Biochemistry of Metalocenes. The Organ Distribution of Hydroxyacetyl [^{103}Ru]ruthenocene and Its Glucuronide in Mice

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Hydroxyacetyl [^{103}Ru]ruthenocene and its o-glucuronide were prepared in vitro by incubation of acetyl [^{103}Ru]ruthenocene with rat-liver homogenate, NADPH, and UDP-glucuronate. The factors affecting hydroxylation and glucuronidation in vitro were optimized for acetylruthenocene. Hydroxyacetyl [^{103}Ru]ruthenocene glucuronide showed no affinity for the adrenal glands, but after i.v. administration of hydroxyacetyl [^{103}Ru]ruthenocene there was a distinct accumulation of Ru-103 in adrenals, similar to that found after administration of acetyl [^{103}Ru]ruthenocene.

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One of the main difficulties in developing radiopharmaceuticals is to find a suitable combination of a gamma emitter (normally a metal) and an organic moiety that is capable of variation. Metal chelates have been used with success, but the metalocene group of compounds seems to have more potential. Metalocenes consist of a central metal atom covalently bound to two cyclopentadiene rings, which have an aromatic character due to the metal binding. Several metals form metalocenes (iron, ruthenium, and osmium) and gamma emitters such as Fe-59, Ru-97, or Ru-103 can be used.

The chemical nature of the metalocenes can be varied simply, using standard aromatic chemistry. Further, the metalocene derivatives show exceptional chemical stability under the conditions encountered in biological systems.

Previous work has shown that ruthenocene ($C_5H_5RuC_5H_5$) has some derivatives with affinity for specific organs. The estradiol and estrone esters of ruthenocene carboxylic acid are adrenal-seekers (*1*), cinnamoyl ruthenocene accumulates in thymus (*2*), and ruthenocene carboxylic acid shows an affinity for kidneys (*3*).

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Following the observation that acetyl [^{103}Ru]ruthenocene also had an affinity for adrenal glands (*4*), the metabolism of acetylruthenocene was shown to proceed by preliminary hydroxylation of the acetyl methyl group to an alcohol that was then conjugated with glucuronic acid (*5*). Experiments were then performed to determine whether the metabolites of acetylruthenocene could produce a better accumulation of Ru-103 in adrenal glands than acetylruthenocene itself.

The metabolites of acetylruthenocene were prepared by incubating acetyl [^{103}Ru]ruthenocene with a crude liver homogenate fraction (10,000 *g* centrifugation), and some of the parameters that contribute to the efficiency of hydroxylation and conjugation in vitro (substrate and cofactor concentration, barbiturate pretreatment) were optimized for the metabolism of acetylruthenocene.

MATERIALS AND METHODS

Chromatography. Samples were applied to Kieselgel 60 plates* as streaks and were developed either in benzene/ethylacetate (5:1 v/v, = Solvent A) or butan-1-ol/acetic acid/water (4:1:1 v/v/v, = Solvent B). Solvents were freshly prepared.

Detection and determination of Ru-103. The radioactive content of samples was measured either in a NaI well counter with a counting efficiency of 26.5% for

Ru-103, or in an automatic gamma counter with an efficiency of 24.5% for Ru-103. Ru-103-labeled compounds on chromatograms were located and determined by measuring the β^- and Auger electrons of Ru-103 (*6*) with a thin-layer scanner.**

Chemicals. NADPH, UDP-glucuronate,† acetylferrocene,‡ $^{103}RuCl_3$,‖ and SKF 525A (β-diethyl-aminoethyldiphenylpropylacetate§) were obtained commercially.

Experimental animals. Male and female Wistar rats (250-300 g) and female CF-1 mice (20-25 g) were used.

Preparation of acetyl [^{103}Ru]ruthenocene. Acetylferrocene (0.6-1.2 mg), aluminum oxide (10-15 mg; neutral), and $^{103}RuCl_3$ (30-100 μCi) were heated at 180°C in sealed, evacuated ampoules for 30 min (*7*). Reaction products were dissolved in ether and separated on thin-layer plates in Solvent A. Radioactive material corresponding to markers of acetylferrocene was scraped off the plate and eluted with ether. The radiochemical purity of the eluted material was tested by further chromatography in Solvent A.

Incubations. The principles and procedures described by Mazel (*8*) were used as guidelines.

