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Synthesis and distribution of *N*-benzyloxycarbonyl-[^{14}C]-glycine, a lipophilic derivative of glycine.

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SUMMARY

N-benzyloxycarbonyl[^{14}C]-glycine, a lipophilic derivative of glycine exhibiting anticonvulsant properties, was prepared in one step from [U-^{14}C] glycine and benzyl chloroformate in alkali medium. A comparative study of biodistribution was carried on mice between this compound and the parent amino-acid after intravenous administration. Dimethylsulfoxide was used as injection vehicle for *N*-benzyloxycarbonylglycine. The influence of this injection vehicle was studied comparing glycine injected in a saline solution and glycine co-administered with dimethylsulfoxide. No significant difference was found between these two treatments. Compared to glycine, *N*-benzyloxycarbonylglycine reached quickly the central nervous system and exhibited an enhanced brain penetration index, 13-fold superior to the parent aminoacid value.

KEY-WORDS : glycine, ^{14}C, *N*-benzyloxycarbonylglycine

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INTRODUCTION

Although glycine (**1**) is now known as a neurotransmitter both in the inhibitory and excitatory processes (1-2), few pharmacological activities on whole animal have been reported. The very low octanol/water distribution coefficient prevents any transcellular diffusion across the blood-brain barrier and the absence of a small neutral aminoacid transporter at the luminal side of endothelial cells constituting the blood-brain barrier drastically reduces the cerebral penetration of this aminoacid. The most established pharmacological activities are the anticonvulsant effects i.e. in the strychnine, 3-mercaptopropionic acid induced-seizures and in audiogenic mice (3-8). These effects were observed after i.p. or oral administration of very high doses of glycine : 10-40 mmol/kg. Recently (9), we reported the improved anticonvulsant properties of *N*-benzyloxycarbonylglycine (**2**), a lipophilic derivative of glycine (Figure 1).

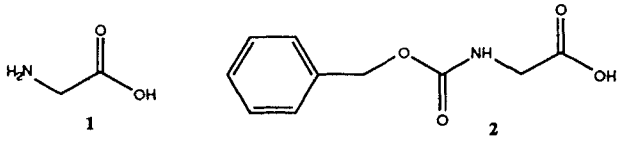


Figure 1. Structures of glycine (**1**) and *N*-benzyloxycarbonylglycine (**2**)

N-benzyloxycarbonylglycine shares and even improves the effects of glycine again the strychnine- and the 3-mercaptopropionic acid-induced seizures. Moreover, *N*-benzyloxycarbonylglycine is also active in the bicucculline and in the Maximal Electroshock Seizure tests. For instance, the ED_{50} in this last test is 0.27 (0.19-0.37) mmol/kg, 3 h after intraperitoneal administration (9).

The aim of this study was to synthesize the radiolabelled *N*-benzyloxycarbonylglycine in order to compare the *in vivo* distribution of *N*-benzyloxycarbonylglycine to that of glycine.

EXPERIMENTAL SECTION

Materials

Chemicals were obtained from commercial sources at the highest purity available. Thin layer chromatography (TLC) was carried on using silicagel 60F254 thin layer plates (Merck,0.25 mm). Spots were visualized under UV light, iodine vapors or ninhydrine treatment .

Radio-thin layer chromatography was carried out using a radioTLC scanner (Bioscan, Auto changer 300, system 200 Imaging Scanner) with similar silicagel plates.

Radioactivity was measured in a liquid scintillation spectrometer (Liquid scintillation counter-Pharmacia Wallac 1410). An automatic correction of quenching and chemoluminescence was included in the counting program.

