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Short communication

# Determination of cytochrome P450 2E1 activity in microsomes by thin-layer chromatography using [2-$^{14}$C]chlorzoxazone

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**Abstract**

A thin-layer chromatographic assay was developed as an alternative method for the determination of cytochrome P450 2E1 (CYP2E1) in microsomes using [2-$^{14}$C]chlorzoxazone. After incubation of microsomes with 0.125 $\mu$Ci/mmol chlorzoxazone, chlorzoxazone and its single metabolite, 6-hydroxychlorzoxazone, were extracted using chloroform–2-propanol (85:15, v/v) and chromatographed on silica gel 60 F254 plates with acetone–hexane (45:55, v/v) as solvent. The plates were then exposed to X-ray film for 2 days to localize the radiolabelled chlorzoxazone and 6-hydroxychlorzoxazone. The metabolite and substrate regions were scraped and counted in a liquid scintillation analyzer. This method is sensitive enough to determine constitutive and induced CYP2E1 activities in liver or kidney microsomes. The precision of the method was similar to that of the HPLC method. The correlation coefficient between both methods was found to be 0.97 ($n$=21). Therefore, the TLC method constitutes a valuable tool for the determination of chlorzoxazone metabolism in microsomes.

*Keywords:* Cytochromes; Enzymes

## 1. Introduction

Cytochrome P450 2E1 (CYP2E1) is a key enzyme in ethanol metabolism. This microsomal enzyme has been shown to be inducible by many compounds such as ethanol, chemicals (benzene, pyridine), drugs (isoniazid, chloramphenicol) or physiopathological states (starvation, obesity, diabetes) [1] and can activate a variety of substrates including toxics and carcinogens [2,3]. It is also involved in the development of alcohol-induced liver disease [4]. Therefore, an assay for the determination of CYP2E1 activity is of great interest to delineate its role in alcohol-induced diseases. The myorelaxant drug, chlorzoxazone was shown to be a useful in vitro or in vivo probe for the determination of CYP2E1 activity [5–9]. CYP2E1 metabolizes chlorzoxazone mainly to a single metabolite, 6-hydroxy-chlorzoxazone, which can be quantified using HPLC [6–8]. The aim of this study was to propose an alternative method for in vitro determination of CYP2E1, using thin-layer chromatography (TLC) and [2-$^{14}$C]chlorzoxazone, a recently commercially available compound. This method was performed in liver and kidney micro-

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somes from control and ethanol- or acetone-induced rats.

## 2. Experimental

### 2.1. Reagents

Chlorzoxazone was purchased from Sigma (St. Louis, MO, USA) and 6-hydroxychlorzoxazone from Ultrafine Chemicals (Manchester, UK). [2-$^{14}$C]-Chlorzoxazone (50 mCi/mmol) was obtained from Amersham (Buckinghamshire, UK). Solvents of analytical grade (Merck, Darmstadt, Germany) were used for extraction and development of TLC plates (silica gel 60 F-254 precoated, 0.25 mm layer thickness, 20 × 20 cm, Merck).

### 2.2. Samples

#### 2.2.1. Microsomes

Liver and kidney microsomes previously prepared from control, ethanol per os, ethanol by inhalation and acetone-treated rats [6] were used in this study. Protein concentrations were determined according to the method of Bradford [10] using bovine serum albumin as standard.

#### 2.2.2. Incubation conditions [6]

Liver microsomal proteins (0.4 mg) were incubated in 0.1 *M* phosphate buffer (pH 7.4) with 1 m*M* NADPH and 400 $\mu M$ chlorzoxazone containing 0.05 $\mu$Ci of labelled chlorzoxazone i.e. 0.125 mCi/mmol for 20 min at 37°C under gentle agitation. Labelled chlorzoxazone, delivered in an ethanol solution, must be evaporated to dryness before adding to unlabelled chlorzoxazone as ethanol is also a substrate for CYP2E1. Kidney microsomal proteins (0.8 mg) were incubated for 120 min with an increased amount of labelled chlorzoxazone (0.625 mCi/mmol), as CYP2E1 activity was quite weak in kidney. The enzymatic reaction was terminated by addition of 50 $\mu$l $H_3PO_4$ 43% (v/v) and the incubation mixture was extracted with 2 ml chloroform–2-propanol 85:15 (v/v). The organic phase was dried by filtration over $Na_2SO_4$ and evaporated to dryness under a nitrogen stream at 37°C.

