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On-line measurements of ^{13}C enrichments in rat breath

Non-invasive method for *in vivo* study of drug enzymatic induction

R. GUILLUY*, F. BILLION-REY and J. L. BRAZIER

L.E.A.C.M. — Laboratoire d'Etudes Analytiques et Cinétiques du Médicament, Institut des Sciences Pharmaceutiques et Biologiques de Lyon, 8 Avenue Rockefeller, 69373 Lyon Cedex 08 (France)

ABSTRACT

A differential kinetic study of $^{13}CO_2$ enrichment of breath after the intake of specific ^{13}C-labelled substrates and co-administration of a drug allows the drug's abillity for enzyme induction to be evaluated *in vivo*. A method and a gas chromatograph–isotope ratio mass spectrometer device for on-line measurements of $^{13}CO_2$ enrichment in the breath of small animals are described. This system allows on-line breath sample collection from a metabolic cage, purification by gas chromatography, determination of CO_2 by thermal conductivity detection and measurement of $^{13}CO_2$ enrichment by isotope ratio mass spectrometry. Two protocols for phenobarbital-inducible P450 and 3-methylcholanthrene-inducible P1-450 isoenzymes are described.

INTRODUCTION

The measurement in the breath of labelled carbon dioxide (CO_2) derived from cytochrome P450-mediated oxidative dealkylation of labelled substrates provides a non-invasive method for measuring the activities of various isoenzymes of the cytochrome P450 system. Various substrates can be used to study the activity of phenobarbital-inducible P450 [1–3] or P1,450 (P 448) [4] arylhydrocarbon hydroxylase. In order to check phenobarbital-inducible P450, antipyrine, phenacetin and aminopyrine can be successfully used as substrates. The methyl groups of these compounds are oxidized to CO_2 and this demethylation leads to the exhalation of CO_2 after an equilibration through the one-carbon formaldehyde–formate pools. Caffeine is a substrate for testing for P1-450 activity. It has been shown [5] that polycyclic aromatic hydrocarbons, including 3-methylcholanthrene [6], have a large inductive effect on hepatic microsomal caffeine N-demethylase activity. It is wel known that the N-demethylation of caffeine produces methylxanthines and that the methyl groups are transformed into formaldehyde, which is then oxidized to CO_2.

A number of studies have been carried out for diagnostic purposes using ^{14}C-

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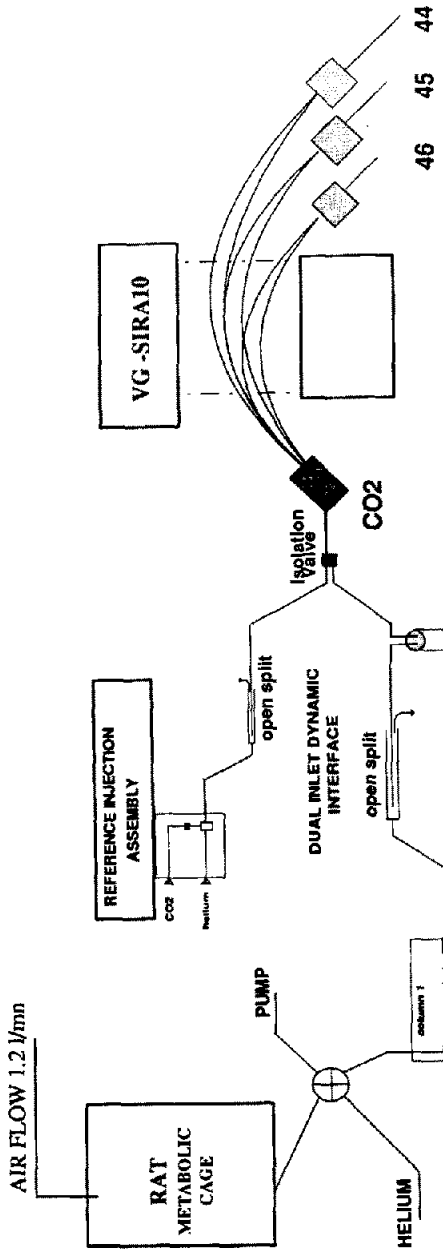

Fig. 1. Scheme of the on-line gas chromatograph–isotope ratio mass spectrometer system connected to a metabolic cage for micro-breath tests on small animals.

labelled phenacetin [7], antipyrine [8], aminopyrine [9], diazepam [10] and caffeine [11,12]. It is obvious that the use of radiolabelled compounds can cause medical and/or ethical problems. Moreover, in various countries, the use of such labelled tracers for *in vivo* experiments is controlled by restrictive regulations. A method using continous-flow isotope ratio mass spectrometry (IRMS) has already been described by Preston and McMillan [13].

