

Determination of Microsomal CYP2A6 Activity by High Performance Liquid Chromatography

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Coumarin has been reported to be a reliable probe for the determination of human microsomal CYP2A6 activity. Coumarin is converted to 7-hydroxycoumarin by CYP2A6. A high pressure liquid chromatographic assay for the estimation of CYP2A6 activity in microsomes was evaluated. A RP C-18 Novapak Waters (15 cm x 3.9 mm, 5 μ m) column was used for the assay. The mobile phase composition was methanol : 1% glacial acetic acid (35:65 v/v) (pH~3.1), with a flow rate of 0.6 ml/min, injection volume of 100 μ l and detection at 320 nm. The retention times for coumarin and 7-hydroxycoumarin were 8.7 min and 5.3 min, respectively. The limit of detection (LOD) was 0.05 μ M, and the limit of quantitation (LOQ) was 0.1 μ M, for 7-hydroxycoumarin. The percent coefficient of variation associated with 7-hydroxycoumarin determination after duplicate estimation was found to be in the range 0.03 to 3.6% for buffer matrix and 0.1 to 6.5% for microsomal matrix. The mean rate of 7-hydroxycoumarin formation in guinea pig liver microsomes was 0.084 nmol/min/nmol P450. Coumarin 7-hydroxylase activity was absent in rat liver microsomes. No interference was observed from incubation mixture components.

Key words: HPLC assay, CYP2A6, coumarin, 7-hydroxycoumarin

Cytochrome P450 (CYP) comprises of a superfamily of heme containing monooxygenases that play an important role in biotransformation of numerous endogenous compounds and xenobiotics¹. The major human CYP isoenzymes that are primarily responsible for hepatic drug metabolism are CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4^{2,3}. The relative levels of these CYP isoenzymes in human liver microsomes can be

determined by monitoring the biotransformation of suitable substrates (termed isoenzyme specific 'probe' substrates) that are selectively metabolized to specific metabolites by the individual CYP isoenzymes⁴. CYP2A6 is the predominant member of CYP2A subfamily. CYP2A6 metabolizes carcinogenic substrates including aflatoxin, N-nitrosodiethylamine, 6-aminochrysene⁵. CYP2A6 is inducible by barbiturates. Coumarin-7-hydroxylation is routinely used as an *in vitro* substrate probe to determine CYP2A6 activity⁴.

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HPLC assay for estimation of CYP2A6 activity in human liver sub-cellular fractions and during *in vivo* studies has been reported previously⁶. We have evaluated the reported procedure with minor modifications for the estimation of coumarin 7-hydroxylase activity under in-house conditions⁶.

Coumarin AR and trichloroacetic acid were purchased from S. D. Fine Chemicals Ltd., Mumbai. 7-Hydroxycoumarin was synthesized in our laboratory⁷. Methanol (HPLC grade) was purchased from Qualigens Chemicals Ltd., Mumbai. The Novapak Waters RP C18 (15 cm×3.9 mm, 5 µm) column was a gift from Wockhardt Ltd., Aurangabad. All other chemicals were of analytical grade.

Microsomes were isolated from the livers of female Sprague-Dawley rats and male Albino guinea pigs. The liver microsomes were prepared by using the calcium aggregation method reported by Schenkman and Cinti⁸ with minor modifications. CYP content of the microsomes was determined by the method of Omura and Sato⁹. Isolated rat liver microsomes and guinea pig liver microsomes showed a CYP content of 12.2 µM and 6.92 µM, respectively. The microsomes were stored at the temperature of -70° until further use.

Stock solution of coumarin (500 µM) and 7-hydroxycoumarin (1 mM) was prepared in methanol : double distilled water (1:9). The sub stock solutions of 7-hydroxycoumarin was prepared by suitable dilution of the stock solution with double distilled water to yield solutions with final concentrations of 0.5, 1, 2.5, 5, 10, 20, 50 and 100 µM. The stock solutions and sub-stock solutions of coumarin and 7-hydroxycoumarin were stored in refrigerator (0-4°) until use.

