SYNTHESIS OF ^{14}C-HYDROGENATED ANALOG OF PHENAZEPAM

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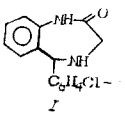
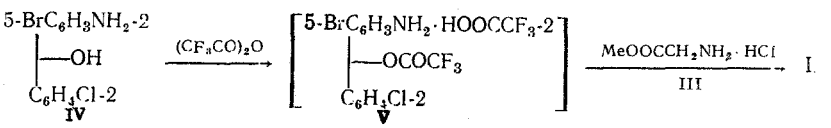
Among the vast group of tranquilizers of the 1,4-benzodiazepine series, their hydrogenated derivatives are especially unique. This is because of the characteristic spectrum of pharmacological activity, possibly explained by the absence of the azomethine bond [1] and unusual course of metabolism of these compounds [2, 4, 11]. The absence of data on pharmacokinetics, and also on the relation between the metabolism of these compounds and the pharmacodynamics let us synthesize a hydrogenated analog of phenazene, 5-o-chlorophenyl-7-bromo-1,3,4,5-tetrahydro-2H-1,4-benzodiazepin-2-one (I), labeled by ^{14}C at the 2-position of the heterocyclic ring. We also studied the optimal conditions for the extraction and quantitative determination of the compound from certain biological substrates.



I

To analyze the chemical purity of the intermediate products of the synthesis of [^{14}C]-I, we separated them radiochromatographically on Silufol UV-254 plates (CSSR). The disposition of the labeled compounds studied was controlled by comparing their R_f values and color in UV light (λ 254 nm) with similar parameters of nonradioactice samples. The radiometric determination of the total and specific radioactivity of the compounds, their chloroform extracts from biological media, and eluates from chromatographic plates, was carried out in a solution of a toluene–alcohol scintillator on the Beckman LS-100C photometer for liquids (US).

We synthesized compound [^{14}C]-I from aminobenzhydrol (IV) and glycine methyl ester hydrochloride (III) according to the following scheme:



In the reaction of IV with $(CF_3CO)_2O$ the intermediate product (V) is formed on cooling. The reaction of V with III proceeds in the presence of Et_3N in an organic solvent. After removal of the solvent, the residue is heated in an aqueous–methanolic solution in the presence of hydrochloric acid, and treated with a base to yield [^{14}C]-I.

There are paths of synthesis of compounds I for which compounds of type III, IV, and bromoacetic acid esters are used as starting materials [7-10]. However, the known methods are multistage, and when small amounts of these compounds are used, the synthesis of I is practically impossible.

[^{14}C]-Glycine (II) with a specific radioactivity of 3.38 mCi/mmole served as the starting material for the synthesis of labeled I. In the radiochromatographic analysis, carried out in the propanol–water (7:3) system 88.04 ± 0.91% of II could be detected out of the total amount of the compound studied at a degree of extraction from the plate of 75.92 ± 1.24% radioactivity (Fig. 1a).

The first stage of the synthesis was the preparation of [^{14}C]-III with a specific activity of 1.93 mCi/mmole. It was found that by using the radiochromatographic method, 72.27 ± 2.65% of III and 87.77 ± 1.94% of the sample out of the total amount deposited on the plate

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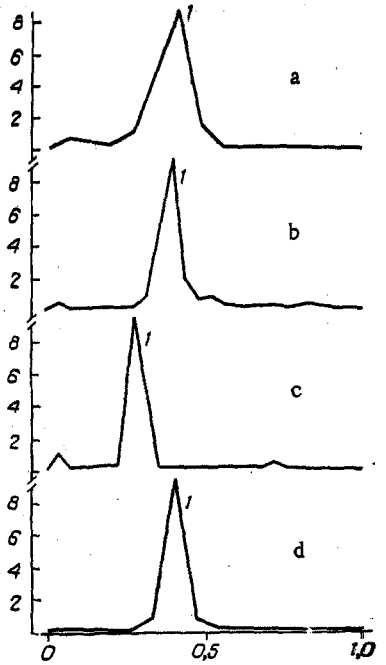

Fig. 1. Radiochromatograms of intermediate products in synthesis of [^{14}C]-I. a) Peak 1 — II (R_f 0.4); b) peak 1 — III (R_f 0.4); c) peak 1 — crude [^{14}C]-I (R_f 0.3); d) peak 1 — purified [^{14}C]-I (R_f 0.74). On abscissa — R_f; on ordinate — content of products (in counts•min^{-1}•10^{-3}).