The development of IRMS allows the stable isotope of carbon, ^{13}C, to be used for substrate labelling. The administration of ^{13}C-labelled substrates is then followed by the measurement of the ^{13}C enrichment of breath CO_2 by IRMS. Before isotope ratio measurement, CO_2 has to be carefully purified from the expired air. Generally, manual or automatic cryodistillation is used for CO_2 purification. When using such a method, the CO_2 is recovered and purified using liquid nitrogen traps under vacuum. This technique needs large volumes of air, is time consuming and has a low practicability.

In order to perform accurate, precise and rapid measurements of ^{13}C enrichment of breath CO_2, we have developed a continuous-flow device by coupling a gas chromatograph with an isotope ratio mass spectrometer. This analytical gas chromatographic (GC)–IRMS system allows, on-line, purification of CO_2 from expired air (10–300 μl) by GC, the determination of CO_2 by thermal conductivity detection (TCD) and the determination of ^{13}C enrichment by IRMS. The system can be fully automated and the run time per determination is 3 min.

EXPERIMENTAL

Apparatus for GC–IRMS

Isotopic ratio measurements were made on a VG Isotech SIRA 10 isotope ratio mass spectrometer (VG Isotech, Middlewich, Cheshire, U.K.) fitted with a 300 l/s diffusion pump. Fig. 1 shows a scheme of the gas chromatograph–isotope ratio mass spectrometer system and its dual-inlet dynamic interface.

GC purification of breath CO_2 samples was performed on a model HP 5710A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.). A dual-column device is used to provide the reference control helium flow to the thermal conductivity detector and to the mass spectrometer inlet interface. High-purity helium (99.9995%) from Airgaz (Lyon, France) was used as the carrier gas at a flow-rate of 12 ml/min. The glass column (3 m × 6 mm O.D. × 2 mm I.D.) was packed with Hayesep Q Porapak (50–80 mesh) (Chrompack, Les Ulis, France). The temperatures of the injector, detector and column oven were set at 110°C.

A laboratory-made dual-inlet continuous-flow interface was used to connect the gas chromatograph to the mass spectrometer. The main features of this device are two capillary lines fitted to the common inlet mass spectrometer source and a continuous flow of helium which is collected inside two open splits. These open splits isolate the mass spectrometer from any pressure transients which may occur in other parts of the system. The first helium flow is used as a carrier to inject

reference CO_2 gas pulses via an original device from VG Isotech (reference injection assembly). The second helium flow arises from the reference gas chromatograph helium flow (column 2). The purified CO_2 from the analytical GC column (column 1) is injected in this flow via a four-port switching valve (Valco Instruments, Houston, TX, U.S.A.).

This dual inlet allows any time-programmed alternating injections of either reference CO_2 samples or CO_2 peaks eluting from the GC column into the mass spectrometer. It also protects the reference CO_2 gas from any pollution from the gas chromatograph.

Device for micro-breath test on small animals

Animals were housed in a tight all-glass metabolic cage (180 mm × 90 mm) swept by a continuous flow of air at 1.2 l/min in order to ensure physiological breathing conditions. A capillary line linked to a membrane pump allowed the removal of a 20 ml/min flow of air from the cage to a six-port sampling valve (Valco Instruments) located at the entry to the GC column. The loop of the valve received a continuous flow of air from the metabolic cage so that a microsample from this air (250–300 μl) could be injected into the chromatograph at any time or according to a preprogrammed sequence.

Labelled compounds and chemicals

Phenobarbital and 3-methylcholanthrene were purchased from Sigma (St. Louis, MO, U.S.A.). Phenobarbital was administered as a solution in sodium chloride (9 g/l) and 3-methylcholanthrene as a solution in corn oil. N,N-[$^{13}C_2$]Dimethylaminopyrine (Tracers Technologies, Somerville, MA, U.S.A.), $^{13}C_2$ isotopic enrichment 99%, was administered as a solution in sodium chloride (9 g/l). [1,3,7-$^{13}C_3$]Caffeine was purchased from CEA (Gif sur Yvette, France), $^{13}C_3$ isotopic enrichment 99%, and administered as a solution in sodium chloride (9 g/l).