### 2.3. Chromatographic separation

#### 2.3.1. Thin-layer chromatography

Dry samples were dissolved in 100 $\mu$l of methanol and spotted on TLC plates. Unlabelled chlorzoxazone and 6-hydroxychlorzoxazone (50 $\mu$g) were co-chromatogaphed and revealed by UV light. TLC plates were run (15 cm) in a glass chamber (25 × 25 × 12 cm) saturated with solvent system, acetone–hexane (45:55, v/v). The TLC plates were dried completely by hot air and then exposed for 2 days to an X-ray film (Kodak X O. Mat K50) to localize the radiolabelled chlorzoxazone and 6-hydroxychlorzoxazone blots. The metabolite and substrate regions were scraped from the glass support and 10 ml of scintillation liquid (Picofluor 40, Packard, Meriden, CT, USA) was added. The radioactivity of each sample was counted during 10 min in a liquid scintillation analyzer (Tricarb, Packard).

#### 2.3.2. HPLC

As previously reported [6], dried samples were dissolved in 200 $\mu$l of a mobile phase which consisted of 0.5% acetic acid in water–acetonitrile (75:25, v/v). Samples (20 $\mu$l) were applied onto a column packed with reversed-phase 5 $\mu$m $C_{18}$ Nucleosil, 250 × 4.6 mm I.D. (Interchim, Montluçon, France). The flow-rate was 1.0 ml/min and detection was performed at 287 nm (SpectroMonitor II, LDC, Riviera Beach, FL, USA). Retention times of 6-hydroxychlorzoxazone and chlorzoxazone were about 7 and 22 min, respectively. In some experiments, samples were also analyzed with a double detection system, i.e. UV and radiometric (Flo-one beta, Packard).

#### 2.3.3. Quantitation

The enzymatic rates of 6-hydroxychlorzoxazone formation were calculated from the percentage of labelled metabolite to the total radioactivity following TLC analysis or from the percentage of metabolite area to the total product area (metabolite + parent drug) following HPLC analysis. It was previously verified that 6-hydroxychlorzoxazone and chlorzoxazone were equally extracted using chloroform–2-propanol as solvent mixture. Data were expressed as nmol/min/mg protein. Statistical analysis was performed using Student's *t* test.

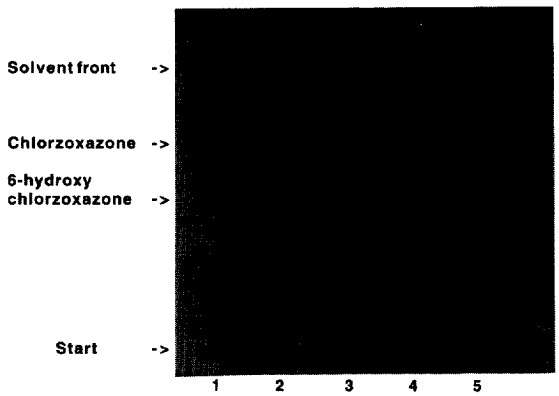


Fig. 1. Autoradiograph of a TLC plate developed in an acetone–hexane (45:55, v/v) solvent. Kidney microsomes from ethanol-induced rats were incubated with [2-$^{14}$C]-chlorzoxazone (0.25 $\mu$Ci) and co-chromatographed with non-radioactive standards as described in Section 2. Lanes 1,4: with NADPH; lanes 2,5: without NADPH; Lane 3: unlabelled chlorzoxazone and 6-hydroxychlorzoxazone.

