November 2009

Methods of P450, FMO and UGT assays used for Human Liver Microsomes in BD Gentest

Total P450 assay, Spectrophotometric Quantitation

The standard method of Omura, T, and R Sato, (1964) J. Biol. Chem. 239, 2379-2385.

P450 Oxidoreductase, Cytochorme c Reductase

A 1.0 ml reaction mixture containing 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.95 mg/ml cytochrome c in 250 mM potassium phosphate (pH 7.4) was prepared and prewarmed to 37° C. The reaction was initiated by the addition of 0.1 mg/ml protein, and the absorbance change at 550 nm was recorded as a function of time. An extinction coefficient for reduced (ferrous) cytochrome c at 550 nm of 19.6 mM-1 cm-1 was used to calculate the reductase activity.

Cytochrome b5, Spectrophotometric Quantitation

The standard method of Estabrook, R.W. and J. Werringloer, (1978) Meth. Enz. 52, 212-220.

HPLC Analysis

All HPLC analysis was performed using Waters HPLC instruments and software. HPLC columns used are Nucleosil C18, 4.6 x 250 mm, 5 um particle size heated to a constant temperature of 45oC. Most C18 columns and HPLC systems should be adequate for the analyses below using the conditions described. However, some adjustment in mobile phase may be required. Column temperature can range from room temperature to 45oC. The use of a controlled, elevated temperature provides greater reproducibility in retention times and lower column back pressures.

CYP1A2, Phenacetin O-deethylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.2 mM phenacetin (delivered as a MeOH solution, final concentration = 1% MeOH) in 100 mM potassium phosphate (pH 7.4) was incubated at 37° C for 20 min. After incubation, the reaction was stopped by the addition of 50 ul acetonitrile and centrifuged (10,000 x g) for 3 minutes. 75 m l of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C, at a flow rate of 1.0 ml per min, with a mobile phase initially of 10% methanol increasing to 25% methanol over 6 min, then increased to 100% methanol to elute the parent compound. The product was detected by its absorbance at 244 nm and quantitated by comparing to the absorbance of a standard curve for acetamidophenol.

CYP2A6, Coumarin 7-hydroxylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 0.065 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.2 mM coumarin (delivered as a concentrated buffer solution) in 100 mM Tris (pH 7.5) was incubated at 37° C for 20 minutes. After incubation, the reaction was stopped by the addition of 0.1 ml 20% trichloroacetic acid and centrifuged (10,000 x g) for 1 minute. 100 ul of the

supernatant was added to 1.9 ml of 100 mM Tris (pH 9) and the fluorescence was determined with excitation at 368 nm and emission at 456 nm in a spectrofluorometer. The activity was quantitated by subtracting the fluorescence of the blank and comparing to a standard curve for umbelliferone (7-hydroxycoumarin).

CYP2B6, (S)-Mephenytoin N-demethylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.1 mM [14C]-(S)-mephenytoin (delivered in 2.5 ul acetonitrile, final concentration = 1% acetonitrile) in 50 mM potassium phosphate (pH 7.4) was incubated at 37° C for 20 min. After incubation, the reaction was stopped by the addition of 40 ul acetonitrile and centrifuged (10,000 x g) for 3 minutes. A portion of the supernatant (100 ul) was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase initially of 27% methanol increasing to 100% methanol over 15 min. and at a flow rate of 1.0 ml per min. The product, nirvanol, (retention time of approximately 10 minutes) was detected by liquid scintillation counting.

CYP2C8, Paclitaxel 6(alpha)-hydroxylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 20 uM paclitaxel (delivered as a 5mM paclitaxel stock in ethanol, final concentration = 0.4% EtOH) in 100 mM potassium phosphate (pH 7.4) was incubated at 37° C for 10 min. After incubation, the reaction was stopped by the addition of 75 ul acetonitrile and centrifuged (10,000 x g) for 5 minutes. 100 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase initially of 60% methanol increasing to 70% methanol over 20 min. and at a flow rate of 1.0 ml per min. The product was detected by its absorbance at 230 nm and quantitated by comparing to the absorbance of a standard curve for 6(alpha) -hydroxypaclitaxel. (GENTEST Catalog No. 451656 (Old B656)).

CYP2C9, Diclofenac 4'-hydroxylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.2 mM diclofenac (delivered as a concentrated buffer solution) in 100 mM Tris (pH 7.5) was incubated at 37° C for 10 min. After incubation, the reaction was stopped by the addition of 50 ul of 94% acetonitrile/6% glacial acetic acid and centrifuged (10,000 x g) for 3 minutes. 100 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase initially of 20% acetonitrile, 30% methanol with 1 mM perchloric acid in water, changing to 100% methanol over 20 min. at a flow rate of 1.0 ml per min. The retention times were approximately 11 min for the 4'-hydroxydiclofenac and 15 min for diclofenac. The product was detected by its absorbance at 280 nm comparing to the absorbance of a standard curve for 4'-hydroxydiclofenac (GENTEST Catalog No. 451443 (Old B443)).