Animals

For each series of experiments, five OFA male rats (IFFA CREDO, L'Arbresle, France) were used. They were kept for 12 h per day under artificial light and housed at a constant temperature of 22°C. They were fed *ad libitum* with controlled artificial food. The mean weights were 269.8 ± 30.0 g in the experiment with [^{13}C]aminopyrine and 340.4 ± 40.0 g in the experiment with [^{13}C]caffeine.

Study of phenobarbital-inducible P450

Control study: sequence I. Five fasted rats were used in this study. Prior to the breath test, breath samples were collected 20 and 10 min before [$^{13}C_2$]aminopyrine administration. Each rat received a single intraperitoneal (i.p.) dose of 4 mg/kg of [$^{13}C_2$]aminopyrine, then was housed individually in all-glass metabolic cages swept by an air flow. Exhaled air samples were automatically collected from

the metabolic cage every 10 min for 180 min and injected into the gas chromatograph for CO_2 purification and determination of ^{13}C enrichment.

Induction study: sequence II. The same rats were then treated with phenobarbital for 6 days (one i.p. 40 mg/kg dose of phenobarbital per day). On the sixth day they received a 4 mg/kg dose of [$^{13}C_2$]aminopyrine and the micro-breath test was performed again as described for sequence I.

Study of methycholanthrene-inducible P1-450

The study of methylcholanthrene-inducible P1,450 was performed according to the same design as above. The labelled substrate used for the breath test was administered as a single i.p. injection of a 10 mg/kg dose of [1,3,7-$^{13}C_3$]caffeine. The determination of ^{13}C enrichment was performed for 225 min. Cytochrome P1-450 induction was carried out by a single i.p. injection (30 mg/kg) of 3-methylcholanthrene 24 h before the second micro-breath test (sequence II).

Procedure for the determination of breath CO_2 enrichment

After injection of the labelled substrate, each rat was kept free in the all-glass metabolic cage. The air sample was then automatically injected into the Porapak QS column of the gas chromatograph where the separation of the gaseous components of the sample (O_2, N_2, CO_2 and H_2O) was performed. The effluent from the chromatographic column was continuously monitored by TCD, which allows the determination of CO_2. After TCD, the purified CO_2 flowed through the switching valve to the isotope ratio mass spectrometer, where the ^{13}C enrichment was measured by comparison with a reference CO_2 sample in the same way as for classical isotopic analysis.

Fig. 2 shows a scheme of the analytical procedure. (i) When the GC analysis of the air sample starts, a pulse of reference CO_2 is admitted into the mass spectrometer and measured for its $^{13}C/^{12}C$ ratio. (ii) The CO_2 peak leaving the detector is switched into the mass spectrometer and measured for its $^{13}C/^{12}C$ ratio. (iii) The switching valve is closed and the water eluted from the column is removed through a vent. (iv) The computer calculates the ^{13}C enrichment of the sample: $\delta^{13}C$/PDB. The system is then ready for a new injection. The overall run time is 3 min.

RESULTS

Fig. 3 shows a typical profile of the $^{13}CO_2$ enrichment profile ($\delta^{13}C$/PDB *vs.* time) from a rat after administration of [$^{13}C_2$]aminopyrine (sequence I) and of the same substrate after a 6-day administration of phenobarbital (sequence II). It can be observed that the $\delta^{13}C$/PDB values are not significantly different for the two sequences (-23.70).

Immediately after [$^{13}C_2$]aminopyrine injection, the $^{13}CO_2$ enrichment of the expired air increases rapidly, reaching -4.96 $\delta^{13}C$/PDB 20 min after adminis-

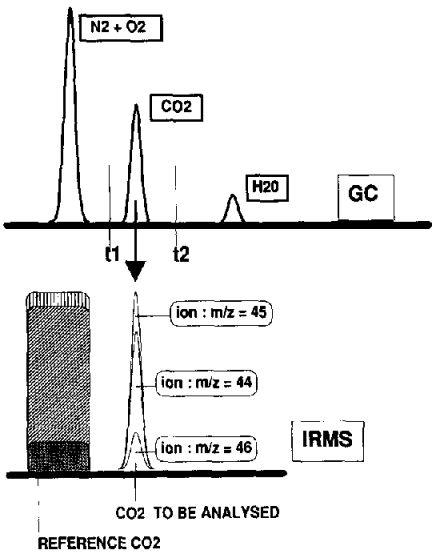

Fig. 2. Scheme of the procedure for micro-breath tests by GC–IRMS. Chromatographic purification of CO_2 from the expired gas (upper part: TCD detection). The switching valve is open between t_1 and t_2 to send the CO_2 peak into the mass spectrometer where ions at m/z = 44, 45 and 46 are measured in the reference CO_2 pulse and in the CO_2 chromatographic peak.