### 2.4. Method validation

The intra-day and the inter-day precision were determined by analyzing a high and a low activity sample, five times on the same day and daily for 5 days, respectively. In addition, samples ($n$=21) were run in duplicate in HPLC and TLC and the correlation studied between both methods using regression analysis based on the least squares method.

## 3. Results and discussion

Fig. 1 displays the autoradiograph of a TLC plate corresponding to the incubation of kidney microsomes with labelled chlorzoxazone and with or without NADPH as described in Section 2. When NADPH was omitted, the 6-hydroxychlorzoxazone band was absent as this cofactor is essential for P450 enzymatic activity. A satisfactory separation of chlorzoxazone and 6-hydroxychlorzoxazone was achieved on silica gel 60 F-254 plates using acetone–

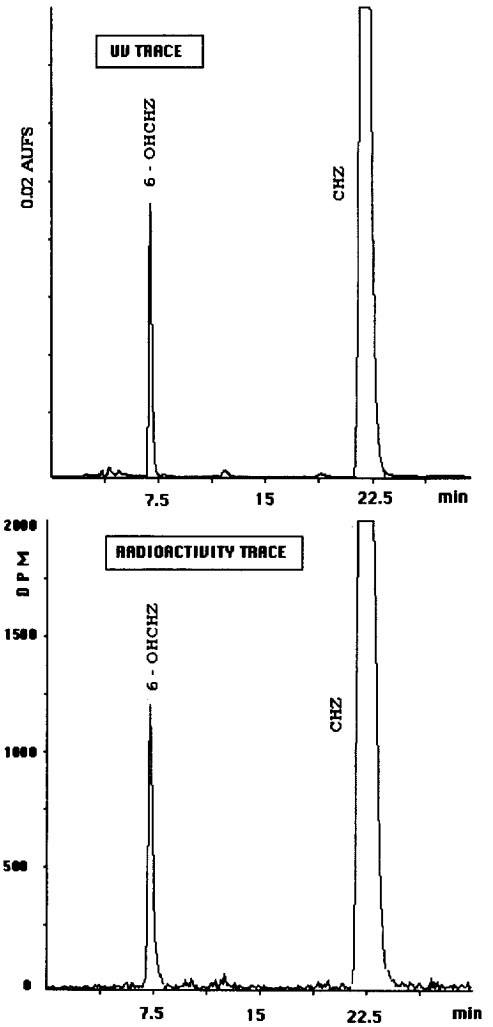


Fig. 2. HPLC profile of chlorzoxazone metabolism in ethanol-induced liver microsomal sample. Detection by either UV (287 nm) or radiometry (on-line radioactivity counter).

Table 1
$R_F$ values of chlorzoxazone and 6-hydroxychlorzoxazone for various solvent mixture

| Ratio acetone–hexane | Chlorzoxazone $R_F$ value | 6-Hydroxychlorzoxazone $R_F$ value |
|---|---|---|
| 70:30 | 0.91 | 0.88 |
| 60:40 | 0.89 | 0.84 |
| 50:50 | 0.64 | 0.47 |
| 45:55 | 0.75 | 0.55 |

hexane 45:55 (v/v) as migrating solvent. However, various acetone–hexane mixtures were tested and the corresponding $R_F$ values are given in Table 1. The optimal amount of labelled substrate was studied by incubating control liver microsomes with various concentrations of [2-$^{14}$C]chlorzoxazone (0.0125, 0.05, 0.1, 0.5, 1 $\mu$Ci i.e. 0.031, 0.125, 0.25, 1.25 and 2.5 mCi/mmol). As only ca.1% of chlorzoxazone is metabolized into 6-hydroxychlorzoxazone in control liver microsomes, the first concentration was too low as only about 250 dpm for 6-hydroxychlorzoxazone could be counted after extraction. However, following addition of 0.125 mCi/mmol, sufficient levels of radioactivity (>1000 dpm) were extracted to obtain reliable data. Thus, 0.05 $\mu$Ci was chosen for the liver. It was necessary to increase this amount to 0.25 $\mu$Ci, i.e., 0.625 mCi/mmol in kidney samples, where only 0.1–0.5% of the substrate was metabolized. It should be noted that when using the chloroform–2-propanol mixture as extraction solvent, recoveries of radioactivity were about 82 ± 5%. Analysis of the same samples using TLC or HPLC with either UV or radioactive detection (Fig. 2) was performed. No additional radioactive metabolite products were observed either with TLC or HPLC.

