In vivo Metabolism of [4-^{13}C]Phenacetin in an Isolated Perfused Rat Liver Measured by Continuous Flow ^{13}C NMR Spectroscopy

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Z. Naturforsch. **39 c**, 859–862 (1984); received March 14, 1984

Metabolism, [4-^{13}C]Phenacetin, Continuous Flow ^{13}C NMR

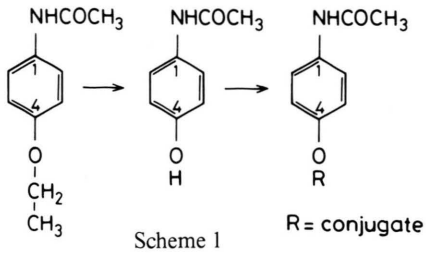
Continuous flow ^{13}C NMR spectroscopy has been used for the first time to monitor the metabolism of a ^{13}C labeled drug in an isolated liver. Continuous and almost immediate information on the metabolite formation could be obtained using ^{13}C labeled phenacetin without alteration of the biological system. The data are consistent with those observed by conventional techniques (HPLC, aliquot ^{13}C NMR measurements). From the biological point of view the sensitivity of continuous flow ^{13}C NMR spectroscopy is still low (10^{-3} M). The results presented demonstrate however that non-invasive and non-radioactive real time monitoring of drug metabolism in intact organs is possible.

Introduction

Since ^{13}C NMR chemical shifts are sensitive towards structural changes, ^{13}C NMR spectroscopy represents a powerful technique for metabolic studies. Such investigations have been performed with cell suspensions [1, 2] or mouse livers placed within the NMR tube [3, 4]. Due to the low concentration of the compounds and the low natural abundance of the ^{13}C isotope (1.1%), ^{13}C-enriched substrates are required.

We report herein a new technique for the investigation of drug metabolism. Metabolite formation in an isolated perfused rat liver is detected in the vascular perfusate by continuous flow ^{13}C NMR spectroscopy.

Phenacetin is well known to exhibit an extensive first pass metabolism [5] (Scheme 1) and was therefore chosen to test the validity of the new technique. The ^{13}C-signals of the oxygen bound aromatic carbon of phenacetin and of its main metabolite, acetaminophen are separated by 2 ppm. Thus, this carbon position is qualified as a probe to measure the consumption of phenacetin and the formation of acetaminophen.



Scheme 1

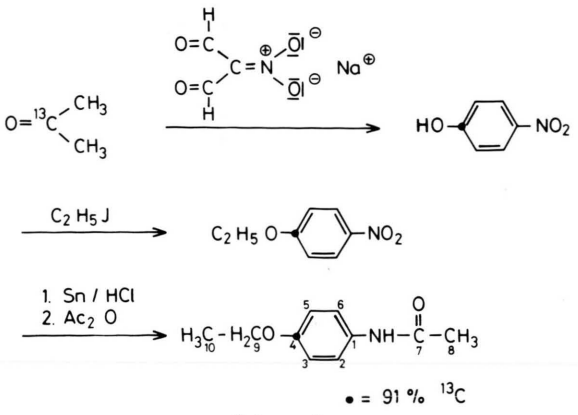
Methods

1. Chemical Synthesis

Phenacetin, ^{13}C-enriched at C-4 (91%), has been synthesized utilising [carbonyl-^{13}C] acetone according to the sequence outlined in scheme 2. Reaction of [carbonyl-^{13}C] acetone with the sodium salt of nitromalondialdehyde yields 4-hydroxy-[4-^{13}C] nitrobenzene [5] which after ethylation of the phenolic function, reduction and acetylation is transformed to the product wanted. Figure 1 shows the NMR spectra of ^{13}C-enriched phenacetin in CD_2Cl_2.

2. Biological System

Isolated perfused rat liver with a fluorocarbon (perfluorotributylamine, 20% w/v; FC-43^{R}, Green Cross Corp., Japan), as artificial oxygen carrier has



Scheme 2

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been used as a biological system that is well controlled and allows investigation of the first pass metabolism [7].

Pretreatment of the animals with phenobarbital, a specific cytochrome-P 450 inducing drug enhances the turn-over-rate of xenobiotics in the liver.

Experiments using HPLC revealed that the metabolism of phenacetin in this system follows strictly first order kinetics and the rate is enhanced 3–5 fold by the phenobarbital pretreatment (Fig. 3).