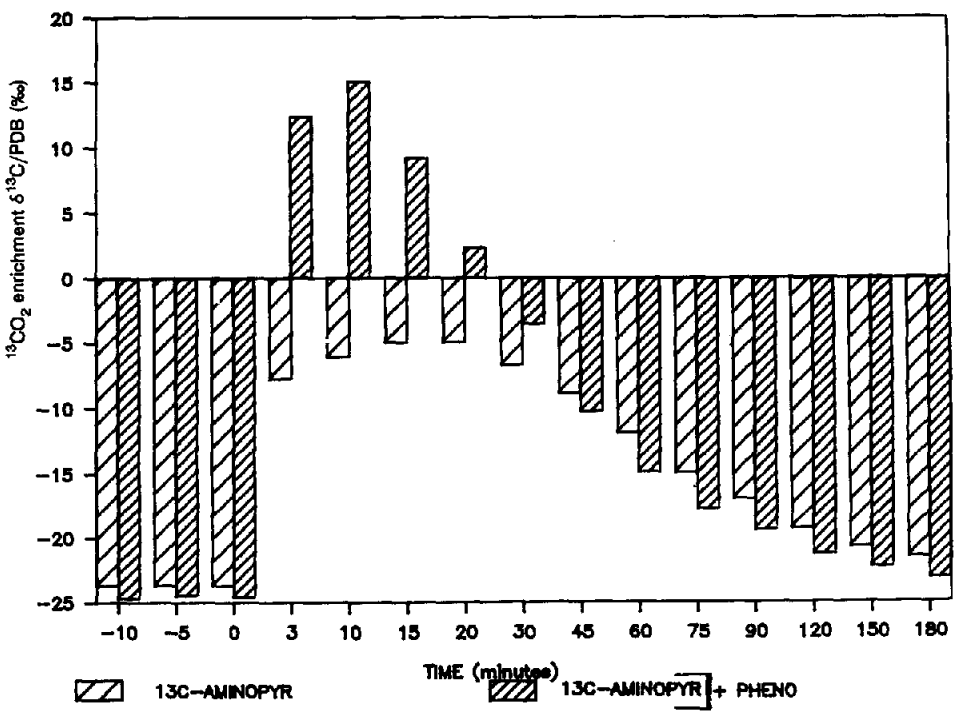

Fig. 3. Variation of the $^{13}CO_2$ enrichment of the air expired by rat No. 4 after administration of [$^{13}C_2$]aminopyrine (sequence I) and [$^{13}C_2$]aminopyrine after a 6-day administration of phenobarbital (sequence II). $\delta^{13}C$/PDB is expressed as ‰ and is defined as $\delta^{13}C/PDB\ (‰) = (R_s - R_{ref}) \cdot 1000/R_{ref}$ where $R_s = {}^{13}CO_2/{}^{12}CO_2$ ratio measured on the studied sample and $R_{ref} = {}^{13}CO_2/{}^{12}CO_2$ ratio measured on a CO_2 sample obtained from an international standard fossil carbonate: the Pee Dee Belemnite limestone.

tration. Subsequently the enrichment reaches the basal level within 180 min. In sequence II, after induction by phenobarbital, the rise in ^{13}C enrichment is both faster and larger: the maximum value is $+15.05$ $\delta^{13}C/PDB$ only 10 min after substrate administration. The maximum enrichment difference between sequence II (induced) and sequence I (non-induced) is $+21.12$ $\delta^{13}C/PDB$ 10 min after [$^{13}C_2$]aminopyrine administration. Such an increase in the rate of excretion of $^{13}CO_2$ derived from N-demethylation of isotopically labelled aminopyrine clearly indicates the inductive effect of phenobarbital.

