Journal of Chromatography, 301 (1984) 282–287
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16.987

Note

Rapid and sensitive assays using high-performance liquid chromatography to measure the activities of phase II drug metabolising enzymes: glucuronyl transferase and sulfotransferase

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(First received June 4th, 1984; revised manuscript received June 27th, 1984)

Phase II enzymes, including glucuronyl transferase and sulfotransferase, are important for: (1) converting drugs, chemicals and endogenous substrates into water soluble compounds excretable via the kidney, and (2) detoxifying potentially toxic reactive intermediary metabolites directly, or providing alternative routes of elimination which bypass the formation of a reactive intermediate[1,2]. Paradoxically, phase II enzymes recently have been shown for a limited group of chemicals to form unstable products which degenerate into a toxic reactive intermediate[2].

Quantification of activity of these enzymes therefore is fundamental to many studies in metabolism and toxicology. Generally such assays involve the addition of a substrate to an incubated enzyme source with subsequent quantification of product per mg of protein per unit of time. Available assays which are specific and sensitive are costly, labour-intensive and lengthy, generally involving the use of differential extractions, costly radiolabeled substrate, thin-layer chromatography and scintillation spectrometry[3–9]. Many of these problems are avoided by the use of high-performance liquid chromatography (HPLC), and this technique is being used increasingly in the kinetic analysis of enzymatic reactions[10]. We report herein simple and sensitive assays using HPLC for quantification of the enzymatic activites of glucuronyl transferase and sulfotransferase, each in less than 10 min.

MATERIALS AND METHODS

Chemicals

Acetaminophen, α-naphthol, β-naphthol, uridine diphosphoglucuronic acid (UDPGA), adenosine-3′-phosphate-5′-phosphosulfate (PAPS), α-naphthol-glucuronic acid and β-naphthol sulfate were obtained from Sigma (St. Louis, MO, U.S.A.).

Biochemical assays

Hepatic microsomal and cytosolic fractions were obtained from male CD-1 mice (Charles River Canada, Quebec, Canada). Glucuronyl transferase and sulfotransferase activities were measured, using the respective substrate α-naphthol and β-naphthol, according to our modifications of published methods[7,9].

For measurement of glucuronyl transferase activity, α-naphthol to a final con-

0021-9673/84/$03.00

centration of 5.0 m*M* was dissolved in a 50 m*M* Tris buffer, pH 7.4, with 10 m*M* magnesium chloride and a range of concentrations of dimethylsulfoxide (DMSO). After addition of UDPGA to a concentration of 4.0 m*M*, the mixture was incubated at 37°C for 5 min, microsomal protein was added in variable amounts and the suspension was incubated in a shaking water bath at 37°C for a variable duration up to 20 min.

For determination of sulfotransferase activity, the reaction contained 0.125 m*M* β-naphthol in 0.25 *M* sodium phosphate buffer at pH 6.5, 5.0 m*M* 2-mercaptoethanol and 0.2 m*M* PAPS in 5.0% (v/v) acetone in phosphate buffer. This mixture was preincubated at 37°C for 5 min before the reaction was initiated by the addition of cytosolic protein from the 100,000 × *g* fraction to give a final concentration of 2.0 mg/ml. This suspension was incubated in a shaking water bath at 37°C for 10 min.

In both assays, the reaction was stopped by the addition of ice cold methanol containing the respective internal standards. The respective standards for the glucuronyl transferase and sulfotransferase assays were β-naphthol and acetaminophen. The suspension was centrifuged at 1000 × *g* for 20 min and the supernatant was

