DETERMINATION OF 4-METHYLUMBELLIFERONE SULFOTRANSFERASE 1A1*1 ACTIVITY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

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ABSTRACT

The determination of sulfotransferase 1A1*1 (SULT1A1*1) kinetic parameters using p-nitrophenol as a substrate is impeded by strong substrate inhibition at higher concentrations. We have developed a method using an alternative substrate, 4-methylumbelliferone (4MU), which allows Km and Vmax to be determined more easily for this enzyme.

A direct isocratic HPLC procedure for the measurement of 4MU-SULT1A1*1 activity was established and bacterial cytosolic fractions containing recombinant human SULT1A1*1 were used to validate the assay procedure.

Formation of 4-MUS was linear with respect to time and protein concentration at a 4MU concentration equal to Km. Values were obtained for the kinetic parameters: Km was approximately 2 µM and Vmax approximately 9 pmol/min/µg protein.

INTRODUCTION

Substrate inhibition was encountered at high concentrations of p-nitrophenol when attempting to measure the kinetic parameters of human sulfotransferase 1A1*1 (SULT1A1*1). As such, it was difficult to obtain an accurate estimate of Km and Vmax for this enzyme. This led to the development of an assay using an alternative substrate.

4-methylumbelliferone (4MU) is also metabolised by SULT1A1*1. A direct isocratic HPLC procedure was set up to separate 4MU and its metabolite, 4-methylumbelliferylsulfate (4MUS). Bacterial cytosolic fractions containing recombinant human SULT1A1*1 were used to validate the assay procedure.

During assay development, some variability in results was attributed to the possibility of the enzyme existing in both reduced and oxidised forms with differing kinetic characteristics (Duffel et at, 2001). Following investigation all assays were carried out in the presence of 10 mM dithiothreitol to ensure the enzyme remained in the reduced form.

MATERIALS AND METHODS

CHEMICALS

4-methylumbelliferone (4MU), 4-methylumbelliferylsulfate (4MUS) and adenosine 3'-phosphate 5'-phosphosulfate (PAPS) were purchased from Sigma-Aldrich; HPLC grade acetonitrile and tetra-n-butyl ammonium hydrogen sulfate came from Fisher. All other chemicals were supplied by BDH.

INCUBATIONS

Cytosol isolated from *E. coli* expressing human SULT1A1*1 (Cypex Ltd, Dundee) was incubated at 37 ˚C in 50 mM phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$, 10 mM dithiothreitol, 20 µM PAPS and substrate in amber tubes. Incubations were stopped by the addition of acetonitrile and centrifuged. Supernatants were injected onto the HPLC column.

HPLC CONDITIONS

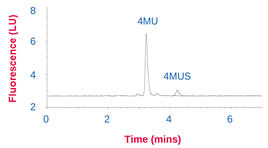
4MU and 4MUS were separated using an Agilent 1100 system with a Hypersil ODS column (5 µm, 250 x 4.0 mm) at a temperature of 30 ˚C. Detection was by fluorescence using excitation and emission wavelengths of 315 and 375 nm, respectively. The mobile phase consisted of 50 mM ammonium acetate containing 50 mM tetra-n-butyl ammonium hydrogen sulfate (final pH 3.6) / acetonitrile at 54/46 (v/v) and the flow rate was 1.0 mL/min.

RESULTS (1)

HPLC separation of 4-methylumbelliferone and 4-methylumbelliferylsulfate was established (Figure 1). Retention times for the compounds were 3.3 and 4.3 minutes respectively.

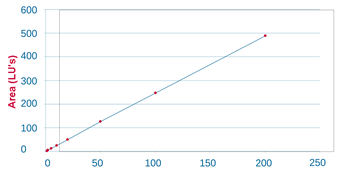
A standard curve of 4-methylumbelliferylsulfate was prepared, with standards ranging from 1 ng/200 µl up to 200 ng/200 µl (Figure 2). The limit of detection was determined as 0.57 ng/200 µl and the limit of quantitation was taken as 0.91 ng/200 µl.

