Biochemical Pharmacology, Vol. 29, pp. 2085-2088.

0006-2952/80/0715-2085$02.00/0

A METHOD FOR THE STUDY OF N-GLUCOSIDATION IN VITRO - AMOBARBITAL-N-GLUCOSIDE FORMATION IN INCUBATIONS WITH HUMAN LIVER

Bing K. Tang and Giovanna Carro-Ciampi

Department of Pharmacology, University of Toronto,
Toronto, Ontario M5S 1A8

(Received 25 March 1980; accepted 5 May 1980)

N-Glucosidation, as a major pathway of the metabolism of barbiturates in man, was discovered in this laboratory during the course of pharmacogenetic investigations with amobarbital [1,2] and phenobarbital [3]. The biochemistry of formation of the newly identified barbiturate-N-glucosides and of N-glucosides in general is at the present time totally unexplored. In fact, the one other report of mammalian N-glucosidation was based on in vivo observations only [4]. The biological significance of the new metabolic pathway is also unknown. Studies of O-glucosidation, an analogous though not identical reaction, suggested that conjugation with glucose may be relevant to the physiological role of steroids [5,6].

Preliminary attempts in this laboratory to study N-glucosidation of amobarbital in vitro [7] revealed the need for a re-examination of the methods developed earlier for the purpose of in vivo measurements. As will be shown, a two-dimensional thin-layer chromatographic separation (2xD TLC) was devised which made use and combined the advantages of two previously worked out TLC solvent systems [8]. Application of this method to the analysis of incubation mixtures with amobarbital, UDP-glucose and human liver microsomes led to the demonstration of in vitro formation of amobarbital-N-glucoside.

Materials and Methods

All non-radiolabelled biochemicals were purchased from Sigma Chemical (St. Louis, Mo.). UDP-[6-^{3}H]glucose, specific activity 3.1 Ci/mmol, was obtained, as the ammonium salt, from Amersham (Oakville, Ontario). [^{14}C]Amo, specific activity 5.0 Ci/mol, and C-OH were synthesized as reported earlier [8].* Amo-N-glu was prepared as follows: 1-(2,3,4,6-tetraacetyl-β-D-glucopyranosyl)amobarbital was synthesized by condensation of N-(2,3,4,6-tetraacetyl-β-D-glucopyranosyl) urea and 2-ethyl-2-(3-methylbuty)malonyl chloride as described previously [3]. Methane CI mass spectrum gave m/e 557 (MH^+), 497 (MH^+-60), 331, 271 and 227. Amo-N-glu was synthesized by acid hydrolysis (0.8 ml 6N HCl) of the tetraacetyl derivative (1.1 mmole in 3 ml of methanol) at 60° for 2 hrs. The mixture after being dried was redissolved in 1 ml of water and extracted with three 4 ml portions of ethyl acetate. After separation and drying of the organic phase, the resulting gum-like substance weighed 320 mg. A white amorphous solid was obtained after recrystallization from ethyl acetate and ether, m.p. 143°-144°C. The methane CI mass spectrum gave m/e 389 (MH^+), 227, 163, 145, 127. Anal.($C_{17}H_{28}N_2O_8$)C,H,N.

Human urine containing [^{14}C]Amo and metabolites was collected during a previous study [8] and stored at -20°C. Extraction with three 5 ml portions of ethyl acetate in the presence of saturating $(NH_4)_2SO_4$ gave greater than 99% extraction efficiency. Human liver specimens

*Abbreviations
Amo = amobarbital; [^{14}C]Amo = [2-^{14}C]amobarbital; Amo-N-glu = amobarbital-N-glucoside or 1-(β-D-glucopyranosyl) amobarbital; [^{14}C] Amo-N-glu = [2-^{14}C] amobarbital-N-glucoside; Amo-N-[^{3}H]glu = amobarbital-N-[6-^{3}H] glucoside; C-OH = 3'-hydroxyamobarbital; G6P = glucose-6-phosphate; G6PDH = glucose-6-phosphate dehydrogenase.

were obtained from kidney donor cases (K_3, K_4, K_5) and autopsy cases (A_{19}, 12 hours after death) at the Toronto General Hospital, quick-frozen with liquid nitrogen as 1 cm^3 cubes and kept in storage at -80°C. All incubations, 5 ml total volume, containing 92 mM KCl, 80 mM KH_2PO_4 (pH 7.4) and microsomes prepared from 1 g of liver according to standard procedures [7], were carried out at 37°C, under air, for periods of 20 to 75 minutes. The reactions were stopped by addition of 0.2 ml 1N HCl and cooling.