Perfusion of the isolated rat liver with a synthetic medium (FC-43^R) has been performed in a flow controlled recirculating system similar to the method used by Schimassek [8]. The perfluorotributylamine suspension was constantly equilibrated with 95% O_2/5% CO_2 in a film oxygenator.

250–300 g liver donor rats (male, Sprague-Dewley) pretreated for three days with phenobarbital (80 µg/g b.w.i.p.) were housed with free access to food and water. Operations were performed between 8–10 a.m. under light ether anesthesia. After canulation of the portal vein, perfusion was started immediately, the liver excised and transferred to the perfusion chamber.

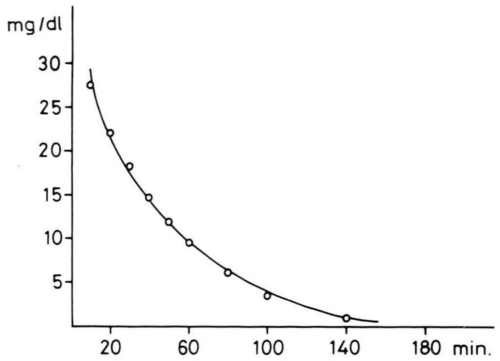

Fig. 2. Phenacetin metabolism in an isolated perfused rat liver stimulated with phenobarbital, detection in aliquots using HPLC. 27 mg Phenacetin dissolved in 0.5 ml ethanol were added to the perfusate (100 ml) at zero minutes.

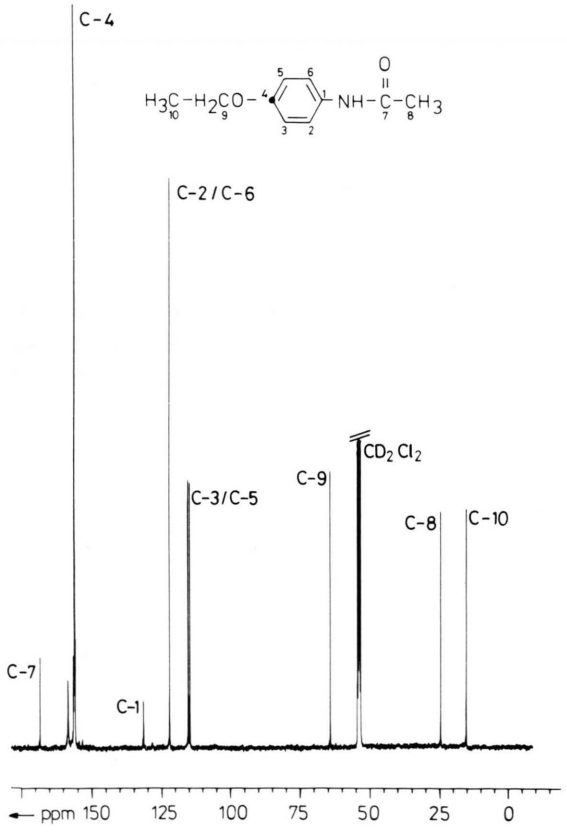

Fig. 1. ^{13}C NMR of phenacetin ^{13}C-enriched at C-4.

Venous flow, arterial pressure, temperature, pH, O_2-consumption, glucose consumption, lactate/pyruvate ratio and bile flow were constantly controlled (Fig. 3). These parameters were shown to be stable for a period of at least 3 h.

Continuous flow ^{13}C NMR spectroscopy

Based on the experience with the registration of continuous flow ^1H-NMR-spectra [9, 10] a ^{13}C-NMR flow cell has been developed. The NMR-measuring-cell consists of a U-shaped glass-tube; the measuring coil and the decoupling coil being mantled at one side. To inhibit coagulation, the inner glass-walls were silylated.