CYP2C19, (S)-Mephenytoin 4'-hydroxylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.1 mM [14C]-(S)-mephenytoin (delivered in 2.5 ul acetonitrile, final concentration = 1% acetonitrile) in 50 mM potassium phosphate (pH 7.4) was incubated at 37° C for 20 min. After

incubation, the reaction was stopped by the addition of 50 ul acetonitrile and centrifuged (10,000 x g) for 3 minutes. A portion of the supernatant (100 ul) was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase initially of 27% methanol increasing to 100% methanol over 15 min. and at a flow rate of 1.0 ml per min. The product, 4'-hydroxymephenytoin, (retention time of approximately 9 minutes) was detected by liquid scintillation counting.

CYP2D6, (+/-)-Bufuralol 1'-hydroxylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 25 uM (+/-)-bufuralol (delivered as a concentrated buffer solution) in 100 mM potassium phosphate (pH 7.4) was incubated at 37° C for 20 minutes. After incubation, 25 ul of 70% perchloric acid was added and the mixture was centrifuged at 12000 x g to pellet the protein. A portion of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase of 30% acetonitrile, 1 mM perchloric acid at a flow rate of 1.0 ml per minute. The retention time of the product was approximately 6 minutes. The fluorescence of the product was measured in the flow cell of a spectrofluorometer with excitation at 252 nm and emission at 302 nm. The response was quantitated by comparing to a standard curve of product (carbinol).

CYP2E1, Chlorozoxazone 6-hydroxylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 2.0 mM chlorzoxazone (delivered as a 8.5 mM solution in 100 mM potassium phosphate (pH 7.4)) in 100 mM potassium phosphate (pH 7.4) was incubated at 37° C for 20 min. After incubation, the reaction was stopped by the addition of 75 ul of 94% acetonitrile/6% glacial acetic acid and centrifuged (10,000 x g) for 3 minutes. 100 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase of 4% methanol, 18% acetonitrile with 1 mM perchloric acid in water at a flow rate of 1.0 ml per min. The retention time was approximately 8.5 min for 6-hydroxychlorzoxazone. The product was detected by its absorbance at 280 nm and quantitated by comparing to the absorbance of a standard curve for 6-hydroxychlorzoxazone.

CYP3A4, Testosterone 6(beta)-hydroxylase

A 0.25 ml reaction mixture containing 0.5 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.2 mM testosterone (delivered as an acetonitrile solution, final concentration = 1% acetonitrile) in 100 mM potassium phosphate (pH 7.4) was incubated at 37° C for 10 min. After incubation, the reaction was stopped by the addition of 125 ul acetonitrile and centrifuged (10,000 x g) for 3 minutes. 100 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C (at a flow rate of 1.0 ml per min) with a mobile phase initially of 58% methanol increasing to 62% methanol over 8 min. and then increased to 100% methanol to elute the parent testosterone. The product was detected by its absorbance at 254 nm (242 nm is optimal) and quantitated by comparing to the absorbance of a standard curve for 6(beta)-hydroxytestosterone.

CYP4A, Lauric acid 12-hydroxylase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.1 mM [14C]-lauric acid (delivered as a concentrated buffer solution) in 100 mM Tris (pH 7.5) was incubated at 37° C for 10 min. After incubation, the reaction was stopped by the addition of 125 ul of 94% acetonitrile/6% glacial acetic acid and centrifuged (10,000 x g) for 3 minutes. 100 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase initially of 30% methanol, 21% acetonitrile with 0.7 mM perchloric acid in water changing to 47% methanol, 16% acetonitrile with 0.5 mM perchloric acid in water over 22 min, and then changing to 100% methanol. The flow rate was always 1.0 ml per min. The retention times were approximately 17 min for the (omega)-hydroxylauric acid and 30 min for lauric acid. The product was detected by liquid scintillation counting.

FMO, Methyl p-tolyl sulfide oxidase

A 0.25 ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride, 1.2 mM diethylenetriaminepentacetic acid, 0.5 mg/ml Triton X-100 and 0.2 mM methyl p-tolyl sulfide (delivered as a MeOH solution, final concentration = 0.4% MeOH) in 0.05 M glycine (pH 9.5) was incubated at 37° C for 10 min. After incubation, the reaction was stopped by the addition of 75 ul acetonitrile and centrifuged (10,000 x g) for 5 minutes. 100 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45° C with a mobile phase initially of 46% methanol increasing to 55% methanol over 7 min. (the substrate was then eluted with 100% methanol) and at a flow rate of 1.0 ml per min. The product was detected by its absorbance at 237 nm and quantitated by comparing the absorbance to a standard curve of methyl p-tolyl sulfoxide.

UGT (UDP-Glucuronosyltransferase)

UGT Glucuronidation assays contained 0.5 mg/ml protein for UGT1A1 and 1A4, 0.1 mg/ml for 1A6, 0.15 mg/ml for 1A9 and 0.8 mg/ml for 2B7, along with 2 mM UDPGA, 10 mM $MgCl_2$, 25 ug/ml Alamethicin in 50 mM Tris-HCl buffer (pH 7.5). UGT1A1 was incubated for 30 minutes, 1A4 for 20 minutes, 1A6 for 15 minutes, 1A9 for 10 minutes and 2B7 for 25 minutes. Activities expressed as pmol product per (mg protein x minute) except cytochrome c reductase which is expressed as nmol product per (mg protein x minute). Substrate concentrations are as follows.
UGT1A1, Estoradiol, 100 uM
UGT1A4, Trifluoperadine, 100 uM
UGT1A9, Propofol, 30 uM
UGT2B7, AZT, 5 mM