Incubations using [^{14}C]Amo as radiolabel contained 25 µM [^{14}C]Amo, specific activity 1 Ci/mol, and were carried out with or without 1-5 mM UDP-glucose, 2 mM $MgCl_2$ or the NADPH generating system. Extraction from incubation mixtures was performed as for urine. Dry extracts were redissolved in 5-100 µl of dioxane and a fraction or all was spotted on pre-coated silica gel TLC plates, 0.25 mm (GF - 254, obtained from E. Merck/B.D.H., Toronto, Ontario). The plates were developed in two different solvent systems, system A: n-butyl chloride and dioxane, 1:1 (v/v), and system B: n-butanol and water, 93:7 (v/v), alone or combined. Half or one centimeter segments of silica gel were scraped from the plate, and the radioactivity measured by scintillation counting and expressed as percent of the total radioactivity on plate, i.e. of the mixture analyzed. Percentage radioactivity values associated with a TLC peak were converted into numbers of picomoles of metabolite formed.

Incubations using UDP-[^{3}H]glucose as radiolabel containing 5-20 µM UDP-[^{3}H]glucose, specific activity 13-25 Ci/mol, were carried out with or without 5 mM Amo. Extraction and TLC were performed as described above. Calculation of number of picomoles of metabolite formed was based on the absolute amount of radioactivity associated with a TLC peak.

Results and Discussion

Preliminary results obtained during *in vitro* testing of human livers with [^{14}C]Amo as a substrate revealed that, due to small yields of Amo-N-glu, the one-dimensional TLC used for *in vivo* studies were not adequate for either identification or quantitation (Table 1). As may be seen in Fig. 1, two-dimensional combination of solvent systems A and B benefited both. It permitted accurate comparison of the R_f value with that of authentic compound and separation of Amo-N-glu from all interferences listed in Table 1. The elimination of inter-

Table 1. Properties of [^{14}C]Amo-N-glu in One-dimensional TLC with Different Solvent Systems

Properties	System A*	System B*	A and B, sequentially†
R_f value	0 - 0.2 (amount-dependent)	0.55	-
Interferences			
polar materials‡	yes	no	no
metabolite "X"§	no	yes	yes
C-OH and/or Amo	no	yes	no

* System A: n-butyl chloride and dioxane, 1:1 (v/v); system B: n-butanol and water, 93:7 (v/v).

† Development first with solvent system A to 15 cm and, secondly with solvent system B to 4 cm.

‡ Unknown materials, see Fig. 1.

§ Non-identified metabolite, see Fig. 1.

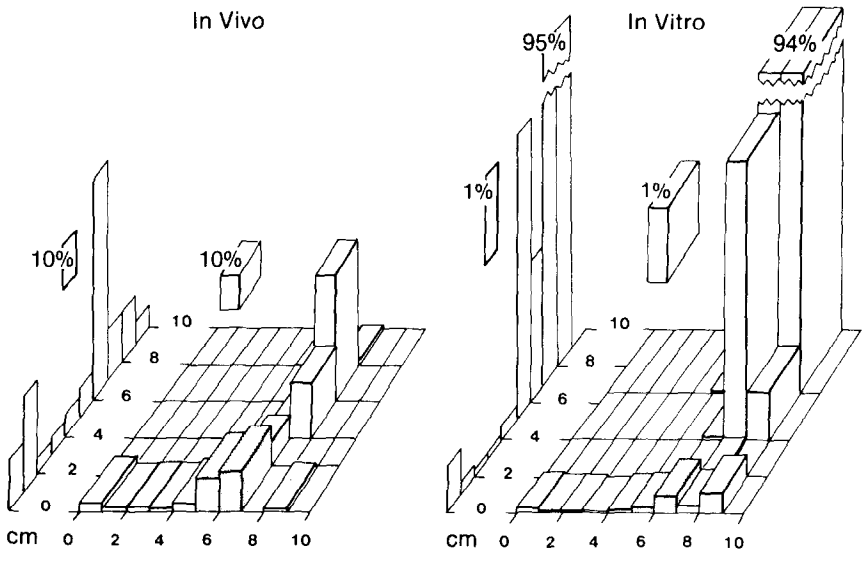

Fig. 1. Two-dimensional TLC separation of [^{14}C]Amo and metabolites: development first with solvent system A (A) to 15 cm (radiochromatograms shown on the left of each plate), and secondly with solvent system B (B) to 11 cm along a direction at right angle to the first. The location of individual radioactivity peaks was as follows: Amo: cm 9-10 (A), 8-9 (B); C-OH: cm 5-7 (A), 8-9 (B); metabolite "X": cm 3-4 (A), 6-7 (B); Amo-N-glu, shown as dotted solid: cm 0-2 (A), 6-7 (B); polar materials: cm 0-2 (A), 0-2 (B), and: 0-2 (A), 8-9 (B). In Vivo: human urine; In Vitro: 60 min incubation with 25 µM [^{14}C]Amo, K_3 human liver microsomes, 1 mM UDP-glucose, 2 mM $MgCl_2$, 0.4 mM NADP, 1 mM NADH, 4 mM G6P and 2 units G6PDH.