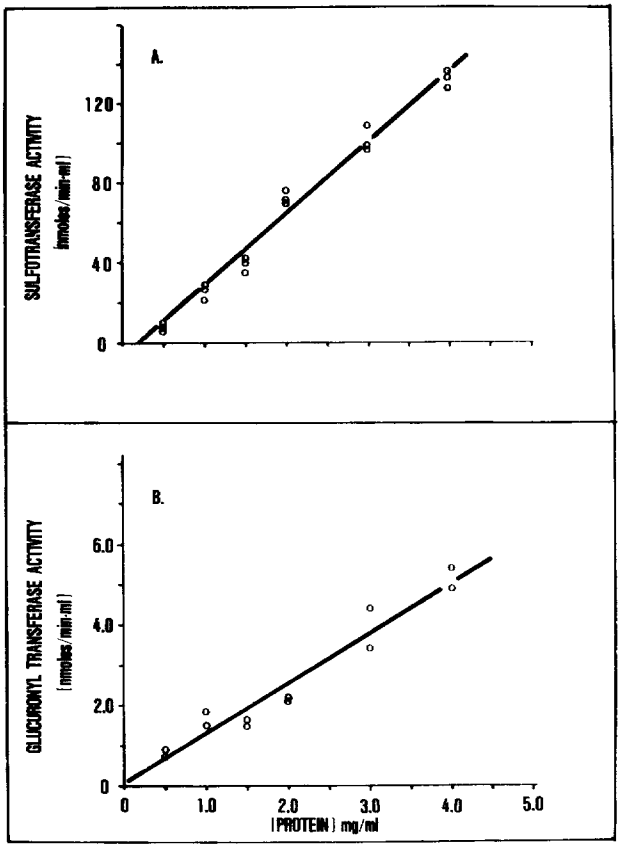

Fig. 1. Effect of protein concentrations on enzyme activity. (A) Glucuronyl transferase activity was measured by the amount of α-naphthol glucuronide produced at varying concentrations of microsomal protein ($r = 0.982$, $p < 0.05$). (B) Sulfotransferase activity was measured by the amount of β-naphthol sulfate produced at varying concentrations of cytosolic protein ($r = 0.942$, $p < 0.05$).

saved. The pellet was rewashed with methanol, centrifuged and this second supernatant added to the first. For the glucuronyl transferase assay, the supernatant was dried under nitrogen, redissolved in methanol and injected into the HPLC. With the sulfotransferase assay, the supernatant was dried under nitrogen and redissolved in water. The unconjugated β-naphthol was extracted out with chloroform to prevent it from interfering with subsequent assays due to its long retention time (30 min). The aqueous layer was injected into the HPLC.

HPLC

HPLC was employed using an automated system (Perkin-Elmer Canada, Toronto, Canada). Samples were injected from microvials using an autosampler (Model ISS-100). The pumping system (Model Series 4) consisted of a quaternary gradient system with on-line helium degassing and a controller. The detector was a double-beam, variable-wavelength, UV–VIS spectrophotometer (Model LC 85) equipped with a 2.4-μl flow cell, automatic baseline compensation (Autocontrol) for gradient conditions, and stop-flow scanning. The detector signal was integrated as the chromatographic peak area-under-the-curve by a 16-bit microcomputer (Model Sigma 15) and stored on-line on floppy disks using an 8-bit microcomputer (Model 3600).

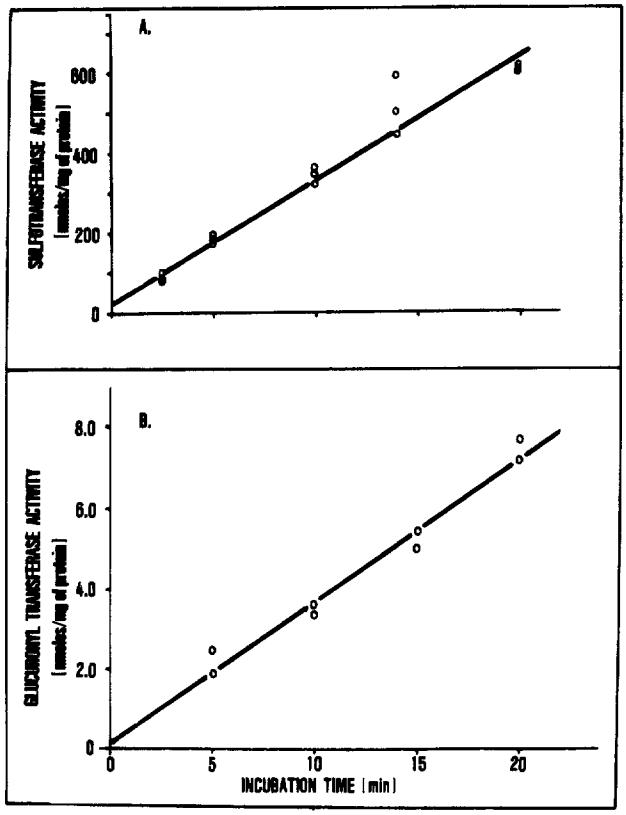

Fig. 2. Effect of incubation time on enzyme activity. (A) Glucuronyl transferase activity was measured by the amount of α-naphthol glucuronide produced after varying incubation time periods ($r = 0.966$, $p < 0.05$). (B) Sulfotransferase activity was measured by the amount of β-naphthol sulfate produced after varying incubation time periods ($r = 0.973$, $p < 0.05$).

