DETERMINATION OF 3,5-DIACETYL-1,4-DIHYDROLUTIDINE USING HPLC WITH FLUOROMETRIC DETECTION

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ABSTRACT

Formaldehyde is formed from the N- and O-demethylation of various substrates and can be measured indirectly via derivatisation to 3,5-diacetyl-1,4-dihydrolutidine (DDL) using Nash Reagent.

We have established high performance liquid chromatography (HPLC) conditions to separate and detect DDL using fluorescence and produced a standard curve of derivatised formaldehyde.

In this study, the activities of Bactosomes expressing single CYPs were determined using this new method of detection and compared to detection of the primary metabolite. We show correlation between formaldehyde formation and the rate of demethylation.

INTRODUCTION

Formaldehyde is formed from the N-demethylation of various substrates and can be measured indirectly using the procedure of Nash, based on the Hantzsch reaction, whereby the formaldehyde is derivatised to 3,5-diacetyl-1,4-dihydrolutidine (DDL). By measuring the amount of DDL produced the activity of any formaldehyde producing N- or O-demethylating enzyme can be measured regardless of the other metabolite.

Previously, the use of high performance liquid chromatography (HPLC) to measure formaldehyde produced has involved separation of the formaldehyde followed by derivatisation to form 3,5-diacetyl-1,4-dihydrolutidine (DDL), which is then detected by fluorescence (1). We have developed an alternative, sensitive method to measure DDL itself, using HPLC with fluorescence detection.

In this study we have determined the N- and O- demethylase activity of Bactosomes expressing cytochrome P450s 2C19, 2D6 and 1A2 using diazepam, dextromethorphan and caffeine as substrates. Results from the measurement of DDL were compared to values obtained from direct measurement of metabolite. A substrate not known to undergo N- or O-demethylation (phenacetin) was used as a control.

MATERIALS & METHODS

3,5-diacetyl-1,4-dihydrolutidine standard was purchased from QMX Laboratories, acetylacetone and HPLC grade acetonitrile were from Fisher. All other chemicals were supplied by BDH.

DETERMINATION OF ACTIVITY USING DIRECT MEASUREMENT OF DDL

Bactosomes co-expressing individual P450 isoforms with P450 reductase (Cypex Ltd, Dundee) were incubated at 37 ˚C in 100 mM phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$, 0.5 mM NADPH and substrate.

Incubations were stopped by the addition of ethanol and centrifuged. All assays incorporated a control incubation without substrate. Supernatants from the incubations were mixed 2:1 with optimised Nash Reagent (4M Ammonium acetate, 1µL/mL acetylacetone, pH 6) and heated at 60˚C for 10 min. Samples were cooled prior to HPLC analysis.

3,5-diacetyl-1,4-dihydrolutidine was separated on an Agilent 1100 System with a Hypersil BDS-C18 column (3 µm, 100 x 4.0mm) at a temperature of 30 ˚C. Fluorescence detection was at 416 nm and 508 nm for excitation and emission wavelengths respectively. The mobile phase consisted of water/acetonitrile 85/15 (v/v) at a flow rate of 1.0 mL/min.

DETERMINATION OF ACTIVITY USING DIRECT MEASUREMENT OF METABOLITES

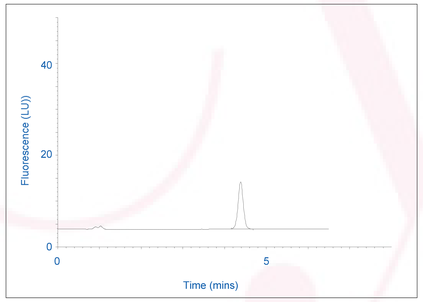
Assays were carried out under conditions previously optimised for each substrate according to Standard Operating Procedures. Metabolites were quantified by HPLC.

RESULTS (1)

HPLC separation of 3,5-diacetyl-1,4-dihydrolutidine (DDL) was established and confirmed by comparison to authentic DDL standard (Figure 1). Retention time for the compound was 4.4 min.

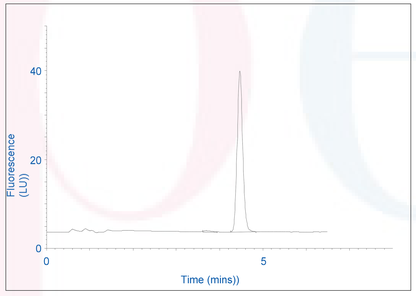
A standard curve of formaldehyde was prepared and derivatised by Nash Reagent (Figure 2). Background fluorescence was attributed to contamination of the acetylacetone with formaldehyde, but was minimised during optimisation of the assay. A background reading of 10 -15 LU was average.