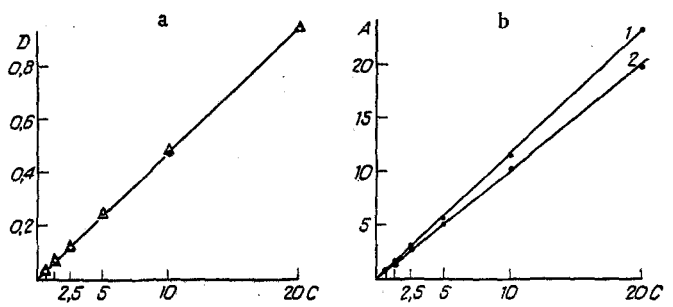

Fig. 2. Calibration graphs of dependence of optical density (a) at λ 257 nm of I (triangles) and [^{14}C]-I (circles) and recorded radioactivity (b) of [^{14}C]-I (1) and [^{14}C]-I extracted from RCG [radiochromatograph] (2), on concentration of solutions. On abscissa — concentration C (in μg/ml); on ordinate — optical density D; b) recorded radioactivity.

could be determined. The analysis of the purity of the compound by chromatography in a methyl ethyl ketone—pyridine—water—AcOH system (7.5:1.5:1.5:0.2) showed a high percentage (more than 96) of the inclusion of II into the synthesis (Fig. 1b).

The synthesis was finished with the production of crude [^{14}C]-I (76.54 ± 0.54% of the total radioactivity). Subsequent purification led to the separation of a white crystalline powder with a yellowish tinge that was insoluble in water, sparingly soluble in $CHCl_3$, ether, and ethanol, with a specific radioactivity of 1.38 mCi/mmole. The melting point and other physical properties of I and [^{14}C]-I are the same. By radiochromatography in a $CHCl_3$—hexane—acetone (1:1:0.5) system, 93.12 ± 54% of the compound studied, or 99.47 ± 2.11% of total amount of the labeled compound were determined (Fig. 1c, d).

The synthesized [^{14}C]-I was identified and its quantitative characteristics were determined by comparing the UV spectra (Specord UV Vis, SF-26 spectrophotometers) with similar

characteristics of nonradioactive I. It was found that solutions of weighed samples of I and [^{14}C]-I (0.32-20 μg/ml) in 95% ethanol have an average-wave absorption maximum at 247 nm (ε $1.7 \cdot 10^4$) and that at this concentration, the Lambert–Beer's law holds (Fig. 2a). Figure 2b shows a plot of the dependence of the total radioactivity of [^{14}C]-I on the concentration in the range of 0.32-20 μg/ml. Figures 2a and b show that there is a direct dependence between the concentration, the recorded radioactivity and the optical density of the [^{14}C]-I solutions.

EXPERIMENTAL CHEMISTRY

2-Amino-5-bromo-4'-chlorobenzhydrol (IV). A 0.37-g portion (0.01 mole) of $NaBH_4$ in 10 ml of water is added to a solution of 3.1 g (0.01 mole) of 2-amino-5-bromo-4'-chlorobenzophenone. The solution is stirred at 25-30°C for 1.5-2 h, diluted with water to separation of a precipitate, and filtered. The precipitate is washed with water to pH 7.0, dried, recrystallized from an acetone–hexane mixture (1:8) to yield 3 g (96%) of IV, mp 94-95°C.

Glycine Methyl Ester Hydrochloride (III). Glycine for the synthesis of III was obtained by mixing 0.018 g of II with 0.26 g of glycine.

A 0.278-g portion (0.0037 mole) of the prepared mixture of glycines is added to a solution of 0.86 ml (0.012 mole) of $SOCl_2$ in 10 ml of MeOH, and the mixture is stirred for 2 h at 20-22°C. The solution is evaporated, and the residue is washed with 10 ml of Et_2O to yield III, mp 173-174°C.

