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**Short communication**

# Microplate Assay Measurement of Cytochrome P450-Carbon Monoxide Complexes

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**Cytochrome P450 in microsomes can be quantitated using the characteristic 450 nm absorption peak of the CO adduct of reduced cytochrome P450. We developed a simple microplate assay method that is superior to previous methods. Our method is less laborious, suitable for analyzing many samples, and less sensitive to sample aggregation. Microsome samples in microplate wells were incubated in a CO chamber rather than bubbled with CO gas, and then reduced with sodium hydrosulfite solution. This modification allowed a reliable and reproducible assay by effectively eliminating variations between estimations.**

**Keywords:** Carbon monoxide, Cytochrome P450, Microplate

## Introduction

Cytochromes P450 (P450) are the principal enzymes that are involved in the oxidative metabolism of drugs and other xenobiotics. Knowledge of the effect of drugs on P450 activity is essential if we are to avoid drug interactions and improve therapeutic efficacy (Park *et al.*, 1995). The expression of fish P450 was suggested as an environmental bioindicator because it is affected by many environmental xenobiotics (Gupta and Abou-Donia, 1998; Williams *et al.*, 1998; Snyder, 2000). Recently, a change in the P450 level was also shown to be related to the anticarcinogenic activity of garlic (Park *et al.*, 2002).

Reduced P450 forms a complex with carbon monoxide to produce a unique 450 nm absorption peak (Garfinkel, 1958; Klingenberg, 1958). This spectral property was employed for the specific estimation of P450 content (Omura and Sato, 1964). Typically, the reduced P450-CO complex shows an absorption spectrum that is different than that of the reduced P450. The P450 concentration can be determined using a formula that incorporates the change in absorbance at 450 nm relative to 490 nm, and a millimolar difference extinction coefficient of 91 (Omura and Sato, 1964).

However, previous applications of this methodology had some drawbacks. First, the absorption spectrum is extremely sensitive to various environmental factors, primarily due to changes in the aggregation state of microsome (Estabrook and Werringloer, 1978). Second, current protocols tend to be laborious and time-consuming, making them unsuitable for high throughput analysis of multiple samples. Moreover, reproducibility is a problem. For example, in experiments examining the effect of xenobiotics on the fish P450 expression, we found it difficult to obtain identical results when different aliquots of the same microsome sample were successively analyzed (data not shown). It appeared that even minute differences in processing samples led to significantly different results.

To overcome these drawbacks, we developed a microplate-based assay method for the P450-CO complex. In this method, samples in microplate wells were incubated in a CO chamber and reduced by the addition of a sodium hydrosulfite solution, resulting in the reduced P450-CO complex. Absorbance differences were measured using a microplate reader. These modifications made it possible to assay many samples simultaneously and minimize any discrepancy between the estimations.

## Materials and Methods

**Preparation of rat liver microsome** Five rats were injected daily with 75 mg/kg sodium phenobarbital (Sigma, St. Louis, USA) for four days. Blood was removed by perfusion with an ice-cold 0.9% NaCl solution. Livers were removed and homogenized in a 0.25 M sucrose solution at a ratio of 10 ml/g of tissue. Phenylmethylsulfonyl fluoride (PMSF, Sigma; 0.67 mM) was

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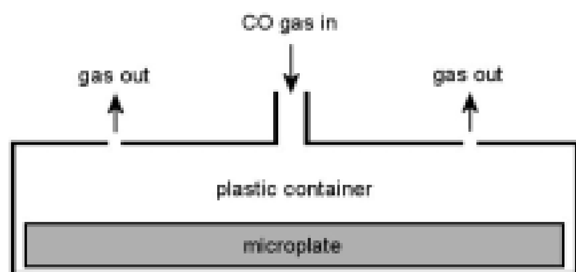


**Fig. 1.** The CO chamber. The CO chamber was a $16 \times 11 \times 4$ $cm^3$ ($W \times D \times H$) plastic box. CO gas was injected through a hole in the center, and escaped through two holes located on both sides.

added to inactivate serine proteases. The homogenate was centrifuged at $600 \times g$ for 5 min, and the supernatant was centrifuged again at $12{,}000 \times g$ for 10 min to remove any broken mitochondrial fragments. The supernatant was collected and centrifuged at $100{,}000 \times g$ for 1 h, and the resultant pellet was resuspended in 0.25 M sucrose. The protein concentration of the microsome preparation was adjusted to 20 mg/ml, as determined using the BCA reagent (Pierce Biotechnology Inc., Rockford, USA) and bovine serum albumin as a standard.