Fig. 4 shows the $^{13}CO_2$ enrichment profiles obtained from a rat after an i.p. injection of [1,3,7-$^{13}C_3$]caffeine before (sequence I) and after (sequence II) 3-methylcholanthrene administration. The basal levels are not significantly different according to the two sequences (-23.36 $\delta^{13}C/PDB$). After [^{13}C]caffeine administration the increase in $^{13}CO_2$ enrichment of the expired air is slow, and 225 min after dosing this enrichment is still increasing slowly. After 3-methylcholanthrene induction, the $^{13}CO_2$ excretion profile is different. Immediately after [^{13}C]caffeine administration, the $^{13}CO_2$ enrichment rises very rapidly up to $+30.74$ $\delta^{13}C/PDB$ within 20 min; 10 min after the [^{13}C]caffeine injection the $\delta^{13}C/PDB$ difference value is already $+42.81$ and the maximum ($+53.88$ $\delta^{13}C/PDB$) is reached after only 30 min. This large change in CO_2 excretion clearly

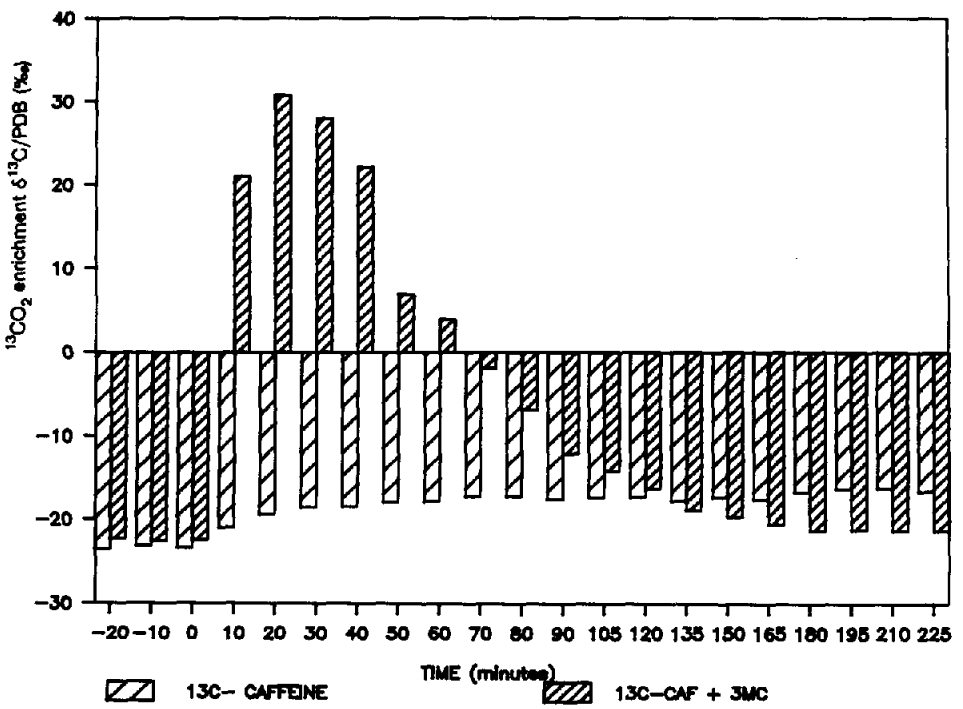

Fig. 4. Variation of the $^{13}CO_2$ enrichment of the air expired by rat No. 5 after administration of [$^{13}C_3$]caffeine (sequence I) and [$^{13}C_3$]caffeine after a single administration of 3-methylcholanthrene (sequence II).

TABLE I

$\delta^{13}C$/PDB DIFFERENCES BETWEEN PHASE II (INDUCTION WITH PHENOBARBITAL) AND PHASE I (CONTROL) AFTER [$^{13}C_2$]AMINOPYRINE ADMINISTRATION TO RATS ($n = 5$)

Time (min)	Mean	S.D.
−10	−0.0300	0.5827
−5	−0.0540	0.4806
0	−0.0420	0.4256
3	17.7380	2.8837
10	15.6840	4.1051
15	9.6400	2.7198
20	5.2080	1.3882
30	1.9240	1.0323
45	−1.4880	1.0268
60	−2.3240	1.2871
75	−1.6940	1.6530
90	−1.7900	0.7420
120	−1.1140	0.8430
150	−0.5176	0.5597
180	−0.5580	0.5163