Substrate. The desired amount of acetyl [^{103}Ru]ruthenocene (maximum 2 μmol) was added as an ethereal solution to a 20-ml vial containing 50 μl of 1,2-propyleneglycol. Ether was removed under nitrogen to leave the acetylruthenocene in solution in propyleneglycol.

Homogenate. Male and female rats were injected intraperitoneally with a single daily dose of sodium phenobarbital, 80 mg/kg, on 3 consecutive days. On the fourth day they received no phenobarbital, and on the fifth day they were killed and the livers removed. A homogenate was prepared in 1.15% aqueous KCl (KCl/liver = 3:1 w/w) using a Potter homogenizer, and cell debris was removed by centrifuging at 10,000 *g* for 10 min at 4°C.

Content of incubates. To the propyleneglycol solution 50 μl of acetyl [^{103}Ru]ruthenocene were added the cofactor solutions (NADPH 50 μl ≡ 1.3 μmol; UDP-glucuronate 100 μl ≡ 1.0 μmol), 0.1 *M* phosphate buffer 800 μl (pH 7.4), and finally 1.0 ml of homogenate. Each vial was thoroughly mixed and then incubated for 30 min at 37°C.

Separation of metabolites from the incubation mixture. Each sample was extracted twice with 3 ml ether and the amounts of radioactivity in the ether and aqueous phases were determined. The ether phases were concentrated, applied to thin-layer plates, and developed in Solvent A to separate hydroxyacetylruthenocene (R_f = 0.26) from unchanged acetylruthenocene (R_f = 0.47). The plates were scanned and zones corresponding to hydroxyacetylruthenocene were scraped off and eluted with ether. The ether extracts were stored at −20°C until required. Analysis of the extracts by thin-layer chromatography showed that no decomposition took place over the 10-day storage period, since the R_f values remained the same and no inorganic Ru-103 (a typical sign of metalocene breakdown) was detectable. The extracted aqueous phase was diluted to 15 ml with water and applied to a column of Amberlite XAD-2¶ (*9*). After washing the column with water (20 ml), methanol (40 ml) eluted the bulk of the radioactivity. The methanol eluate was concentrated in vacuo and the residue taken up in 25% aqueous methanol and applied to thin-layer plates, which were developed in Solvent B. After scanning, zones corresponding to hydroxyacetylruthenocene glucuronide (R_f = 0.44) were scraped off and eluted with the aqueous methanol mixture. The glucuronide was stored in aqueous methanol at −20°C until required, and was still pure 14 days after synthesis.

Characterization of metabolites prepared in vitro. Samples of hydroxyacetylruthenocene separated from the incubation mixture were mixed with authentic samples and co-chromatographed in Solvent A. Thin-layer chromatography of hydroxyacetylruthenocene glucuronide with authentic samples in Solvent B was supplemented by hydrolysis of the authentic and test samples with β-glucuronidase and chromatography of the aglycones in Solvent A. In all cases, the metabolites produced in vitro were identical with those produced in vivo. The characterization of the compounds by mass spectrometry and NMR has already been described, as has the hydrolysis of the glucuronide with β-glucuronidase (*5*).

Preparation of solutions suitable for injection. The ethereal solution of hydroxyacetyl [^{103}Ru]ruthenocene was first filtered through a Millipore filter (0.2 μ) to remove any remaining Kieselgel, and ether was then removed under nitrogen in the presence of the required amount of propyleneglycol (50 μl/mouse). A solution of hydroxyacetyl [^{103}Ru]ruthenocene glucuronide was prepared by removing the methanol/water solvent in vacuo and dissolving the residue in the appropriate amount of physiological saline, which was filtered through a Millipore filter (0.2 μ) before injection.

Administration to animals. Groups of seven female CF-1 mice received the compounds orally (by stomach intubation), intraperitoneally, or intravenously (tail vein). Whole-body activity was measured in the well of the NaI well counter. At the appropriate time, the mice were killed by dislocation of the neck, and organs were removed, blotted free of blood, weighed, and counted in the automatic gamma counter. The amount of radioactivity in each organ was calculated as percentage of the dose per gram organ. The tissues and organs studied were: muscle, blood, thymus, heart, lungs, liver, spleen, kidneys, adrenals, stomach, and the intestinal tract. In the case of i.v. administration, the tail was also removed and the amount of radioactivity remaining at the site of injection was determined.