o-Chlorophenyl-7-bromo-1,3,4,5-tetrahydro-2H-1,4-benzodiazepin-2-one (I). A 0.53-ml portion (0.0038 mole) of $(CF_3CO)_2O$ is added to a solution of 0.94 g (0.003 mole) of IV in 15 ml of $CHCl_3$ at a temperature of from −18 to −20°C. The mixture is stirred for 15 min, and then, at −14 to −16°C, compound III in 10 ml of $CHCl_3$ and 3 ml of Et_3N are introduced. The temperature of the solution is raised to 20-22°C, the mixture is stirred for 2 h, and then boiled for 2.5 h. The solution is evaporated *in vacuo*, and to the residue 15 ml of Et_2O and 15 ml of 12% HCl are added. The ether layer is decanted, and to the residue 20 ml of MeOH are added. The mixture is heated on a water bath for 30 min, then cooled to 2-3°C, and 25% NH_3 is added to pH 7.8. The precipitate is separated and dried to yield 0.76 g of diazepinone I. Chromatographically homogeneous I is obtained by recrystallization from MeOH or an acetone–diethyl ether (1:3) mixture, mp 211-212°C. Found, %: C 51.32; H 3.36; N 8.00. M^+ 351. $C_{15}H_{12}BrClN_2O$. Calculated, %: C 51.24; H 3.44; N 7.96. M 351. IR spectrum, ν_{max}, cm^{-1}: 3420-3440, 3280 (NH), 1675 (C=O).

EXPERIMENTAL PHARMACOLOGY

To find the optimal methods for extraction and determination of [^{14}C]-I in biological media, we set up a series of model experiments.

A quantitative determination of the radioactive compounds studied and their metabolites is often based on their extraction from biological fluids, followed by chromatographic analysis [3, 5]. We introduced a solution of a weighed sample of [^{14}C]-I in 95% ethanol into a 0.2 Na-phosphate buffer at pH 3.0-11.0, followed by a threefold extraction by $CHCl_3$ (1:1). It was found that the most suitable condition for the quantitative extraction of [^{14}C]-I is pH in the range of 7.0-8.0 (degrees of extraction 98.43 ± 0.15%).

Since the degree of extraction of the compounds from biological substrates is often determined by the ability to bind them with proteins present in the probe [6], we used a 0.1% solution of bovine serum albumin in a Na-phosphate buffer (0.2 M, pH 7.0), into which an alcoholic solution of 0.5-4 μg/ml of [^{14}C]-I was added. The mixture was incubated for 30 min at 37°C, and then was extracted by $CHCl_3$ (1:1). The degree of extraction of [^{14}C]-I from probes containing albumin was in all cases less than that from probes without albumin, and a linear dependence was observed between the logarithms of the extracted [^{14}C]-I on the number of extractions, and also no dependence of the degree of extraction on the [^{14}C]-I concentration in the probe (Fig. 3a).

The determination of the degree of extraction of [^{14}C]-I from homogenates of various tissues of animals, and an occasional determination of the kinetics of this process are indispensable conditions for the quantitative detection of free metabolites of the compound in a biological probe. A solution of [^{14}C]-I in 95% ethanol was introduced into the corresponding media. After it had been incubated at 37°C for 30 min, the pH of the probe was adjusted to 8.0, and the mixture was successively extracted by four portions of $CHCl_3$ (1:1). Under these conditions, we were able to extract 84.59 ± 1.31% of [^{14}C]-I from the homogenates. The degree

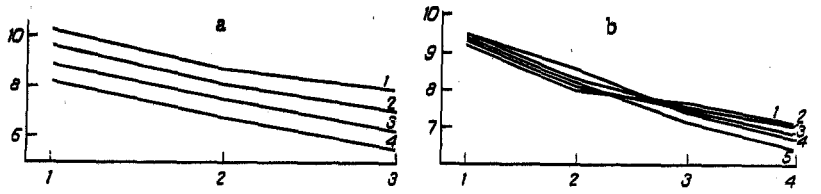

Fig. 3. Logarithmic dependence of extractability of ^{14}C-I on number of extractions during successive extractions from 0.1% solution of bovine serum albumin (a) and homogenates (b) of brain (1), liver (2), kidneys (3), muscular tissue (4), and blood plasma (5). For a) 1, 2, 3, 4 — concentration of ^{14}C-I: 20, 10, 5, 2.5 μg/ml, respectively. On abscissa — number of extraction; on ordinate — concentration.