Synthesis of N-benzyloxycarbonyl- [^{14}C]-glycine

0.5 g of unlabelled glycine (Janssen Chimica, p.a.) and 0.5 ml of [U-^{14}C]-glycine (Amersham, 106 mCi/mmol, aqueous solution containing 2 % of ethanol) were dissolved in 1 ml of 5M sodium hydroxide (Union Chimique Belge, p.a.) under vigorous stirring. The reaction mixture was placed in an ice-bath. A large excess of benzylchloroformiate (Aldrich-Chemie, 95 %) was added dropwise. After addition, the reaction mixture was stirred at 0-4°C for 30 min and then allowed for 1 h to come to room temperature. 2 ml of diethyl ether were added and the layers were separated by centrifugation. The ethereal phase was discarded and two additional extractions of the aqueous phase with 2 ml of diethyl ether were performed. The aqueous phase was cooled again in an ice-bath and acidified by 6M HCl until pH 1. Three extractions of 5 ml of diethyl ether and evaporation under reduced pressure of the organic phase allowed to obtain *N*-benzyloxycarbonyl- [^{14}C]-glycine. The obtained white product has similar TLC properties than the unlabelled product (Rf methanol/ acetone 1:1 = 0.64, ethyl acetate/n-butanol/acetic acid/water 2:1:1:1 = 0.85).

Distribution in mice

NMRI male mice (15-20 g) from the University facilities have been used. Each experiment was repeated three times and all radioactive countings were performed in triplicates.

Distribution of [U-14C]-glycine

0.4 mmol of glycine (Janssen Chimica) containing an amount of [U-14C]-glycine (Amersham, 3.93 GBq/mmol, 106 mCi/mmol) affording ~ 10^6 dpm /animal was intravenously administered in saline solution to mice via one of the tail veins. Two minutes before the required time (5 min, 30 min and 180 min) the mice were anesthetized with diethyl ether and sacrificed by cardiac puncture. Several organs and biological fluids were collected : brain, liver, kidneys, spleen, lungs, heart, tail (site of injection), bone, muscle, blood and urine. Three tissue aliquots were collected, carefully washed with a saline solution and then dissolved during 18 h at 40° C in 1 ml Soluene 350 ® (Camberra-Packard). Samples were discolored by addition of 1 ml hydrogen peroxide (UCB) by small portions (0.1-0.2 ml) and then diluted by 10 ml of scintillation liquid Hionic fluor ® (Camberra-Packard). Radioactive samples were counted 24 h after this addition. The radioactivity of the injected solution was measured on three aliquots and the volume injected was determined by weighing.

The results are expressed as circulating radioactivity i.e. the injected radioactivity minus the part remaining at the site of injection.

In order to study the influence of dimethylsulfoxide required for the administration of *N*-benzyloxycarbonylglycine, a co-administration of glycine in saline solution and dimethylsulfoxide was performed into two different tails veins, as glycine is not soluble in dimethylsulfoxide.

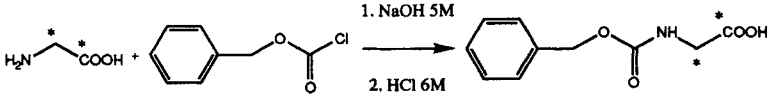
Distribution of N-benzyloxycarbonyl[U-14C]-glycine

The same procedure was followed to study the distribution of *N*-benzyloxycarbonylglycine. 0.4 mmol of *N*-benzyloxycarbonylglycine (Sigma)

containing sufficient *N*-benzyloxycarbonyl[^{14}C]-glycine to obtain 10^6 dpm/animal was administered intravenously in a dimethylsulfoxide solution (volume : 2 ml/kg).

RESULTS

The synthesis of *N*-benzyloxycarbonyl-[^{14}C]-glycine was performed under the same experimental procedures as the synthesis of unlabelled *N*-benzyloxycarbonylglycine, from glycine dissolved in sodium hydroxide solution to which benzyl chloroformate was added dropwise (Scheme 1).



Scheme 1. Synthesis of *N*-benzyloxycarbonylglycine

* indicated the labelled atoms

The radiochemical and chemical yields are presented in Table I.

Chemical yield	37 %
Chemical purity	> 95 %[a]
Radiochemical yield	98 %
Specific activity	0.343 MBq/mmol

Table I. Purity and yields of *N*-benzyloxycarbonyl-[^{14}C]-glycine

[a] determined by TLC analysis

The *in vivo* distribution of glycine and *N*-benzyloxycarbonylglycine was carried out at three times i.e. 5 min, 30 min and 3 h after intravenous administration to mice. 3 hours is the maximum of anticonvulsant activity of *N*-benzyloxycarbonylglycine (9).