TABLE I

EFFECT OF DIMETHYLSULFOXIDE (DMSO) ON GLUCURONYL TRANSFERASE ACTIVITY IN MICROSOMAL SUSPENSION*

DMSO concentration (%, v/v)	*Glucuronyl transferase activity** (% of maximal activity)*
0.3	100
1.0	91.3
5.0	95.3
10.0	48.5
15.0	44.6

* Microsomal protein concentration was 1.0 mg/ml.
** Activity was calculated as μg of α-naphthol glucuronide produced per min per mg protein.

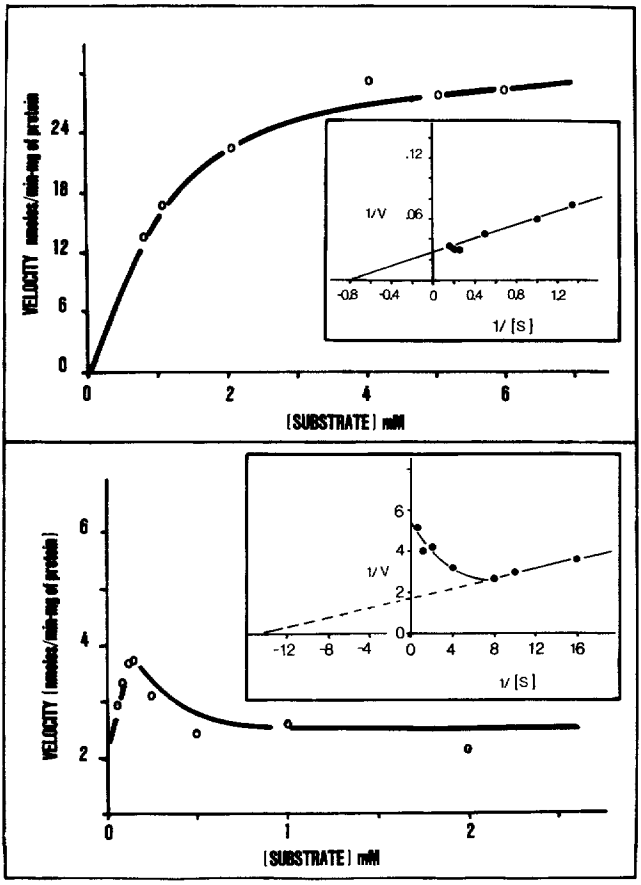

Fig. 3. Effect of substrate concentration on enzyme activity. (A) Glucuronyl transferase activity was measured by the amount of α-naphthol glucuronide produced at varying concentrations of α-naphthol. The apparent V_{max}, 38 nmoles/min/mg of protein, and the apparent K_m, 1.25 mM, was determined from the double reciprocal plot with a correlation coefficient of 0.944 ($p < 0.05$). (B) Sulfotransferase activity was measured by the amount of β-naphthol sulfate produced at varying concentrations of β-naphthol. The apparent V_{max}, 0.59 nmoles/min/mg of protein, and the apparent K_m, 0.07 mM, were determined by extrapolating the linear part of the double reciprocal plot ($r = 0.850$, $p < 0.05$).