TABLE 1. Kinetics of Extraction of [^{14}C]-I and Its Metabolites (total amount) from Brain of Mice by Successive Extractions

No. of extraction	Time, min							
	5		10		15		30	
	C, counts/min	[eff]	C, counts/min	[eff]	C, counts/min	[eff]	C, counts/min	[eff]
1	1301	1,01	4874	0,82	9772	0,87	14 145	0,86
2	458	0,99	2090	0,89	3544	0,87	10 105	0,85
3	138	0,97	484	0,92	528	0,80	3 773	0,87
4	83	0,96	176	0,90	149	0,92	794	0,88
5	60	1,07	...	...	140	0,91	282	0,91
Metrological characteristics								
a	8,568		9,856		10,630		10,715	
B	1,454		1,137		1,262		0,806	
$n_{0,95}$	2,05		2,63		2,37		3,72	

Note. [eff]) counter-efficiency of samples, a) calculated values of logarithms of amount of [^{14}C]-I extracted.

and the kinetic parameters of the extraction are independent of the nature of the biological substrate. There is always a linear dependence of the logarithms of the [^{14}C]-I content in the successive chloroform extracts of the homogenates on the number of extraction (the process is monoexponential (Fig. 3b).

Since the model experiments cannot give a complete idea on the metrological characteristics of the methods used, we studied the kinetics of extraction of [^{14}C]-I and its metabolites (total amount) from the brain of mice. We introduced [^{14}C]-I in a dose of 7 mg/kg (20 μmole/kg) intraperitoneally in a Tween emulsion to male SVA mice. The animals were decapitated after 5, 10, 15, 20 and 30 min of the experiment, the brain was removed, weighed, ground with dry Na_2SO_4 to a dry powder, and was successively (five times) extracted by 7 ml portions of $CHCl_3$. The extracts were placed in measuring flasks and evaporated in a vacuum exsiccator. The residue was dissolved in 7 ml of a toluene–alcoholic scintillator, and the content of ^{14}C-products was determined.

Table 1 shows that the extraction of ^{14}C-products from the brain of mice is a monoexponential process, and the calculated value of the parameters studied is commensurable with the experimental data. We can use the calculated data to determine the degree of extraction of the compounds studied (n) by successive extractions with a given volume of the extractant and the number of extractions required to reach the given degree of extraction (95%).

$$n = \frac{\ln\left\{\frac{\sum[S_\infty]-\sum[S_n]}{\sum[S_\infty]}\right\}}{B},$$

$$\sum[S_n] = (1-e^{-nB})\cdot\sum[S_\infty],$$

where $\Sigma[S_n]$ is the sum of the extracted ^{14}C material during n successive extractions, B is a constant.

A combination of methods of extraction from biological substrates, chromatographic separation, and identification can be thus used for studying the metabolism of [^{14}C]-I.

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HIGHLY PURIFIED CHORIONIC GONADOTROPIN: BIOLOGICAL, IMMUNOLOGICAL, AND PHYSICOCHEMICAL PROPERTIES

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Highly purified chorionic gonadotropin (CG) with specific activity no less than 10,000 units/mg is now finding wide use in the creation of diagnostic agents used in immunological and radioimmunological investigations of the hormone level in human blood in the normal state and in gynecological diseases. The medicinal preparation with activity 3000-4000 units/mg is used in the treatment of infertility in men and women. However, a preparation of this degree of purification cannot be used for fine biological and biochemical investigations. In view of this, we undertook the development of a method of isolating highly purified CG.

A whole complex of modern biochemical methods of isolation and purification is used for the production of pure hormones. As a rule, at the concluding stages of the isolation of highly purified CG a combination of methods of ion exchange chromatography and gel filtration is used [4, 6, 8]. These methods do not always permit the production of a homogeneous preparation of CG that could be used in immunochemistry for the production of monovalent antisera. Such a preparation forms more than one fraction in electrophoresis in polyacrylamide gel (PAAG); they are separated according to electrophoretic mobility and biological activity [5, 9]. Isoelectric focusing (IEF) is a method with high resolving power for the separation of proteins with a high value not only of the molecular weight, but also of the isoelectric point (pI) [2, 3, 7].

In this work we used the method of IEF in conjunction with gel filtration of the fraction containing CG on domestic dextran gel ED-10 for the isolation of highly purified CG. The amino acid composition, physicochemical and biological properites of pure CG were studied and refined. A monovalent antiserum was produced on the basis of highly purified CG. The proposed scheme for obtaining a pure hormone can be used in semiindustrial manufacture.

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