Use of specific radiolabelled metabolite standards in the optimisation of drug metabolising enzyme assays

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Abstract

Radiolabelled (14C) lauric acid is routinely used to assay for specific CYP450 activity in liver microsomes, for example as part of ex vivo enzyme induction studies. The compound undergoes hydroxylation at the 11- and 12- positions, reactions which are catalysed by CYP2E1 and CYP4A, respectively. Radiolabelled samples from this assay are routinely analysed by HPLC with online radiodetection. However, the lack of a suitable chromophore in the parent compound and the metabolites hinders the absolute assessment of the elution order of the two major hydroxylated metabolites. We have now prepared 14C-radiolabelled forms of both 11- and 12-hydroxylauric acid for use as definitive markers for the products of lauric acid metabolism. We present data showing the chromatographic characteristics of each radiolabelled metabolite standard and also a comparison of the overall metabolism of 14C-lauric acid using rat, dog, Cynomolgus monkey and human liver microsomes. Furthermore, the use of radiolabelled metabolite standards lends it self to investigate possible species-differences in the formation of each enantiomeric form of 11-hydroxy lauric acid.

Introduction

Lauric acid 11- and 12-hydroxylation is a commonly used reaction(s) to measure CYP2E1 and CYP4A activity in liver microsomes. However, in most HPLC analyses these two metabolites tend to elute close together (similar polarity) and the absolute assignment of either the 11- or 12-hydroxylauric acid can be ambiguous because of the lack of suitable UV-absorbing standards. Historically, the elution order has been assumed to be 11-hydroxylauric acid first followed by the 12-hydroxylauric acid. This assumption has been made based upon studies using refractive index detection.

At BioDynamics, ^{14}C-lauric acid hydroxylation is a commonly conducted assay which is used during classical *ex vivo* enzyme induction studies. Therefore, we decided to synthesise radiolabelled standards of the two hydroxylated lauric acid metabolites in order to confirm the HPLC elution order.

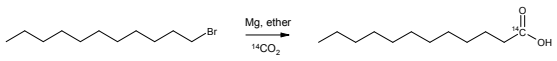
As part of our ongoing development of the classical CYP450 assays, we also compared the metabolism of ^{14}C-lauric acid using rat, dog, Cynomolgus monkey and human liver microsomes.

Materials and Methods

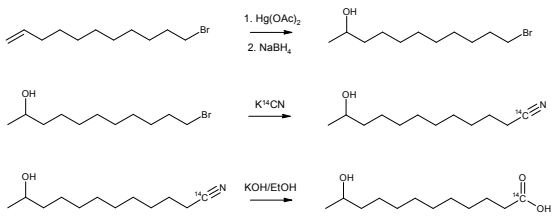
Radiosynthesis

Lauric acid-[1-^{14}C] (47.2 mCi mmol^{-1}), 11-Hydroxylauric acid-[1-^{14}C] (51.8 mCi mmol^{-1}) and 12-Hydroxylauric acid-[1-^{14}C] (50.8 mCi mmol^{-1}) were prepared by the isotope synthesis group at BioDynamics using the methodology shown:

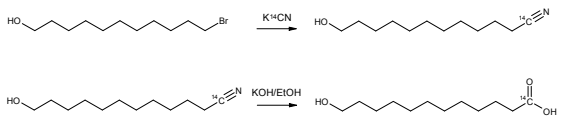
Lauric acid-[1-^{14}C]



11-Hydroxylauric acid-[1-^{14}C]



12-Hydroxylauric acid-[1-^{14}C]



Microsomal incubations

Rat, dog and Cynomolgus monkey microsomes were prepared at BioDynamics using livers from naïve male animals and incubated with ^{14}C-Lauric acid (100 µM) at a final protein concentration of 1 mg/mL.) at 37°C in an oscillating water bath. Pooled human liver microsomes (n = 20 donors) were purchased from Human Biologics International (Arizona, USA). Following extraction with ether, the samples were dissolved in mobile phase for analysis by HPLC.