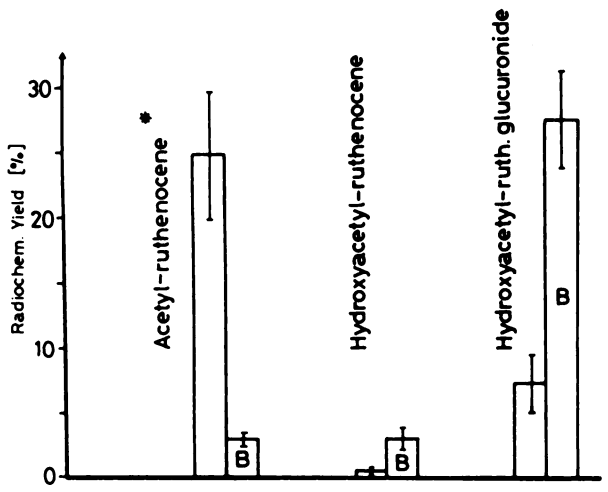

FIG. 1. Effect of phenobarbital pretreatment on the in vitro metabolism of acetylruthenocene in rat (♀) liver homogenate procedure (see Materials and Methods). Means of five experiments ± s.d.; 100% ≙ initial radioactivity of acetyl [^{103}Ru]ruthenocene ☐ control experiment; [B] Pretreatment, with phenobarbital; * Recovery of acetyl ruthenocene after incubation.

RESULTS

Optimization of metabolite synthesis in vitro. Experiments with liver homogenates from untreated and phenobarbital-treated rats showed that the homogenate from treated rats hydroxylated and conjugated appreciably more acetylruthenocene in the 30-min incubation period than homogenate from untreated rats (Fig. 1). The use of a 100,000-*g* microsome fraction from either group proved no advantage. Substrate concentration was optimal at 2 μmol acetylruthenocene per 250 mg liver (equivalent to 1 ml homogenate) and the optimal time was found to be 30 min. Longer incubations lead to partial breakdown of the product. With 2 μmol of acetylruthenocene, 1.3 μmol NADPH, and 1 μmol UDP-glucuronate per 1 ml homogenate, only 3% of unchanged acetylruthenocene was found and the yield of hydroxyacetylruthenocene glucuronide was 20–30% of the start activity.

It has been suggested (*11*) that addition of UDP-glucuronate in five to six times excess is necessary to obtain maximum yields of glucuronides, but such excesses did not appreciably increase (+ 5%) yields of the metalocene glucuronide. The various losses during extraction of the glucuronide can be summarized as follows. Start 100%, ether extract 5%, aqueous phase 87%, approximately 35% is either washed through the XAD-2 column or remains bound to it, 52% of the start activity is obtained in the methanol eluate, and after TLC purification 32% remains (typical values).

Organ distribution of Ru-103. Only those tissues and organs with appreciable accumulation of Ru-103, or those that are important in determining the contrast ratio, are included in the Results.

Hydroxyacetyl [^{103}Ru]ruthenocene glucuronide. Administration of this compound to mice by intraperitoneal, oral, or intravenous routes produced no accumulation of Ru-103 label in adrenal glands. The organs with the greatest concentration of Ru-103 were the kidneys and the liver, in that order, and, 24 hr after injection, 89–95%

TABLE 1. CONCENTRATION OF Ru-103 IN ORGANS FOLLOWING i.v. ADMINISTRATION OF ACETYL [^{103}Ru]RUTHENOCENE AND ITS METABOLITES

