protein, respectively. Using 0.2 mg of microsomal protein and an incubation time of 30 min, 4NC formation at the lowest substrate concen-

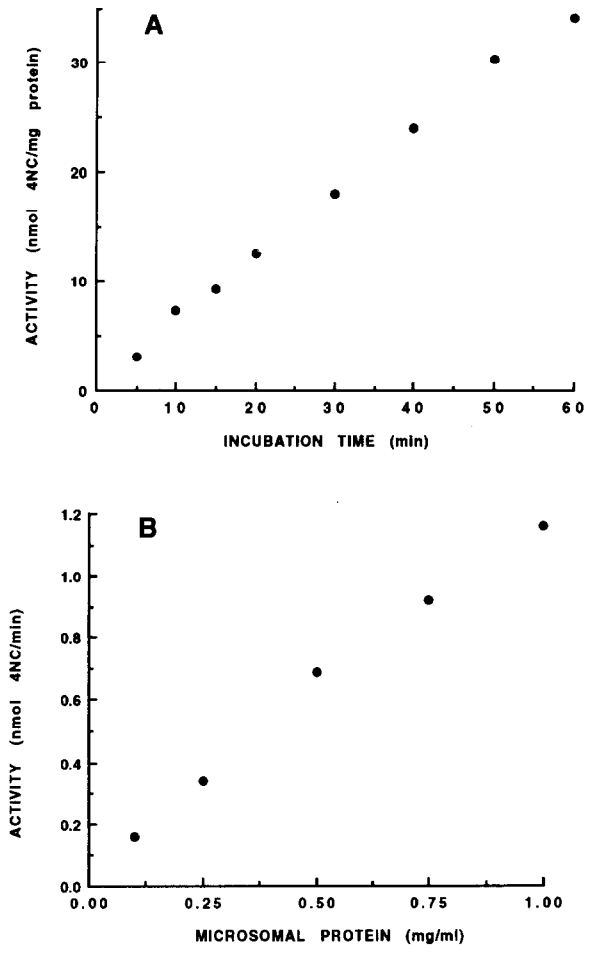

Fig. 3. (A) Time dependence, using 0.2 mg of protein and (B) protein dependence with 30 min incubation of 4NP hydroxylation by human liver microsomes (100 μM 4NP).

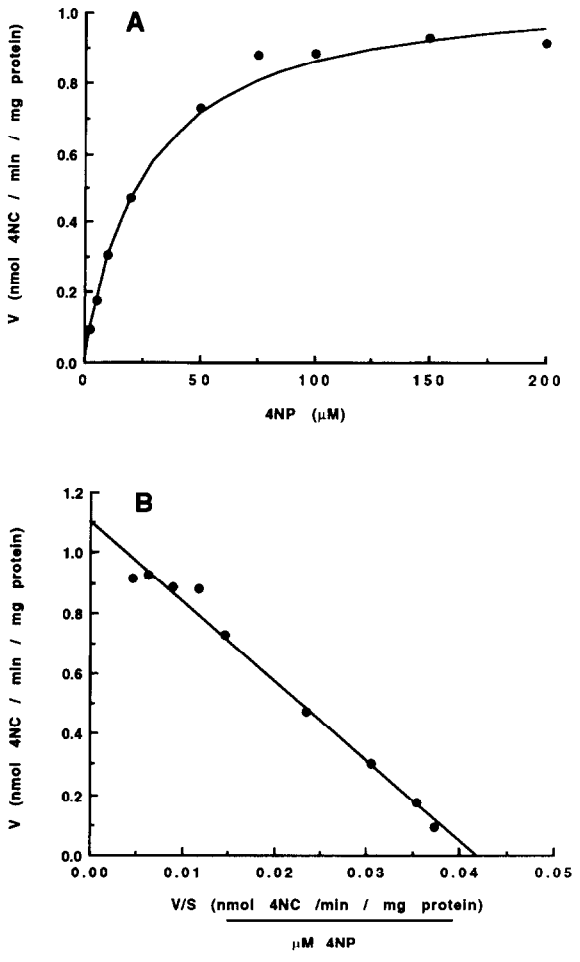

Fig. 4. Human liver microsomal 4NP hydroxylation kinetics. (A) Plot of substrate concentration *versus* product formation. (B) Eadie-Hofstee plot: V = velocity; S = substrate. Points are experimentally determined values while the solid lines are the computer-generated curves of best fit.

tration (*viz.* 2.5 μM) was approximately ten times the limit of assay sensitivity.