As shown in the schematic diagram (Fig. 3), the NMR flow cell was placed in a venous bypass circuit (total volume 32 ml). At a flow-rate of 1 ml/min, the sensitivity and resolution of test NMR spectra approached conventional routine-spectra. Higher flow-rates resulted in a dramatic decrease of signal intensity and increase of half width. Due to the experimental design and the different flow-rates in the systemic circuit (20 ml/min) and NMR-circuit (1 ml/min), the metabolic event and the detection are separated by a total delay-time of 15 min. A transient increase of the NMR flow-rate equalizes the substrate and metab-

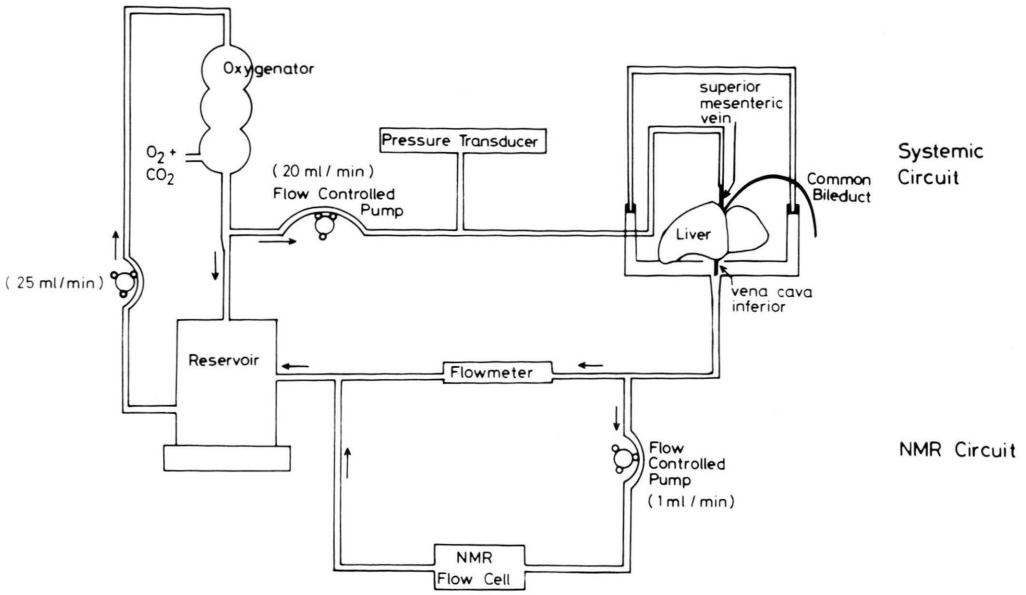

Fig. 3. Schematic diagram of the perfusion apparatus.

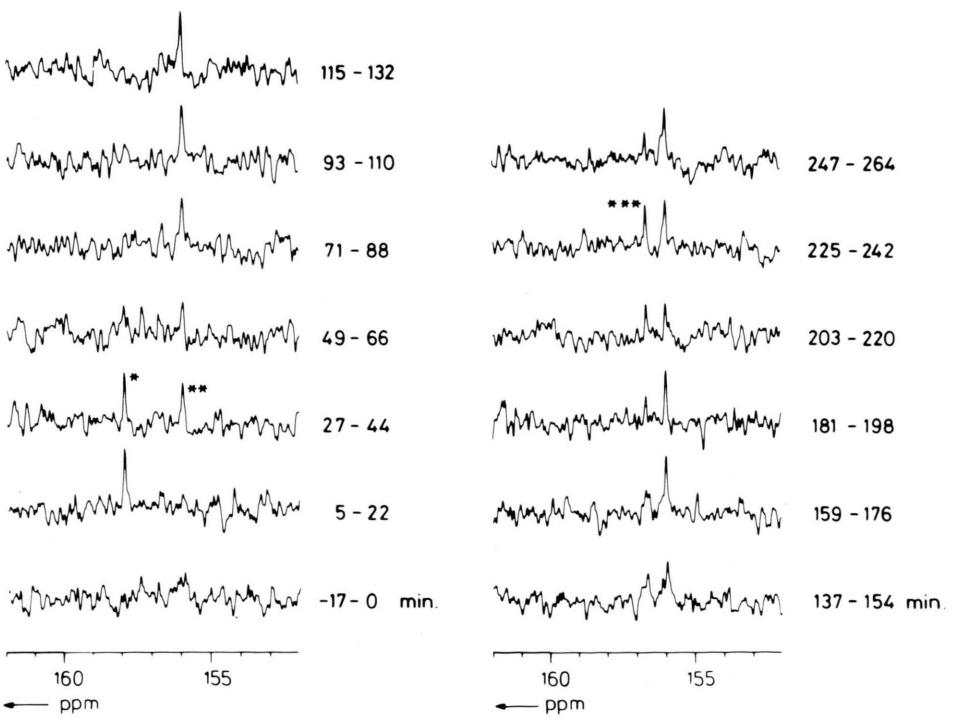

Fig. 4. Continuous flow ^{13}C NMR spectra (100.6 MHz, Bruker WM 400). Time dependent metabolic formation from ^{13}C phenacetin (10^{-3} molar) in an isolated rat liver perfused with 20% (w/v) fluorocarbon FC-43^{R} (C4-phenacetin*, C4-acetaminophen**, conjugate***).