**Standard assay method** The microsome preparation was diluted with a PG buffer (10 mM potassium phosphate, pH 7.4, 20% glycerol) as required. Two 200 µl aliquots were placed in microplate wells; one was designated as reduced P450 (P) and the other as reduced P450-CO complex (PC). The PC well was placed in the CO chamber, while the P well was sealed with tape and placed outside the chamber (because the two wells should be separated during the incubation period, strip plates are more convenient than 96-well plates). The CO chamber was prepared with a plastic container (Fig. 1). CO gas was allowed to flow into the chamber at a rate of about 0.5 l/min, and both wells were shaken for 2 min. All of the samples were reduced by adding 5-10 µl of a 0.5 M sodium hydrosulfite (SHS) solution (made fresh), with the final SHS concentration being 12.5-25 mM. At this stage, the yellow color of the PC sample was visible, whereas the P sample remained colorless or turned pale pink. The difference in absorbance of the samples at 450 and 490 nm was measured with a SpectraCount microplate reader (Packard Instrument Company, Meriden, USA). The P450 concentration was calculated using the following formula; $[P450](mM) = (\Delta A_{PC} - \Delta A_P)/91$, where $\Delta A_{PC}$ is the absorbance difference of the PC sample, and $\Delta A_P$ is the absorbance difference of the P sample.

## Results and Discussion

**Difference spectrum** The presence of P450 in the microsome preparation, and the effectiveness of the microplate method for measuring the P450-CO complex formation, can be verified by the 450 nm peak in the absorbance difference spectrum. Although some other hemoproteins are known to react with CO, they could be distinguished from P450 by their spectral properties. For example, the carbon monoxide complex of reduced hemoglobin has a characteristic peak at 420 nm (Waterman, 1978). To analyze the spectral property of the microsome, P and PC samples were prepared according to the standard microplate method, transferred into cuvettes, and their difference spectrum was measured using a spectrophotometer. We found that the spectrum showed the characteristic 450 nm peak, indicating that the microsome preparation contained P450 and the reduced P450-CO complex was properly formed in the microplate assay (Fig. 2). The small peak at 420 nm may represent the presence of contaminating hemoglobin, or the breakdown product of P450 (Waterman, 1978; Schenkman and Jansson, 1998). The absorbance difference in the spectrum was usually lower than that obtained by reading directly with a microplate reader. This may be due to a change in the microsome aggregation state when transferring samples with a pipette.

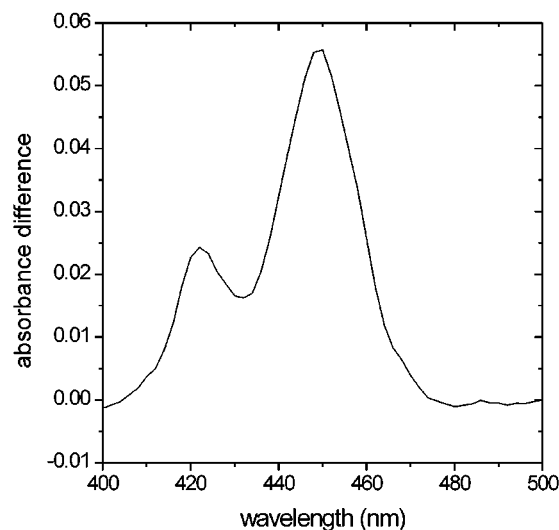


**Fig. 2.** Absorbance difference spectrum. Microsome was diluted with a PG buffer to a protein concentration of 1.5 mg/ml. The P and PC samples were prepared following the standard microplate assay method and transferred into two different spectrophotometer cuvettes. Cuvettes were placed in the reference and sample holders of a Shimadzu UV-1601 double beam spectrophotometer (Shimadzu, Tokyo, Japan) and the spectrum difference was recorded.

**Optimization of assay conditions** Three parameters in the assay procedure were optimized: the CO incubation time, SHS volume, and reaction time after the SHS addition. To determine the optimal incubation time in the CO chamber, diluted microsome samples were treated according to the standard method, and the incubation time in the CO chamber was varied. The data presented in Fig. 3 show that the absorbance difference reached a maximum after a 2 min incubation. This is six times longer than the time that is required to saturate a sample by bubbling with CO gas

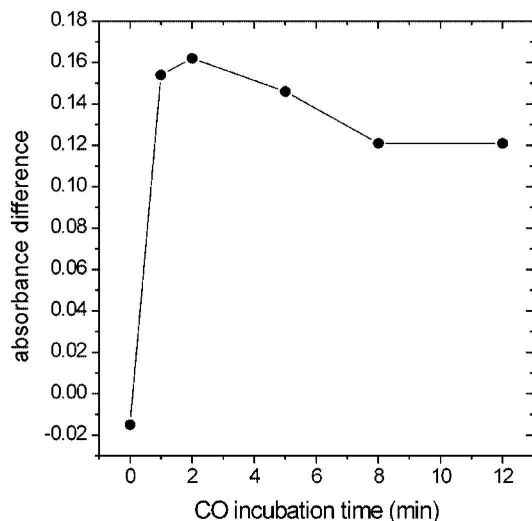


**Fig. 3.** CO incubation time. Six pairs of P and CP samples were prepared using 1 mg/ml microsome in a PG buffer. Each pair was processed by the standard method, but the incubation time in the CO chamber varied.