The intra-day and inter-day precision was 10.5 and 18.7% respectively for a low activity sample (0.5 nmol/mg/min) and 9.75 and 15.5% for a high activity sample (2.7 nmol/mg/min). It was compared to that of the HPLC method which gave similar values (9.9 and 18.2%, 7.5 and 13.8%, respectively).

Table 2

| Sample | Chlorzoxazone 6-hydroxylation (nmol/min/mg protein) |
|---|---|
| *Liver* | |
| Control | 0.43 ± 0.09 ($n$=5) |
| Ethanol per os | 1.27 ± 0.62 ($n$=5)* |
| Ethanol by inhalation | 2.30 ± 0.26 ($n$=5)*** |
| Acetone | 1.12 ± 0.28 ($n$=5)*** |
| *Kidney* | |
| Control | 0.016 ± 0.01 ($n$=8) |
| Ethanol per inhalation | 0.092 ± 0.08 ($n$=7)* |

Measure of 6-hydroxylation of chlorzoxazone in rat microsomes after induction by ethanol or acetone using TLC procedure. Results are expressed as mean ± S.D.
*$p$<0.05; ***$p$<0.001 (Student's $t$ test).

This method was used for the determination of CYP2E1 activity in liver and kidney microsomes from control and induced rats. Twenty-one samples were processed either by TLC or by HPLC. The correlation coefficient between both techniques was found to be 0.97 (Fig. 3). CYP2E1 activities were found to be systematically lower using TLC analysis rather than HPLC. This could be explained by an increased detection specificity of the method using the radioactive substrate.

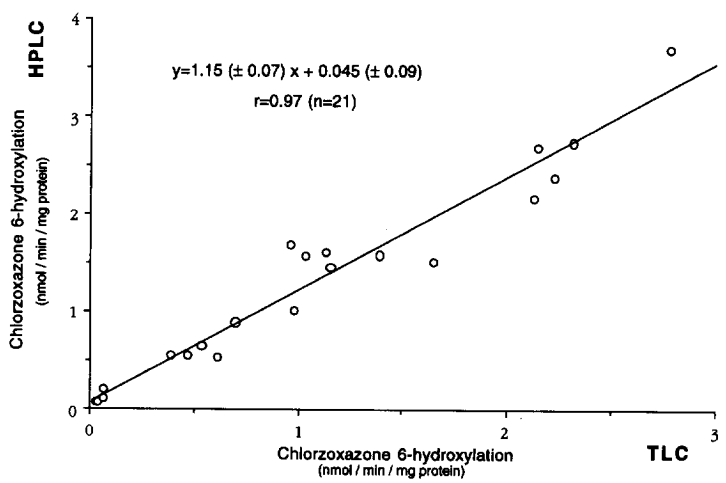


Fig. 3. Correlation between HPLC and TLC determinations of chlorzoxazone 6-hydroxylation in 21 rat microsomal samples.

Treatments with ethanol per os, ethanol by inhalation or acetone resulted in a 3-, 5- and 2.5-fold increase of activity in the liver. Following ethanol inhalation, a 8-fold increase was seen in the kidneys (Table 2). Kidney appears to be more sensitive to induction in our experiments compared to liver. These data are in good agreement with previously published results obtained using the same microsomes and HPLC determination [6]. Thus, labelled chlorzoxazone and TLC method constitutes an alternative method to determine CYP2E1 activity in control or induced microsomes.

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