olite concentration in the NMR- and systemic circuits. Therefore, basically two measuring modes are possible:

1. Continuous monitoring produces results with an inherent delay-time of 15 min.
2. The actual data are obtained by increasing the NMR flow rate during the intervals between two measurements.

Results and Discussion

A representative sequence of NMR spectra of a perfusion experiment is demonstrated in Fig. 4. Each spectrum consists of 3000 scans with a total acquisition-time of 17 min. During the waiting-interval of 5 min between two measurements, the concentration ratio in the NMR-circuit is adjusted to the systemic circuit by increasing the flow rate to 10 ml/min. In Fig. 4 the spectra are restricted to the region of interest for the labeled carbon atoms. The resonances of the non-labeled carbon atoms (phenacetin, metabolites, perfusion medium) are not shown. Zero minutes represent the time when [^{13}C]phenacetin (concentration 10^{-3} M) was introduced. Thus, the first spectra (−17−0 min) shows statistical noise. The signal in the second spectra (5−22 min) at 158.0 ppm is due to the labeled carbon of [4-^{13}C]phenacetin. During the next measuring interval (27−44 min) a second signal at 156.0 ppm appears. This signal corresponds to the labeled carbon C-4 in the main metabolite acetaminophene. In the following spectra another signal at 156.7 ppm with varying intensity is found. The chemical shift value and the time dependent behaviour suggest that this signal is presumably due to a secondary metabolite. A comparison with synthetic [4-^{13}C]phenacetin-sulfate excluded this metabolite. Precise assignments by comparison with chemical shift values of further authentic compounds are under progress.

The results presented show that it is possible to obtain continuous flow ^{13}C NMR spectra of metabolic events. The still low sensitivity (10^{-3} M) of the continuous flow ^{13}C NMR spectroscopy is in part (for metabolites) compensated by the induction of the drug metabolising enzymes of the biological system used. Currently we study new probehead designs to achieve an increase in sensitivity. By reducing the void volume of the NMR circuit the time delay between metabolic events and detection will be reduced. Thus, we do expect that in further experiments the drug concentrations may be lowered to a normal therapeutical range.

Acknowledgment

The authors gratefully acknowledge the valuable technical assistance of D. Vieillard-Baron (Medizinische Universitätsklinik Tübingen), E. Grom, A. Paulus (Institut für Organische Chemie, Tübingen) and P. Dejon (Bruker Analytische Meßtechnik, Karlsruhe).

[1] A. I. Scott and R. L. Baxter, Ann. Rev. Biophys. Bioeng. **10,** 151 (1981).
[2] K. Ugurbil, T. R. Brown, J. A. Den Hollander, P. Glynn, and R. G. Shulman, Proc. Natl. Acad. Sci. USA **75,** 3742 (1978).
[3] S. M. Cohen, R. G. Shulman, and A. C. McLaughlin, Proc. Natl. Acad. Sci. **76,** 4808 (1979).
[4] S. M. Cohen, Hepatology **3,** 738 (1983).
[5] G. Margetts, J. Int. Med. Res. **4,** Suppl. 4, 55 (1976).
[6] G. L. Swartz and W. M. Gulick, J. Labelled Comp. **11,** 525 (1975).
[7] W. Krone, W. B. Huttner, S. C. Kampf, B. Rittich, H. J. Seitz, and W. Turnowski, Biochim. Biophys. Acta **372,** 55 (1974).
[8] H. Schimassek, Biochem. Z. **336,** 460 (1963).
[9] E. Bayer, K. Albert, M. Nieder, E. Grom, and T. Keller, J. Chromatogr. **186,** 497 (1979).
[10] E. Bayer, K. Albert, M. Nieder, E. Grom, G. Wolff, and M. Rindlisbacher, Anal. Chem. **54,** 1747 (1982).

Verantwortlich für den Inhalt: A. KLEMM
Satz und Druck: Konrad Triltsch, Würzburg