In order to study the influence of dimethylsulfoxide, the solvent required for the administration of *N*-benzyloxycarbonylglycine, the distribution of

glycine was studied first in saline solution and then, in the presence of dimethylsulfoxide (2 ml/kg). The results are presented in Table II and are expressed as percentages of circulating radioactivity both per organ and per g of organ.

	% circulating radioactivity 5 min after iv administration		% circulating radioactivity 30 min after iv administration		% circulating radioactivity 180 min after iv administration	
	per org.	per g org.	per org.	per g org.	per org.	per g org.
a.Glycine						
Blood	3.58 ± 0.11	2.66 ± 0.24	1.67 ± 0.16	1.05 ± 0.01	1.22 ± 0.4	0.79 ± 0.3
Brain	0.19 ± 0.02	0.41 ± 0.04	0.30 ± 0.1	0.68 ± 0.18	0.23 ± 0.04	0.30 ± 0.03
Liver	13.5 ± 2.48	14.97 ± 3.9	9.77 ± 2.13	8.46 ± 2.41	5.12 ± 0.96	4.67 ± 0.87
Lung	1.22 ± 0.28	7.30 ± 0.39	1.32 ± 0.27	6.24 ± 0.49	0.45 ± 0.18	2.18 ± 0.87
Kidney	3.59 ± 0.2	14.8 ± 2.25	2.7 ± 0.56	8.52 ± 2.6	1.0 ± 0.21	2.49 ± 0.72
b.Glycine + DMSO						
Blood	2.46 ± 0.18	1.59 ± 0.07	2.06 ± 0.68	1.32 ± 0.37	2.31 ± 0.8	2.36 ± 0.36
Brain	0.18 ± 0.01	0.37 ± 0.05	0.27 ± 0.05	0.65 ± 0.12	0.32 ± 0.08	0.56 ± 0.7
Liver	12.11 ± 1.9	10.27 ± 1.8	11.22 ± 1.8	11.32 ± 1.2	5.26 ± 0.99	6.5 ± 4.42
Lung	1.09 ± 0.29	5.28 ± 1.21	5.93 ± 1.34	5.93 ± 1.34	0.48 ± 0.01	3.46 ± 0.26
Kidney	2.35 ± 0.82	6.18 ± 1.84	7.82 ± 3.14	7.82 ± 3.14	1.88 ± 0.44	5.85 ± 4.07
c.*N*-benzyloxy-carbonylglycine						
Blood	9.97 ± 1.67	5.76 ± 0.91	3.76 ± 0.86	2.65 ± 0.84	0.63 ± 0.18	0.44 ± 0.10
Brain	1.30 ± 0.18	3.21 ± 0.51	0.19 ± 0.04	0.53 ± 0.09	0.14 ± 0.04	0.27 ± 0.05
Brainstem	-	2.14 ± 0.46	-	0.56 ± 0.09	-	0.29 ± 0.05
Spinal cord	-	6.12 ± 1.71	-	1.35 ± 0.24	-	0.38 ± 0.01
Liver	9.8 ± 3.28	6.27 ± 2.04	1.49 ± 0.21	1.29 ± 0.16	0.36 ± 0.01	0.39 ± 0.01
Lung	0.36 ± 0.06	2.0 ± 0.35	0.12 ± 0.02	0.73 ± 0.09	0.03 ± 0.01	0.23 ± 0.03
Kidney	4.90 ± 0.45	11.77 ± 1.7	2.31 ± 0.1	8.47 ± 1.62	0.37 ± 0.09	1.47 ± 0.24

Table II : Distribution of radiolabelled glycine, administered alone (a), in co-administration with dimethylsulfoxide (b) and *N*-benzyloxycarbonylglycine (c). Results are expressed by the mean ± s.e.m. ($n \geq 3$).

As seizures originate from the central nervous system, our attention was focused on the crossing of the blood-brain barrier by glycine and *N*-benzyloxycarbonylglycine. The determination of the Brain Penetration Index (BPI) according to the method of Shashoua *et al.* (10-12) was carried out. The BPI is defined as the following ratio :

$$BPI = \frac{\text{radioactivity in the brain} \times 100}{\text{radioactivity in the liver}}$$

measured 5 min after the administration.