The effects of glycerol (used to stabilize frozen microsomes) on 4NP hydroxylation is illustrated in Fig. 5. Glycerol concentrations of 1.0% (v/v) or less had a negligible effect on 4NC formation over the 4NP concentration range 10–100 μM.

DISCUSSION

Preliminary studies in this laboratory of 4NP hydroxylation by human liver microsomes using the previously published spectrophotometric method [6,7] indicated that the procedure suffered limitations of sensitivity (lower limit approximately 5 nmol of 4NC), specificity and stability of 4NC under the alkaline conditions necessary to measure product formation. Moreover, the spectrophotometric assay had a high requirement for human liver microsomal protein (1 mg per incubation). In contrast, the HPLC assay described here is sensitive, reproducible and once extracted 4NC was stable for at least 3 h when reconstituted in the mobile phase. Furthermore, the HPLC assay has a low requirement for microsomal protein, is free from interference by

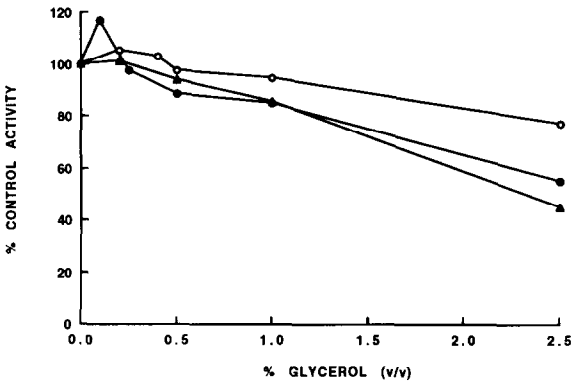

Fig. 5. Effects of glycerol on human liver microsomal 4NP hydroxylation. Concentrations of 4NP were (●) 100 μM, (○) 25 μM, and (▲) 10 μM.

other potential CYP2E1 probes (*e.g.* chlorzoxazone and diethyldithiocarbamate) and is straightforward; about 40–50 samples may be analyzed in a single day.

A number of compounds besides 4NP have been used as *in vitro* substrate probes for CYP2E1 activity in various species. These include acetone [2], aniline [12], chlorzoxazone [3], ethanol [13] and N-nitrosodimethylamine [14]. Acetone and ethanol are both volatile compounds and incubations with these substrates must be performed in sealed containers. Measurement of product formation with these substrates is similarly complicated by metabolite volatility. Although aniline hydroxylation has been used as a marker for CYP2E1 activity *in vitro*, this compound is not specific for CYP2E1 [12]. N-Nitrosodimethylamine is a known carcinogen and interpretation of activity with this substrate is complicated by its non-linear kinetics [15]. The low substrate concentration of N-nitrosodimethylamine required for the specific measurement of CYP2E1 activity necessitates the use of a radiometric procedure [16]. An HPLC assay for chlorzoxazone hydroxylation, a reaction linked to human CYP2E1, has been described recently [3] but the metabolic product, 6-hydroxychlorzoxazone, is not commercially available. The various complications associated with all of these methods are not apparent with the procedure developed for 4NP hydroxylation.

A preliminary study of 4NP hydroxylation using the newly developed HPLC method has shown that 4NC formation by human liver microsomes follows Michaelis–Menten kinetics, consisted with the involvement of a single cytochrome P-450 isoform in this reaction (Fig. 4). Further studies are under way to confirm the specific involvement of CYP2E1.

Glycerol is used widely to stabilize frozen liver microsomes but it has been reported recently that this compound may inhibit CYP2E1 [16,17]. Over the 4NP concentration range 10–100 μM, glycerol concentrations $\leq 1.0\%$ (v/v) were shown to have a negligible effect on product formation. Thus, the 4NP hydroxylation activity of liver microsomes stabilized with glycerol may be determined as long as the final incubation of glycerol does not exceed this value.

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