Route (n)*	Dose (μmol/kg)	Time (hr)	Excretion (% dose)	Ru-103 content (% dose/g) Muscle†	Blood	Liver	Kidneys	Adrenals†	Ratio Adrenal/muscle
				Acetyl-ruthenocene					
i.v. (6)	0.1	24	88	0.045 ± 0.014	0.091	5.6	2.0	8.98 ± 1.7	200
				Hydroxyacetyl-ruthenocene glucuronide					
i.v. (6)	5 8	24	89	0.076 ± 0.015	0.46	1.7	4.1	0.62 ± 0.28	8
i.p. (5)	6.9	24	87	0.16 ± 0.04	0.55	1.8	5.2	0.44 ± 0.29	3
oral (6)	5.0	24	99	0.008 ± 0.004	0.012	1.9	2.0	0.16 ± 0.20	20
				Hydroxyacetyl-ruthenocene					
i.v. (7)	0.8	6	80	0.074 ± 0.014	0.23	5.6	4.6	9.04 ± 3.1	122
i.v. (7)	0.8	24	91	0.057 ± 0.011	0.12	2.7	3.3	9.51 ± 2.6	166
i.v. (7)	0.8	48	93	0.026 ± 0.004	0.03	3.5	2.1	5.73 ± 1.6	220
i.v. (7)	2.4	72	96	0.021 ± 0.006	0.03	2.0	1.3	6.82 ± 1.6	336
i.p. (5)	0.8	24	87	0.025 ± 0.016	0.12	2.2	3.0	1.35 ± 1.2	54
oral (6)	8.4	24	94	0.020 ± 0.011	0.03	3.4	1.8	0.54 ± 0.4	27

* n = number of mice (♀).
† $\bar{x}$ ± s.d.

of label had been excreted (Table 1).

Hydroxyacetyl [^{103}Ru]ruthenocene. The organ distributions observed after administration of this compound are represented as percentage dose per gram tissue (Table 1). Oral and intraperitoneal administration produced accumulation of Ru-103 in liver and kidneys, but not in adrenals. After i.v. administration of this agent, however, Ru-103 concentrations similar to those seen after administration of acetylruthenocene (*4*) were obtained. The organ distribution was studied at 6, 24, 48, and 72 hr to determine whether better adrenal-to-kidneys and adrenal-to-liver ratios could be achieved with time. The 72-hr values are the best, namely, adrenal-to-liver = 3.4; adrenal-to-kidney = 5.2; and adrenal-to-muscle = 336. It has already been established that acetylruthenocene is hydroxylated in vivo to hydroxyacetylruthenocene, which is then conjugated with glucuronic acid (*5*). The glucuronide of hydroxyacetyl [^{103}Ru]ruthenocene does not accumulate in adrenals following any route of administration (Table 1). Consequently, after injection of hydroxyacetylruthenocene, the part that becomes glucuronidated will not be accumulated in adrenals.

Attempts to improve the accumulation of hydroxyacetylruthenocene in adrenals by administering an inhibitor of glucuronidation (SKF 525A) were not successful, although no experiments were performed to prove that SKF 525A did indeed inhibit the glucuronidation of hydroxyacetylruthenocene.

DISCUSSION

The administration of acetylruthenocene metabolites did not improve the adrenal accumulation of Ru-103 compared with the values obtained with acetylruthenocene. When compared with the best iodine-labeled cholesterol radiopharmaceuticals (*12*) both acetyl- and hydroxyacetylruthenocene have lower affinities for adrenals and worse adrenal-to-liver or adrenal-to-kidney ratios. They do have the advantage, however, that the Ru-103 label remains bound in the metallocene sandwich structure and does not become hydrolyzed and accumulated in a nontarget organ (thyroid) as is the case with radioactive iodine.

Comparing the metabolism of acetylruthenocene with that of the acetylbenzene analog, acetophenone, the degradation of acetylruthenocene in vivo proceeds by a pathway that plays only a minor role in acetophenone metabolism (*5*). The metabolism of the two compounds is therefore dissimilar. Acetylruthenocene metabolism can better be compared with the interconversions of a steroid ring system with an acetyl side-chain, progesterone. This steroid is converted by a 21 β-hydroxylase (in adrenals) to 11-desoxycorticosterone, which has the hydroxyacetyl side-chain. Furthermore, the hormonal properties of 11-desoxycorticosterone are inactivated by glucuronidation of the C-21 hydroxy group (13). The metabolism of acetylruthenocene therefore more closely resembles that of the acetyl steroid ring analog, progesterone, than that of the acetyl benzene-ring analog, acetophenone.

FOOTNOTES

* Merck, Darmstadt, Germany.
† Boehringer, Mannheim, Germany.
‡ Alfa Europe Products, Rotterdam, The Netherlands.
‖ The Radiochemical Centre, Amersham, England.
§ Smith, Kline & French Laboratories Ltd., Welwyn Garden City, Herts, England.
¶ Serva, Heidelberg, Germany.
** Berthold, Wildbad, FRG.

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