The standard curve for determination of coumarin 7-hydroxylase activity was developed in duplicate, in guinea pig liver microsomal matrix and in 0.1 M phosphate buffer containing 0.001 M EDTA, pH 7.4. The sample preparation protocol for development of standard curve in microsomal matrix and buffer matrix is given in Tables 1 and 2, respectively. The sample work-up was done by using the following procedure. To each of the samples tubes, 250 µl of 50% trichloroacetic acid was added (for the precipitation of microsomal proteins) and the samples were transferred into 1.5 ml capacity Eppendorff

TABLE 1: PROTOCOL FOR STANDARD CURVE PREPARATION IN MICROSOMAL MATRIX

Final conc. (µM)	Concentration of sub stock 7HC solution added (50 µl)	Vol. of 500 µM CMN solution (µl)	Vol. of buffer (µl)	Vol. of micro-somes (µl)
Blank	-	-	400	100
10	100 µM	50	300	100
5	50 µM	50	300	100
2.5	25 µM	50	300	100
1	10 µM	50	300	100
0.5	5 µM	50	300	100
0.25	2.5 µM	50	300	100
0.05	0.5 µM	50	300	100

The abbreviations used are 7HC= 7-hydroxycoumarin and CMN = coumarin. The buffer used was 0.1 M sodium phosphate buffer containing 0.001 M EDTA, pH 7.4. All samples were prepared in duplicate.

TABLE 2: PROTOCOL FOR STANDARD CURVE PREPARATION IN BUFFER MATRIX

Conc. (µM)	Concentration of sub stock 7HC solution added (50 µl)	Vol. of 500 µM CMN solution (µl)	Vol. of Buffer (µl)
Blank	-	50	500
10	100 µM	50	400
5	50 µM	50	400
2.5	25 µM	50	400
1	10 µM	50	400
0.5	5 µM	50	400
0.25	2.5 µM	50	400
0.1	1 µM	50	400
0.05	0.5 µM	50	400

The abbreviations used are 7HC= 7-hydroxycoumarin and CMN = coumarin. The buffer used was 0.1 M phosphate buffer containing 0.001 M EDTA (pH 7.4). All samples were prepared in duplicate.

tubes. The samples were centrifuged at 13000×g for 10 min at 4°. The supernatant was separated and stored at -70° till HPLC analysis.

The microsomal incubation conditions for coumarin 7-hydroxylase activity determination were adapted from reported procedures^{6,10}. The incubations were conducted in a total volume of 0.5 ml of 0.1 M sodium phosphate buffer containing 0.001 M EDTA, pH 7.4 and contained 100 µl microsomes, coumarin at a final concentration of 50 µM, and 0.6 mM NADPH. Incubations were initiated by addition of NADPH and were performed at 37° for 15 min. After incubation, the reactions were stopped with 250 µl of 50% trichloroacetic acid and samples processed as mentioned for the standard curve samples. Control incubations were also performed either without microsomes or without coumarin, in order to evaluate possible interference from incubation components.

HPLC analysis was done with minor modifications of the method reported previously⁶. Analysis was

performed with a Jasco HPLC with PU-980 intelligent pump, equipped with a UV-975 detector and Jasco-Borwin software (version 1.5) with Hercule-2000 interface. HPLC analysis was done on A RP C-18 Novapak Waters (15 cm x 3.9 mm, 5 μ m) column. The mobile phase composition was methanol:1% glacial acetic acid (35 : 65 v/v) (pH~3.1), with a flow rate of 0.6 ml/min and detection at 320 nm. One hundred microlitres sample was injected onto the HPLC column for analysis. The standard (calibration) curve was plotted using area of 7-hydroxycoumarin on the ordinate and concentration of 7-hydroxycoumarin on the abscissa.

Drug metabolism studies (*in vitro*, *in vivo* and/or *in silico*) are very important in the assessment of safety of pharmaceuticals, individualization of drug therapy and prediction of drug-drug interactions^{4,11-16}. The present study illustrates the evaluation of the suitable HPLC assay to determine coumarin 7-hydroxylase activity in liver microsomal preparations for drug metabolism studies. The conversion of coumarin to 7-hydroxycoumarin in humans is selectively mediated by CYP2A6⁶. The concentration of the substrate i.e. coumarin, used for determination of CYP2A6 activity in liver microsomes is 50 μ M¹¹. Since the concentration generally used for determination of the V_{max} is five times the K_m , the projected K_m would be 10 μ M. Further, considering $\leq 5\%$ conversion [for adherence to Michaelis-Menten steady state assumptions] in the microsomal incubations^{6,10}, the maximum expected concentration of 7-hydroxycoumarin at the end of incubation would be 2.5 μ M or 0.5 μ M, if incubations are performed at V_{max} or K_m conditions respectively. Thus, a standard curve with range from 0.05 to 10 μ M (0.05, 0.1, 0.25, 0.5, 1, 2, 5 and 10 μ M) should be sufficient for application of this assay for CYP2A6 activity determination and drug-drug interaction studies that are performed either at saturating and non-saturating substrate concentrations, respectively.

