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A Qualitative Method For Assessing The ^{14}C Labelling Position In [^{14}C]-Dimethyl Aminoantipyrine (Aminopyrine)

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Summary

[^{14}C]-Aminopyrine is a well known compound which is widely used for the evaluation of the hepatic function (breath test). As this breath test is based upon N-demethylation, it is essential to verify the labelling position in order to guarantee relevant results. We propose an enzymatic method based on collection of $^{14}CO_2$ produced after specific in vitro N-demethylation in a medium containing horseradish peroxidase, formaldehyde dehydrogenase and formate dehydrogenase. This assay allows for a qualitative monitoring of the labelling position.

Key words: breath test, aminopyrine, quality control,

Introduction

Hepatic metabolism in man can be assessed by breath analysis of the $^{14}CO_2$ output after administration of [^{14}C]- dimethyl aminoantipyrine (aminopyrine), as demonstrated by Hepner in 1974 (1, 2). This technique, now best known as the CO_2 breath test, has been widely used, and extended to other radiopharmaceuticals such as caffeine (3), erythromycin (4) and phenacetin (5). It has also been extended to other pathologies such as gastritis and peptic ulcer disease using [^{14}C]- urea (6).

Because [^{14}C]- aminopyrine is not manufactured as a radiopharmaceutical but as a radiochemical, the injectable solution must be locally prepared by a radiopharmacist. This involves quality control for radiochemical purity, which is routinely done by means of radio TLC. However TLC techniques do not allow for control of the labelling position which is of critical importance. Indeed, in vivo, the main metabolic route is two sequential N-demethylations to give 4-monomethyl aminoantipyrine, 4-aminoantipyrine and formaldehyde which is further oxidised to CO_2 (see figure 1) (7). It is essential to verify the labelling position in order to guarantee an effective recovery of $^{14}CO_2$ and relevant results.

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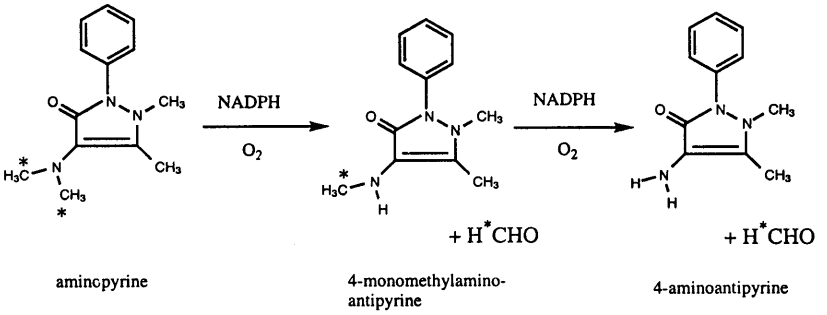
In this paper, we report an enzymatic method based on the collection of $^{14}CO_2$ produced after specific in vitro N-demethylation of aminopyrine which allows for the delineation of the labelling position. We compare this assay with more complex metabolism systems using microsomes or S9 fraction.



$$2\ H^*CHO \longrightarrow 2\ ^*CO_2$$

Figure 1: In vivo oxidative metabolism of aminopyrine

Material and methods

Chemicals

-[^{14}C]- aminopyrine was purchased from Amersham. Two specific activities were used: 3.81 GBq/mmol (103 mCi/mmmol), the specific activity supplied by Amersham, and 8.55 MBq/mmol (231 μCi/mmol) which is the specific activity of the solution prepared in our radiopharmacy and routinely used for breath tests.

-Horseradish peroxidase (HRP), formaldehyde dehydrogenase (FLDH) from yeast and formate dehydrogenase (FTDH) were obtained from Sigma; NAD, NADP and NADPH were from Fluka Biochemika.

-Hydrogen peroxide (H_2O_2, 30%), sodium hydrogencarbonate, potassium dihydrogenphosphate and other chemicals were purchased from Merck, and were all of analytical grade.

- The liquid scintillation cocktail used was Hionic Fluor (Packard).

Microsomes and S9 fractions

Microsomes were prepared by ultra centrifugation of Wistar rat liver homogenate as described by Amar-Costesec (8). Protein concentrations were determined by the Bradford method (9) with Bovine Serum Albumin (Sigma) as standard. P450 activity was 0.3 nmol/mg protein as assayed according to Omura and Sato (10).

The supernatant fraction after 10000g centrifugation supplemented with coenzymes (NADH and NADPH 1mM each) was used as S9 fraction.

Enzymatic systems

Various enzymatic systems were used to achieve N-demethylation of [^{14}C]-aminopyrine and subsequent oxidation into $^{14}CO_2$.