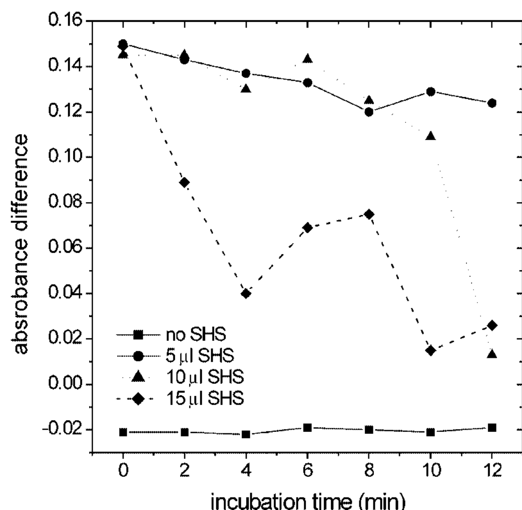


**Fig. 4.** SHS concentration and reaction time. Four pairs of P and CP samples were prepared using 1 mg/ml microsome in a PG buffer. Different volumes of the SHS solution were added to each pair (0, 5, 10, 15 μl). All of the samples were processed following the standard method, and the absorbance difference was measured at different time points.

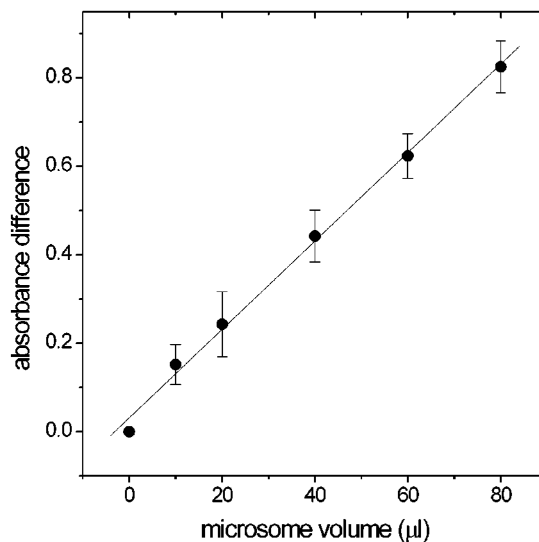


**Fig. 5.** Dose response. Different volumes of the microsome solution (0, 10, 20, 40, 60, or 80 μl) were mixed with a PG buffer to make a total volume of 200 μl. The protein concentration of each sample was 0, 1, 2, 4, 6, or 8 mg/ml respectively. The samples were processed following the standard method. The experiment was separately performed three times. Each data point and error bar represents the mean and standard deviation.

(Omura and Sato, 1964). However, when many samples are analyzed, the microplate method saves time and CO gas.

Next, the volume of the SHS solution was varied and the absorbance difference measured at different time points. The absorbance difference was relatively stable when 5-10 μl of SHS was added (Fig. 4). However, at higher SHS concentrations we observed the formation of large aggregates and the absorbance difference was unstable. The absorbance difference was greatest when measured immediately after the addition of SHS. This seems to contradict the report by Schenkman and Jansson (1998), which indicated that the P450 reduction is slow even with the strong reductant SHS; therefore, at least a 1 min incubation time is necessary. In the microplate assay, we concluded that it usually took 1-2 min to complete the measurements after the addition of SHS, and this time lag provided sufficient time for the P450 reduction.

**Dose response** Microsomes at different concentrations were processed by the standard procedure. We found that the absorbance difference range of 0-0.8 showed a good correlation with the microsome concentration (Fig. 5). Moreover, there were little variations between the different assays when the experiment was repeated three times. Therefore, the P450 concentration of any sample can be calculated from the measured absorbance difference. Increasing the intensity of the yellow color was also visible with increasing microsome content. After a more than 80 μl microsome solution had been added, the absorbance difference did not increase. This is probably because the sensitivity of the instrument was limited when measuring the highly turbid samples. The P450 concentration of the undiluted microsome sample was calculated to be about 1 nmol/mg protein, similar to typical rat microsome preparations (Estabrook and Werringloer, 1978; Ryan *et al.*, 1978).

In conclusion, we have developed a very simple method that is suitable for the rapid measurement of cytochrome P450

in multiple samples. Typically, it took less than 30 min to process 48 samples. Most importantly, it was possible to obtain consistent results because there was very little variability between the assays.

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