The liver was chosen as reference organ as no physiological barrier restricts the hepatic uptake. The Table III shows the BPI values of glycine alone, glycine in co-administration with dimethylsulfoxide and *N*-benzyloxycarbonylglycine.

Compounds	BPI
Glycine	1.40 ± 0.5
Glycine + dimethylsulfoxide	1.49 ± 0.3
N-benzyloxycarbonylglycine	19.7 ± 6.6[a,b]

Table III : Brain Penetration Index of glycine , glycine co-administered with dimethylsulfoxide and *N*-benzyloxycarbonylglycine.

The values are expressed by the mean ± s.e.m.

[a] Statistically different from glycine value ($p<0.05$, t test)

[b] Statistically different from glycine + dimethylsulfoxide value ($p < 0.05$, t test)

No significant difference was found between the BPI of glycine and the BPI of glycine + dimethylsulfoxide. However, the lipophilic derivative of glycine, *N*-benzyloxycarbonylglycine exhibited a significant increase of BPI, 13-fold superior to the parent aminoacid.

DISCUSSION

The synthesis of *N*-benzyloxycarbonyl-[^{14}C]-glycine was conducted in order to study the biodistribution of this lipophilic derivative of glycine. A comparison was thus made after intravenous administration to mice

between the distribution of glycine itself and *N*-benzyloxycarbonylglycine.

Glycine is a water soluble small neutral aminoacid, which does not significantly cross the blood-brain barrier (13,14). The brain uptake index (BUI) indicates the ability of a compound to penetrate readily the brain. The BUI value for glycine is very low : 2.5 (15) compared for example to a large neutral aminoacid like phenylalanine (BUI = 55). However, it was shown that the administration of very high doses of glycine (≥ 10 mmol/kg) led to a significant increase of glycine in the CNS (16-18).

In this study, a low cerebral penetration of glycine was indeed found after i.v. administration of 0.4 mmol/kg of glycine. Either when glycine is administrated in saline alone or co-administered with DMSO, the maximal value observed in the brain was only 0.32 % of circulating radioactivity (expressed per organ); while in the liver, values in the range of 11-13 % were measured. Intravenous administration of *N*-benzyloxycarbonylglycine gave an increase of measured radioactivity up to 1.30 %. This value was measured five minutes after intravenous administration. These results confronted with the pharmacological time-curve of *N*-benzyloxycarbonylglycine are compatible with a prodrug mechanism. On the one hand, the radiolabelled study indicates that *N*-benzyloxycarbonylglycine quickly reaches the brain. On the other hand, *N*-benzyloxycarbonylglycine presents a delayed anticonvulsant activity. In this regard, *N*-benzyloxycarbonylglycine might slowly release glycine as the active compound in the brain.

In the case of the co-administration of glycine and dimethylsulfoxide , only no significant differences were found compared to glycine administered in saline alone regarding the brain penetration of this amino-acid.

Conflictual reports exert about a possible influence of dimethylsulfoxide on the cerebral penetration of drugs. A cerebral increase was reported in the case of ketoconazole (19), pemoline (20) and L-aspartate (21). No influence was noted with barbiturics, dopamine (22) and thiorphan (23). An transient opening of the blood brain barrier was evidenced by injection of horseradish peroxydase (24). However, DMSO does not seem to alter morphological properties of endothelial cells constituting the BBB (24).

In conclusion, the synthesis and the distribution of *N*-benzyloxycarbonyl[^{14}C]glycine allows to provide new information about this compound. *N*-benzyloxycarbonylglycine quickly reaches the brain and more efficiently than the parent compound. Lipophilic derivatives of aminoacids such as *N*-benzyloxycarbonylglycine seem to be interesting candidates for an improvement of the crossing of the blood-brain barrier. In this precise case, the enhanced brain penetration of *N*-benzyloxycarbonylglycine could be attributed to its increased lipophilicity (25) susceptible to facilitate a diffusion process.

In addition, no significant influence of DMSO on the cerebral penetration of glycine was observed in the present study. No definitive conclusion can be drawn about DMSO as facilitating agent for CNS penetration.

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