System 1 consisted of:

HRP, formaldehyde dehydrogenase, formate dehydrogenase 0.05 IU each, hydrogen peroxide 0.45mM, NAD 1mM, aminopyrine with various specific activities in a concentration ranging from 1μM to 80μM in a phosphate buffer 0.05M pH=7 to make a final volume of 250 μL.

System 2 consisted of:

rat liver microsomes ranging from 1 to 4 mg protein, NAD and NADPH 1mM each in a phosphate buffer pH=7. System 2b is system 2 supplemented with FLDH and FTDH 0.05 IU each.

System 3 consisted of:

S9 fraction, NADPH and NADH 1mM each in the same phosphate buffer. System 3b is system 3 supplemented with FLDH and FTDH 0.05 IU each, and NAD 1mM.

CO_2 trap medium

Two CO_2 traps were compared: KOH 5mM in water or methanol saturated with KOH.

Assay procedure

1 Ml of CO_2 trap (either KOH or methanol/KOH) was placed in a 10 Ml glass vial (outer vial). Radiolabelled aminopyrine and enzymatic medium were introduced in a smaller vial (inner vial), covered with Parafilm® pierced by a few holes.

The inner vial was introduced into the larger one, the latter being tightly closed with a rubber stopper and placed in a water bath at 37°C for one hour.

After this incubation period, hydrogen carbonate solution (0.1 ml, 0.1 M) was added to the enzymatic medium (inner vial) using a syringe and a needle through the rubber stopper, and 200 μL of 1M HCl were then slowly added to produce cold CO_2 as carrier. After a few minutes, the vials were sonicated for 10 minutes to ensure that all CO_2 was expelled (this procedure allowed for more than 90% $^{14}CO_2$ recovery). An aliquot of the CO_2 trap solution and of the reaction medium was then counted in a Wallac 1410 liquid scintillation counter (Pharmacia).

All experiments were repeated at least 4 times.

Results and discussion

The CO_2 trap medium had no influence on the recovery of radioactive carbon dioxide. Neither did the specific activity of the starting material influence the recovery.

When rat liver microsomes were used (system 2), the amount of $^{14}CO_2$ produced was very low, whatever the concentrations of microsomes or aminopyrine used (Table 1). This is probably due to the lack of active oxidative enzymes in this highly purified

preparation, leading to a very low rate of conversion of formaldehyde into CO_2. More surprisingly, the $^{14}CO_2$ recovery was not improved when formaldehyde dehydrogenase and formate dehydrogenase were added to the microsomes preparation (system 2b).

Table 1: Recovery of radioactivity as $^{14}CO_2$ in various incubation media

System used	description	[protein]	% recovery of $^{14}CO_2$ (mean ± sem)		
			1 µM aminopyrine	10µM aminopyrine	80µM aminopyrine
System 1	HRP, FLDH, FTDH + coenz.	0.05 IU	35.2 ± 1.2	-	34.1 ± 0.8
System 2	Microsomes + coenz.	1mg	0.75 ± 0.07	0.70 ± 0.05	0.35 ± 0.06
		4mg	2. 4 ± 0.10	0.89 ± 0.06	0.75 ± 0.22
System 2b	Microsomes + FLDH + FTDH + coenz.	4mg	2.76 ± 0.15	-	4.05 ± 0.3
System 3	S9 fraction + coenz.		0.79 ± 0.16	0.58 ± 0.12	0.39 ± 0.11
System 3b	S9 + FLDH + FTDH + coenz.		28.4 ± 2.6	-	28.7 ± 1.5

When the same procedure was carried out using S9 fraction alone (system 3), the production yield of $^{14}CO_2$ was also very low, but markedly improved if formaldehyde dehydrogenase and formate dehydrogenase were added (system 3b). Within the range of concentrations tested the recovery of $^{14}CO_2$ was not influenced by the concentration of aminopyrine.

Using the system 1 where HRP is used to complete the N-demethylation, the $^{14}CO_2$ collected reached 35% of the initial dose (Table 1), this percentage being lower if the enzymes were added in a sequential way rather than at the same time (data not shown).

Neverthelesss, we were unable to obtain a full recovery of the activity as $^{14}CO_2$. This is probably due to a limiting step in the kinetics of one reaction, most likely the conversion of formate to CO_2.

Conclusion

We propose that the system made of the three enzymes quoted above (System 1: HRP, FLDH and FTDH 0.05IU each, H_2O_2 0.45mM, NAD 1mM in a phosphate buffer pH=7 0,05M) could serve as a qualitative complementary quality control method in order to assess the labelling position of aminopyrine. This method is convenient and easy to perform. All chemicals and enzymes are commercially available and can be used without further purification. It is likely that this method could be adopted to verify the labeling position of others [^{14}C]- labelled compounds which are metabolised by N-demethylation.

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