Figure 1a: Chromatogram showing separation of DDL standard



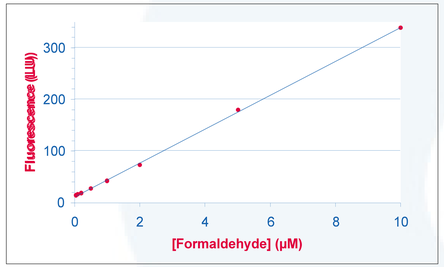
A sample of 2 µM DDL (in ethanol) in 100 mM potassium phosphate buffer, pH 7.4, 5mM magnesium chloride (1 mL) was prepared, then 500 µL ethanol and 20 µL 6M HCl was added. 50 µL was injected onto the HPLC using the conditions as described in materials and methods.

Figure 1b: Chromatograph of derivatised formaldehyde



A sample of 10 µM formaldehyde in 100 mM potassium phosphated buffer, pH 7.4, 5 mM magnesium chloride (1 mL) was prepared, then 500 µL 20% trichloroacetic acid added. Following centrifugation 1 mL supernatant was incubated with Nash Reagent as described in Materials and Methods prior to injection of 50 µL onto the HPLC.

Figure 2: Standard curve of derivatised formaldehyde



Formaldehyde standards in 100 mM potassium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 1% ethanol, 40 pmol P450/mL (1 mL) were prepared, then 1 mL ethanol was added. Following centrifugation, 1 mL supernatant was incubated with Nash Reagent prior to separation by HPLC as described in Materials and Methods.

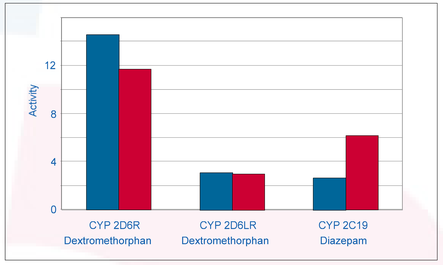
RESULTS (2)

Assays at a saturating concentration of dextromethorphan (10 µM) were used to assess the activity of CYP2D6R and CYP2D6LR Bactosomes. A comparison was made between results taken from direct measurement of dextrorphan produced and derivatisation of formaldehyde produced (Figure 3). The activity measured is similar in both cases for direct and indirect measurement. (CYP2D6R 14.6 and 11.7 pmol product formed/min/pmol P450 for direct and indirect measurement respectively; CYP2D6LR 3.1 and 3.0 pmol product formed/min/pmol P450 for direct and indirect measurement respectively).

Kinetic analysis of CYP2C19R using diazepam as substrate resulted in a similar value for the K_m (16 µM) as obtained from direct measurement of desmethyldiazepam (22 µM). However the v_{max} obtained by derivatisation (6.2 min^{-1}) was double that previously measured (2.7 min^{-1}; Figure 3).

To validate the methodology a substrate not known to undergo N-demethylation was tested as a negative control. The metabolism of phenacetin by CYP1A2R (an O-deethylation reaction) did not result in detectable formation of formaldehyde (not shown). In contrast, metabolism of caffeine by CYP1A2R (positive control) gave an activity of 0.94 pmol HCHO formed/min/pmol P450 at 5 mM caffeine and 20 pmol P450/200µL. It should be noted that as caffeine has 3 N-methyl groups this activity represents the sum of all N-demethylations taking place.

Figure 3: Comparison of activity measured by derivatisation with direct measurement



CYP 2D6 incubations were carried out as described in the Materials & Methods section using
CYP 2D6R 1 pmol P450/200 µL, 10 µM dextromethorphan
CYP 2D6LR 5 pmol P450/200 µL, 10 µM dextromethorphan
CYP 2C19 activity was determined from a kinetic analysis of diazepam at 10 pmol P450/200 µL

CONCLUSIONS

Indirect measurement of P450 activity via formaldehyde formation and derivatisation correlates with the production of metabolite by demethylation. As such, HPLC separation of DDL provides a useful alternative method for measuring the activity of any O- or N- demethylation reaction.

There is no formation of formaldehyde if the substrate does not undergo demethylation (e.g. phenacetin) but appropriate control assays must be included to negate the effect of background formaldehyde formation in the absence of substrate.

This method is sensitive enough to detect the demethylation of substrates via as yet uncharacterised routes. The activity measured gives an overall picture for total demethylase activity - where a substrate may be undergoing multiple demethylations individual routes of metabolism cannot be quantified.

REFERENCE

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