HPLC analysis

Radiolabelled metabolite standards and samples were analysed by reversed-phase HPLC with on-line radiodetection (β-RAM, model 3 with Laura 3 data acquisition software) using a method developed at BioDynamics. **Figure 1 shows the elution of both 11– and 12-hydroxylauric acid radiolabelled metabolite standards.**

Figure 1 Radiolabelled metabolite standards

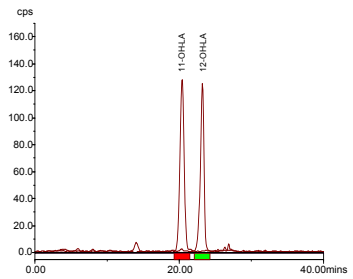
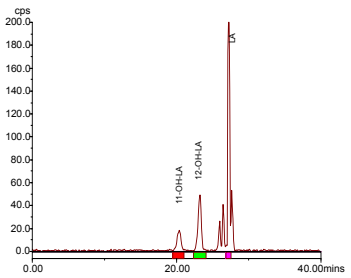
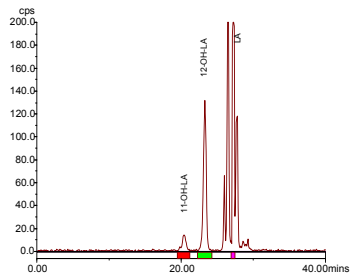


Figure 2 ^{14}C-Lauric acid metabolic profiles

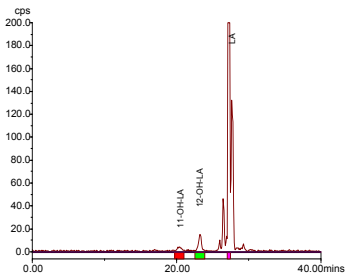
RAT, 30 min incubation



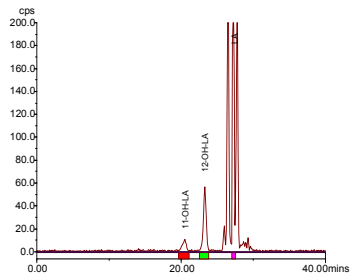
DOG, 30 min incubation



CYNO MONKEY, 30 min incubation



HUMAN, 30 min incubation



Results

The retention times of the authentic radiolabelled standards were:

Standard	Retention time (min)
11-hydroxylauric acid	20.5
12-hydroxylauric acid	23.2
Lauric acid	27.0

In microsomes from all species, 12-hydroxylauric acid was formed in greater amounts than 11-hydroxylaruc acid. The major species-difference was the marked reduction in overall lauric acid hydroxylation by Cynomolgus monkey microsomes.

Species	Rate of metabolite formation (nmoles/min/mg)	
	11-hydroxylauric acid	12-hydroxylauric acid
Rat	0.232	0.530
Dog	0.098	0.691
Cynomolgus monkey	BLQ	BLQ
Human	0.081	0.336

Conclusions

Using authentic radiolabelled metabolite standards we have confirmed that the elution order of radiolabelled components resulting from the microsomal hydroxylation of ^{14}C-lauric acid using our reversed phase HPLC system is:

1) 11-hydroxylauric acid, 2) 12-hydroxylauric acid, 3) Lauric acid

In rat, dog, Cynomolgus monkey and human liver microsomes, 12-hydroxylation of lauric acid dominates over 11-hydroxylation. We have also identified very low overall lauric acid hydroxylase activity in Cynomolgus monkey microsomes.

The rank order for metabolite formation was:

11-hydroxylauric acid: Rat > dog ≥ human >> Cynomolgus monkey

12-hydroxylauric acid Dog > rat >human >> Cynomolgus monkey

Additional Studies

With the availability of the radiolabelled metabolite standards, additional studies will investigate the possible stereo-specific formation of the 11-hydroxylauric acid, which will involve chiral HPLC separation of the enantiomers.