TABLE II

$\delta^{13}C$/PDB DIFFERENCES BETWEEN PHASE II (INDUCTION WITH 3-METHYLCHOLANTHRENE) AND PHASE I (CONTROL) AFTER [$^{13}C_3$]CAFFEINE ADMINISTRATION TO RATS ($n = 5$)

Time (min)	Mean	S.D.
−20	0.2090	1.5821
−10	0.3800	1.1301
0	0.3350	1.2686
10	27.3180	13.0287
20	37.4140	10.4886
30	40.6940	8.8070
40	40.0680	4.6605
50	35.3660	6.2299
60	31.6900	5.3331
70	27.6700	7.0957
80	23.1460	7.7336
90	18.8940	7.7358
105	14.8820	7.4112
120	10.9080	6.5544
135	7.8280	5.5193
150	4.8428	4.2045
165	2.6720	3.1523
180	1.1560	3.1765
195	−0.1800	2.3310
210	−1.0420	2.0848
225	−1.7300	1.8081

indicates the inductive effect of methylcholanthrene on caffeine N-demethylase activity.

Tables I and II give the $\delta^{13}C$/PDB difference values between phase II and phase I for both [^{13}C]aminopyrine and [^{13}C]caffeine micro-breath tests in the five rats. For each individual rat and for both tests a large positive difference can be observed between the $\delta^{13}C$/PDB values after and before the administration of the two inducers. The maximum difference ($+17.73 \pm 2.88$ δ^{13}/PDB) is reached 3 min after [^{13}C]aminopyrine administration. With caffeine the maximum ($+40.69 \pm 8.81$ δ^{13}/PDB) is observed 30 min after substrate administration.

When [^{13}C]aminopyrine is administered as a substrate with phenobarbital as inducer, the $\delta^{13}C$ difference becomes negative as soon as 30 min after administration. This result indicates that the overall $^{13}CO_2$ excretion is faster after induction and that the major part of [^{13}C]aminopyrine has been oxidized and eliminated within the first 30 min when the drug-metabolizing enzymes are induced. When [^{13}C]caffeine is administered after 3-methylcholanthrene induction, the $\delta^{13}C$/PDB difference also becomes negative only 195 min after administration because of a slower metabolism. In this study the caffeine used was labelled with ^{13}C at N-1, N-3 and N-7. As a consequence, all the metabolites, dimethyl- and monomethylxanthines and methyluric acids were labelled at the same positions. As these metabolites undergo the same type of N-demethylation, the overall excretion time of $^{13}CO_2$ is longer.

DISCUSSION

The usefulness of breath tests has been demonstrated when using suitably radiolabelled drugs such as aminopyrine, phenacetin or caffeine. Aminopyrine and caffeine are demethylated in the liver. They are not excreted unchanged (or only at very low levels), so that their N-demethylation reflects their clearance characteristics. To study the modifications of drug-metabolizing liver activities, aminopyrine and caffeine are two useful substrates. Aminopyrine is a substrate of phenobarbital-inducible P450 and caffeine is a substrate of the 3-methylcholanthrene-inducible P1-450 isoenzyme. Hence breath tests with ^{14}C- or ^{13}C-labelled aminopyrine and caffeine as substrates can be used *in vivo* as non-invasive probes of mixed function oxidase activity.

An alternative to the use of radiolabelled compounds consists in using stable isotope-labelled molecules and, for breath-test purposes, tracers specifically labelled with ^{13}C. The limiting phase of ^{13}C breath tests is the CO_2 purification phase before isotope ratio measurement, because isotope ratio mass spectrometers have to work with pure CO_2 only.

The system described here presents several advantages. This gas chromatograph–isotope ratio mass spectrometer system is able to effect automatically, on-line, the purification of CO_2 from small air samples, the determination of CO_2 and the $^{13}C/^{12}C$ isotope ratio with a precision of 0.2 $\delta^{13}C$/PDB and an overall run time of 3 min.

Such a system allows non-invasive repetitive isotopic measurements on small animals *in vivo*. It also allows investigations to be performed using small animals as their own standards in multi-sequence designed studies. The sole operation carried out on the animal is drug administration.

The method described here can be used for studies designed in order to investigate the modifications induced in drug-metabolizing systems by other drugs or xenobiotics. The variations in the excretion rate of $^{13}CO_2$ derived from N-demethylation of the ^{13}C-labelled substrates are significant markers of such modifications.

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