The mean retention times for 7-hydroxycoumarin and coumarin were found to be 5.3 min and 8.7 min, respectively under the stated HPLC conditions. A typical chromatogram in microsomal matrix is shown in fig. 1. Coumarin was included in the standard curve samples to evaluate potential effects on HPLC profile since it would normally be present in microsomal incubation at high concentrations. Linear regression (Microsoft Excel XP) of the standard curve data in

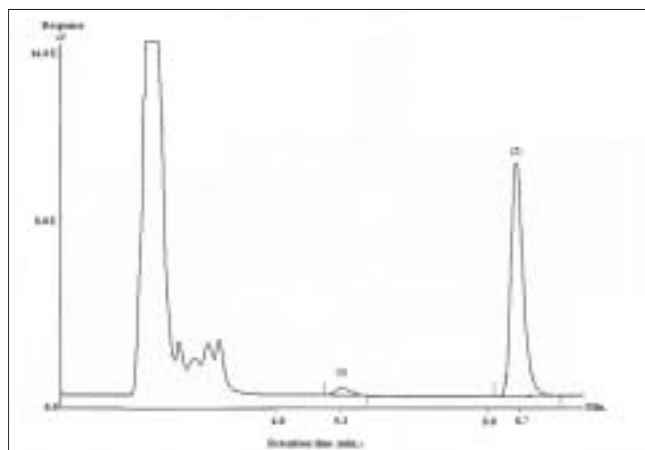


Fig. 1: A typical chromatogram of a microsomal incubation sample (guinea pig liver) showing coumarin 7-hydroxylase activity. The chromatogram shows the microsomal incubation of coumarin for the assay of coumarin 7-hydroxylase activity. 7-Hydroxycoumarin is peak 1 at the retention time 5.3 min, while coumarin is peak 2 at the retention time 8.7 min. These peaks were obtained after incubation of coumarin with guinea pig liver microsomes as described in the text.

microsomal matrix gave the equation $Y=85001X$ with an r^2 of 0.9998. Linear regression (Microsoft Excel XP) of the standard curve in buffer matrix followed the equation $Y=134651X$ with r^2 of 0.9997. The limit of detection (LOD) was 0.05 μ M and the limit of quantitation (LOQ) was 0.1 μ M, in both matrices. The mean coefficient of variation associated with two assay experiments, each done in duplicate was found to be in the range 0.03 to 3.6% for buffer matrix and 0.1 to 6.5% for microsomal matrix, respectively. The above results indicate that the coumarin 7-hydroxylase activity assay developed above is suitable for routine drug metabolism research work. The buffer matrix was evaluated in this study in order to avoid use of microsomes during preparation of standard curve while performing coumarin 7-hydroxylase activity assay on a regular basis. Liver tissues are difficult to obtain and microsomes preparation is tedious and as such use of microsomes just as a matrix for standard curve preparation is unwise.

The biotransformation of coumarin to 7-hydroxycoumarin was observed only in guinea pig liver microsomes, which shows the presence of coumarin 7-hydroxylase activity in the given liver preparation. Control incubations showed no production of 7-hydroxycoumarin. The HPLC profile on incubation of coumarin with guinea pig liver microsomes is shown in fig. 1. No interference was observed from the incubation matrix components. The mean rate of 7-hydroxycoumarin formation in

guinea pig liver microsomes was 0.084 nmol/min/nmol P450. In contrast, metabolism of coumarin to 7-hydroxycoumarin was not observed in rat liver microsomes indicating the absence of this activity. This is not a new finding and it has been reported previously that CYP2A6-like activity shows species differences¹⁷. Coumarin metabolism in monkey, mouse, hamster and rabbit seems to be most similar to humans¹⁷. In mouse, the isoform CYP2A5 is responsible for coumarin 7-hydroxylation while in rats, coumarin is mainly metabolized by opening of lactone ring via hydrolysis by esterase. It is suggested that this may be the reason for coumarin hepatotoxicity in rats (which is not observed in case of guinea pigs or humans)¹⁷ where less than 1% of coumarin is metabolized to 7-hydroxycoumarin.

The above studies indicate the utility of this assay for determination of coumarin 7-hydroxylase activity in animal liver microsomes and potentially, CYP2A6 activity in human liver microsomal preparations.

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