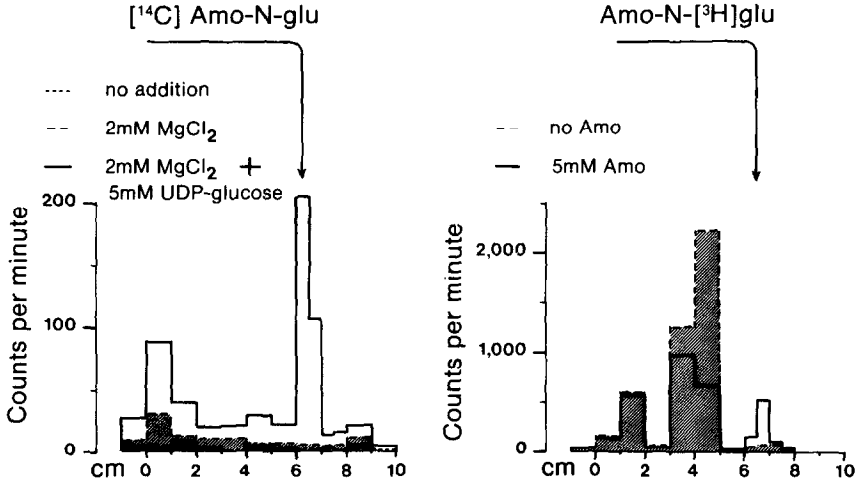

Fig. 2. UDP-Glucose-dependent formation of Amo-N-glu with human liver microsomes. Radiochromatograms were obtained by 2xD TLC and represent: cm 0-2.5 (A) and 0-10 (B) (see Fig. 1). Left panel: 65 min incubations with 25 µM [^{14}C]Amo and A19 liver. Right panel: 75 min incubations with 5 µM UDP-[^{3}H]glucose, 2 mM $MgCl_2$ and K4 liver.

Table 2. Rates of Amo-N-glu Formation with Human Liver Microsomes, as pmol/hr/g liver

Incubations with 25 μM [^{14}C]Amo			Incubations with 5 μM UDP-[^{3}H]glucose		
Liver	no $MgCl_2$ no UDP-glucose	2 mM $MgCl_2$ + 5 mM UDP-glucose	Liver	2 mM $MgCl_2$ no Amo	2 mM $MgCl_2$ + 5 mM Amo
A_{19}	7*	378*	A_{19}	4	12
A_{19}	32	532	K_4	3*	12*
K_4	18 ± 5†	425	K_4‡	11	45
K_3	12	284 ± 20†			
K_5	18	178 ± 25†			
Control (no liver)		19			

* Shown on Fig. 2.

† Average of duplicates within experiment.

‡ 20 μM UDP-[^{3}H]glucose.

ference from polar materials was of particular value since these were relatively more abundant *in vitro* than *in vivo* (Fig. 1) and varied depending on incubation conditions (Fig. 1 and Fig. 2, left panel). UDP-Glucose-dependent formation of Amo-N-glu by human liver microsomes was demonstrated with either [^{14}C]Amo or UDP-[^{3}H]glucose as radiolabel (Fig. 2). Reproducibility of the method is shown on Table 2.

In summary, in order to undertake *in vitro* studies of amobarbital-N-glucosidation, a sensitive and specific method was developed, based on 2xD TLC separation of amobarbital-N-glucoside from over 99.5% other material of lesser or greater polarity. Synthesis of amobarbital-N-glucoside and requirement for UDP-glucose were demonstrated with human liver microsomal preparations. This method will be used in further studies aimed at the optimization and characterization of the enzyme involved in this novel metabolic pathway.

Acknowledgements

We thank Mrs. T. Tam for expert technical assistance, the Bickell Foundation for financial support and Dr. D.S. Layne for helpful discussion. We are especially thankful to Dr. W. Kalow for his support throughout this research.

References

1. B.K. Tang, W. Kalow and A.A. Grey, Res. Comm. Chem. Pathol. Pharmac. 21, 45 (1978).
2. W. Kalow, L. Endrenyi, T. Inaba, D. Kadar and B.K. Tang, Adv. Pharmac. Ther. 6, 31 (1979).
3. B.K. Tang, W. Kalow and A.A. Grey, Drug Metab. Dispos. 7, 315 (1979).
4. D.E. Duggan, J.J. Baldwin, B.H. Arison and R.E. Rhodes, J. Pharmac. Exp. Ther. 190, 563 (1974).
5. D.S. Layne, R.S. Labow and D.G. Williamson, in Conjugation Reactions in Drug Biotransformation (ed. A. Aitio), p. 59, Elsevier North Holland (1978).
6. D.S. Layne, A.M. Comerton and D.B. Williamson, Glycoconjugate Res. 2, 723 (1979).
7. P.A.J. Reilly, Amobarbital Biotransformation in Different Species, Ph.D. thesis, University of Toronto (1978).
8. B.K. Tang, T. Inaba and W. Kalow, Drug Metab. Dispos. 3, 479 (1975).