DETERMINATION OF 4-METHYLUMBELLIFERONE UGT ACTIVITY BY HPLC WITH FLUOROMETRIC DETECTION, AND APPLICATION TO THE STUDY OF HUMAN UGT1A6 EXPRESSED IN *Escherichia coli*

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Abstract

An HPLC-based assay was established for the measurement of UGT activity towards the substrate 4-methylumbelliferone (4MU).

Recombinant human UGT1A6 was successfully expressed in *Escherichia coli*. Bacterial membrane fractions were prepared and used to validate the 4MU-UGT assay procedure.

Formation of 4MU-glucuronide was linear with respect to incubation time and protein concentration at a substrate concentration less than Km. Kinetic parameters for the recombinant UGT1A6 were also determined.

Introduction and Aim

The coumarin derivative, 4-methylumbelliferone (4MU), is glucuronidated by multiple human UGTs. This means that, for enzyme preparations containing a mixture of human UGTs (such as human liver microsomes), 4MU is only suitable as a general marker of total UGT activity, as the contributions of the individual UGTs cannot be determined.

For recombinant UGTs, in contrast, 4MU can be a useful probe substrate. Low levels of UGT activity can be quantified without the need for radiolabelled cofactor or substrate, owing to the fluorescence of the 4MU-glucuronide (4MUG) formed.

The aim of this study was to establish a direct HPLC procedure for the measurement of 4MU-UGT activity. The resulting assay would then be used in the characterisation of recombinant human UGT1A6 expressed in *Escherichia coli*.

Materials and Methods

Recombinant human UGT1A6 was expressed in *E.coli* strain BL21 in 10 l fermenters. Bacterial membrane fractions (Bactosomes) were prepared using standard procedures.

The chemicals 4-methylumbelliferone (4MU), 4-methylumbelliferone glucuronide (4MUG) and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from Sigma. HPLC grade acetonitrile and methanol were from Fisher, as was glucaric acid-1,4-lactone (saccharolactone). All other chemicals were supplied by BDH.

HPLC analysis was carried out using an Agilent 1100 Series instrument. Detection was by fluorescence using excitation and emission wavelengths of 315 and 375 nm respectively. An initial isocratic separation of authentic 4MU and 4MUG standards was established on a Hypersil ODS (5 µm) 250 x 4.6 mm column at 30˚C, using a mobile phase of 0.05% orthophosphoric acid/acetonitrile (79/21; v/v) at a flow rate of 1 ml/min. The injection volume was 10 µl.

For the analysis of samples taken from complete incubations, an acetonitrile gradient was introduced. The percentage of acetonitrile was increased from 21 to 50% between 4 and 7.5 min, held at 50% between 7.5 and 10 min. and then dropped back to 21% between 10 and 11 min. The total run time was 15 min.

Incubations for the determination of 4MU glucuronidation activity were carried out at 37˚C in 50 mM potassium phosphate buffer (pH 7.4) containing 10 mM magnesium chloride, 5 mM saccharolactone and 0.25 mM 4-methylumbelliferone in a total volume of 200 µl. Reactions were started by adding 20 µl of 20 mM UDPGA and terminated by the addition of 20 µl of 1 M hydrochloric acid. Incubations were carried out on UGT1A6 Bactosomes. Kinetic parameters were estimated by fitting the experimental data directly to the Michaelis-Menten equation using Microsoft Excel.

Fig. 1a: Chromatogram showing isocratic separation of 4MU and 4MUG

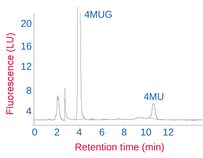
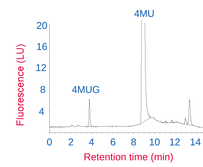


Fig. 1b: Chromatogram showing gradient separation of 4MU and 4MUG



Results 1

An isocratic separation of authentic 4MU and 4MUG standards was established (Fig. 1a). Retention times for the two compounds were 3.9 min (4MUG) and 10.7 min (4MU). Limits of detection and quantitation for 4MUG were 0.17 and 0.28 ng/200 µl, respectively.

However, analysis of samples from complete incubations using these conditions was associated with the appearance of an unknown fluorescent compound with a retention time of 71-72 min (not shown), which interfered with the quantification of 4MUG.

Introduction of an acetonitrile gradient was necessary to decrease the retention time of the interference peak to 13.3 min (Fig. 1b). Retention time of the 4MUG remained essentially unchanged at 3.8 min, and that of 4MU decreased to 8.9 min (Fig. 1b).

A standard curve of 4MUG was linear over the range 10 - 2000 ng/200 µl (Fig. 2).

Fig 2: 4MUG Standard Curve

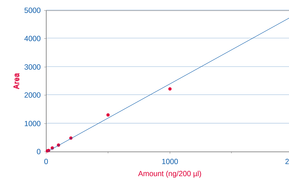
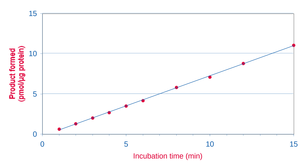


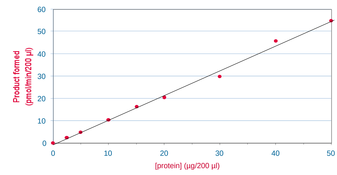
Fig. 3: Product formation with respect to time in UGT1A6 Bactosomes



Results 2

Assays to determine the extent of linear formation of product with respect to incubation time (Fig. 3) and protein concentration (Fig. 4) were performed at a substrate concentration less than Km. In UGT1A6 Bactosomes the reaction was linear to 15 min (Fig. 3) and up to a protein concentration of 250 µg/ml (Fig. 4) at 0.25 mM 4MU.

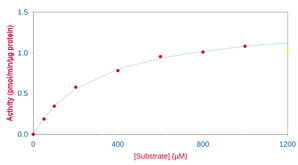
Fig. 4: Product formation with respect to protein concentration in UGT1A6 Bactosomes



Results 3

Michaelis-Menten kinetic parameters for 4MU-UGT activity in UGT1A6 Bactosomes were determined (Fig. 5). The reaction was characterised by Vmax values of 1.4 and 2.2 pmol/min/µg protein for two separate batches. Km (4MU) values were 302 and 305 µM respectively for the two batches. The Km (UDPGA) was 0.36 mM (single determination; data not shown).

Fig. 5: Determination of Michaelis-Menten kinetic parameters for 4MU-UGT activity in UGT1A6 Bactosomes



Conclusions

1) Recombinant human UGT1A6 was successfully expressed in *E. coli*.

2) An HPLC procedure was established to measure 4MU-UGT activity in bacterial membrane fractions.

3) Formation of 4MUG in UGT1A6 Bactosomes was linear for 15 min with respect to incubation time and up to 250 µg/ml with respect to protein concentration.

4) Michaelis-Menten kinetic parameters for two batches were Vmax 1.4 and 2.2 pmol/min/µg protein, and Km (4MU) 302 and 305 µM, respectively.

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