Figure 1 - Chromatograph showing separation of 4MU and 4MUS



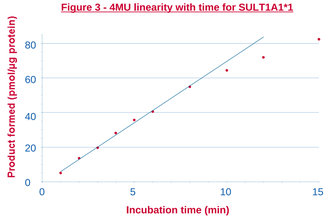
A sample of 4 ng/200 µL 4-methylumbelliferone and 5 ng/200 µL 4-methylumbelliferylsulfate was prepared in 50 mM potassium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 10 mM dithiothreitol with 0.5 µg protein/200 µL (1 mL total volume) then 500 µL acetonitrile added. 20 µL supernatant was injected onto the HPLC using the conditions described in materials and methods.

Figure 2 - Standard Curve of 4-methylumbelliferylsulfate



4-methylumbelliferylsulfate standards in 50 mM potassium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 10 mM dithiothreitol, 0.5 µg protein/200 µL were prepared (200 µL total volume) then 100 µL acetonitrile added. 20 µL supernatant was separated by HPLC as described in Materials & Methods

Figure 3 - 4MU linearity with time for SULT1A1*1



Incubation carried out in 50 mM potassium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 10 mM dithiothreitol, 2 µM 4MU and 20 µM PAPS with 2.5 µg/mL protein.

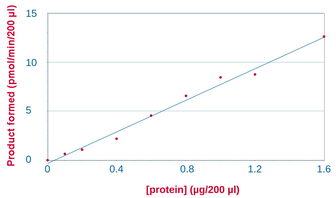
RESULTS (2)

Formation of 4-methylumbelliferylsulfate was linear with respect to time (Figure 3) and protein (Figure 4) when SULT1A1*1 was assayed using a substrate concentration equal to Km (2 µM).

Kinetic analysis of SULT1A1*1 gave results which can be seen in Figures 5a and b showing the Hanes and Michaelis-Menten plots respectively. From the Michaelis-Menten plot Vmax was determined as 8.89 pmol/min/µg protein and Km 2.01 µM.

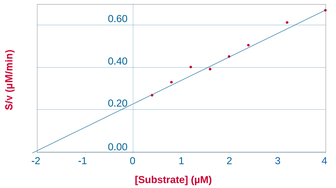
Some substrate inhibition at higher concentrations was noted when optimising the assays - kinetics figures were obtained from assays carried out between 0 and 4 µM 4MU which did not appear to be affected. Substrate inhibition was not seen to affect assays to the same extent as p-nitrophenol, where it was difficult to obtain figures over a range of concentrations sufficient to allow kinetic analysis.

Figure 4 - 4MU linearity with protein concentration for SULT1A1*1



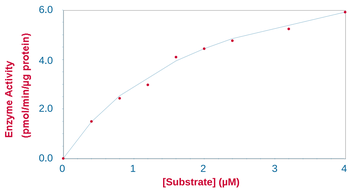
Incubations carried out in 50 mM potassium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 10 mM dithiothreitol, 2 µM 4MU and 20 µM PAPS for 5 minutes.

Figure 5a - Hanes plot for 4MU assay with SULT1A1*1



Incubations carried out in 50 mM potassium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 10 mM dithiothreitol, 20 µM PAPS with 0.5 µg protein/200 µL for 5 minutes.

Figure 5b - Fit of data to Michaelis-Menten equation for 4MU assay with SULT1A1*1



Incubations carried out in 50 mM potassium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 10 mM dithiothreitol, 20 µM PAPS with 0.5 µg protein/200 µL for 5 minutes.

CONCLUSIONS

4-methylumbelliferone (4MU) is metabolised by human SULT1A1*1 producing 4-methylumbelliferyl-sulfate (4MUS) which can be measured using HPLC with fluorometric detection.

Substrate inhibition at higher concentrations of 4MU does not affect the ability to determine kinetic parameters for human SULT1A1*1, and, as such, analysis of 4MUS provides a useful alternative method for characterising this enzyme.

The addition of 10 mM dithiothreitol to assays ensures that the enzyme stays in a reduced form throughout investigations and is useful in eliminating variability.

REFERENCE

1) Duffel MW, Marshal AD, McPhie P, Sharma V, Jakoby WB; Enzymatic Aspects of the Phenol (aryl) Sulfotransferases. Drug Metabolism Reviews 33(3 & 4) (2001): 369-395.