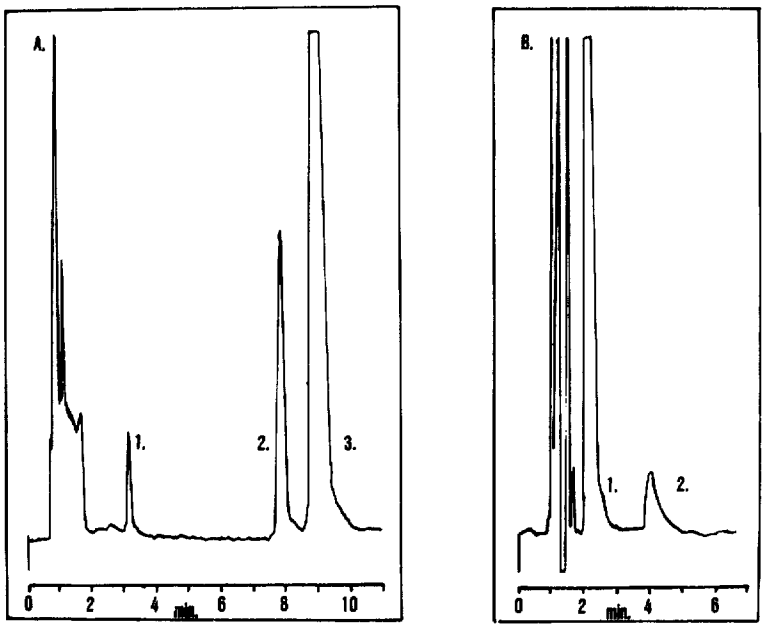

Fig. 4. Chromatograms of the HPLC resolutions of α-naphthol glucuronide and β-naphthol sulfate. (A) Peak identification: 1 = α-naphthol glucuronide; 2 = β-naphthol, the internal standard; 3 = α-naphthol, the substrate. Solvent: 0.1 *M* acetic acid–methanol (55:45, v/v); flow-rate, 1.5 ml/min; wave length, 240 nm. (B) Peak identification: 1 = acetaminophen, the internal standard; 2 = β-naphthol sulfate. Solvent: 0.1 *M* acetic acid–acetonitrile (85:15, v/v); flow-rate, 1.5 ml/min; wave length, 235 nm.

All samples were analysed using a 15 cm × 4.6 mm I.D. reversed-phase (RP) C_{18} column with a particle size of 5 μm (Beckman Instruments, Toronto, Canada). For analysis of the α-naphthol glucuronide the optimal solvent system consisted of 0.1 *M* acetic acid–methanol (55:45, v/v) with a flow-rate of 1.5 ml/min; for β-naphthol sulfate resolution, the optimal solvent system involved 0.1 *M* acetic acid–acetonitrile (85:15, v/v). Absorbance was monitored at 240 nm and at 235 nm respectively. Spectral scans from 180 nm to 300 nm were obtained for α-naphthol glucuronide and β-naphthol sulfate under analytical conditions using HPLC stop-flow scanning. Both compounds exhibited a maximal absorbance at 225 nm. The final wavelength for each assay was chosen to optimise sensitivity and maintain a steady baseline.

RESULTS

For the glucuronyl transferase assay, the substrate α-naphthol required 3.0% (v/v) DMSO for adequate solubilisation in the aqueous medium. The concentration of DMSO had to be kept below 5.0% (v/v) to have a minimal inhibitory effect on enzymatic activity (Table I), and 3.0% (v/v) was used in our assay. The reaction for both enzyme assays were linear for protein concentrations between 0.5 and 4.0 mg/ml (Fig. 1) and linear for incubation times between 2.5 and 20 min (Fig. 2). The final substrate concentration for the glucuronyl transferase activity was chosen according to the apparent K_m (*i.e.* [S] = 4 × K_m = 5 m*M*), since the objective in this instance was to measure the enzyme activity around V_{max} (Fig. 3). The sulfotransferase assay showed substrate inhibition similar to that described by Sekura and Jakoby[9], although the optimal substrate concentration was found to be at 1.25 m*M* in the CD-1 mice (Fig. 3), compared with 2.5 m*M* in rats.

The chromatographs for the resolution and detection of α-naphthol glucuronide and β-naphthol sulfate are shown in Fig. 4. The limit of detection sensitivity, taken as two times the baseline excursion, was 20 ng for α-naphthol glucuronide and 72 ng for the β-naphthol sulfate. The standard curves were linear up to at least 6 μg with a correlation coefficient of 0.980. The recovery efficiency of α-naphthol glucuronide was 84.7 ± 2.9% (mean ± S.D., $n = 7$) and 86.4 ± 3.6 for the internal standard, β-naphthol. The extraction efficiency of β-naphthol sulfate was 91.3 ± 4.8 ($n = 5$) and 92.5 ± 2.3 for the internal standard, acetaminophen.

DISCUSSION

The versatility of HPLC in the separation and detection of a diverse range of enzymatic substate and products has resulted in an increasing use of this technique in the analysis of enzyme kinetics[10]. In this paper, we report that HPLC is a simple and sensitive technique for rapid measurement of the activities of the phase II enzymes, glucuronyl transferase and sulfotransferase. This technique avoids the problems of traditional methods involving differential extractions, costly radiolabeled substrates, thin-layer chromatography with large quantities of solvents, and scintillation spectrometry. The detection sensitivity for naphthol conjugates could be increased if necessary by the use of an electrochemical detector. Even with the more expensive and time-consuming use of radiolabeled substrates and scintillation spectrometric detection, the HPLC method with fraction collection avoids the problems of thin-layer chromatography.

ACKNOWLEDGEMENTS

This research was supported by grants from the Connaught Fund, the Atkinson, Banting Research, and Hospital for Sick Children Foundations, and the Medical Research Council of Canada. E.C.A.T. was the recipient of a University of Toronto Open Fellowship.

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