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High-performance liquid chromatography study of stereospecific microsomal enzymes catalysing the reduction of a potential cytostatic drug, oracin Interspecies comparison

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Abstract

One of the main metabolites of oracin (**I**) {6-[2-(2-hydroxyethyl)aminoethyl]-5,11-dioxo-5,6-dihydro-11H-indeno[1,2-*c*]isoquinoline}, a potential cytostatic drug, is 11-dihydrooracin (**II**) {(+),(−)-6-[2-(2-hydroxyethyl)aminoethyl]-5-oxo-11-hydroxy-5,6-dihydro-11H-indeno[1,2-*c*]isoquinoline}, a metabolite formed by the reduction of oracin's pro-chiral centre on C 11. This metabolite has been found in all laboratory species in vitro and in vivo and it constitutes the main metabolite in man. The stereospecificity of reducing enzymes participating in the oracin biotransformation pathway was investigated using microsomal preparations from standard laboratory animals. Enzyme stereospecificity has been defined as preferential formation by the enzyme of the (+) or (−) stereoisomer of **II**. Significant interspecies differences were observed in the stereospecificity of the respective biotransformation enzymes. HPLC quantitative determinations of both enantiomers were performed using a Chiralcel OD-R column as chiral stationary phase with excellent resolution and stability.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Enzymes; Oracin

1. Introduction

Both oracin {6-[2-(2-hydroxyethyl)aminoethyl]-5, 11-dioxo-5, 6-dihydro-11H-indeno[1, 2-*c*]isoquinoline} (**I**) [1] (Fig. 1) and its hydrochloride display direct cytotoxic effects on Yoshida, Ehrlich and Gardner tumor cells in vitro.

As an inhibitor, it acts more potently on DNA biosynthesis rather than on proteosynthesis in tumor cells [2]. From a chemical viewpoint, **I** remotely resembles mitoxantron. Its basic planar structure with intercalation activity, however, differs from all cytostatics used in clinical medicine.

Studies on the metabolism of the potential cytostatic drug, oracin, have shown that a major metabo-

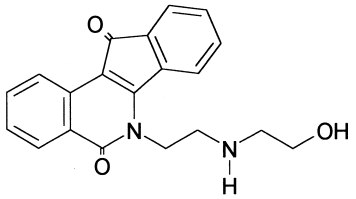

Fig. 1. Chemical structure of oracin (**I**).

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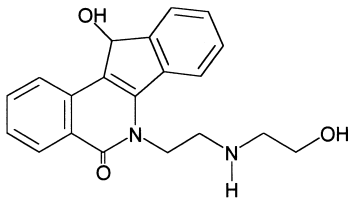

Fig. 2. Chemical structure of 11-dihydrooracin (**II**).

lite of oracin is 11-dihydrooracin {6-[2-(2-hydroxyethyl)aminoethyl]-5-oxo-11-hydroxy-5,6-dihydro-11H-indeno[1,2-*c*]isoquinoline} (**II**) (Fig. 2) which also displays biological activity. The studies were performed in vitro [3] and in vivo [4] using standard experimental animals. Metabolites were assayed for employing non-chiral high-performance liquid chromatography (HPLC). Metabolite **II** has also been identified as the major metabolite in urine and plasma of man.

Metabolite **II** is formed by reduction of the pro-chiral centre of the oxo group of **I**. Biotransformation may thus result in two stereoisomer forms of **II**, which can only be separated with the use of a chiral selector [5]. The chiral stationary phase (CSP) approach proved to be the method of choice for our purposes, mainly because of good reproducibility of results with biological samples. Several CSP types were tested and the best results achieved with Chiralcel OD-R column, with a cellulose derivative as the chiral selector.

Enzymes display two distinct properties in relation to chiral substrates. One of them is stereoselectivity, i.e., the enzyme may prefer one stereoisomer form of its substrate for subsequent biotransformation. The other one is stereospecificity, when the enzyme preferentially generates only one biotransformation product. The situation is simpler in the case of pro-chiral substrates, where we just refer to the stereospecificity of the biotransformation enzymes, as in this case such a substrate has no stereoisomers [6]. The stereospecificity of microsomal reductases was determined on the basis of quantitative determination of the stereoisomers of **II**. Formation of the respective enantiomer of **II** depends on the enzyme pattern of individual animal species. The absolute configuration of individual stereoisomers of **II** has not yet been determined, thus they are referred to simply as (+) and (−) isomers in methanol and in the mobile phase described below.

2. Experimental

2.1. Animals and biological materials

Male Wistar rats (*Rattus norvegicus* var. alba) (180–200 g), mice (*Mus musculus* var. alba) (20–25 g), guinea pigs (*Cavia aperea* var. porcellus) (450–500 g) and rabbits (*Oryctolagus cuniculus* var. chinchilla) (about 3 kg) were obtained from Velaz (Prague, Czech Republic). They were fed with a standard diet, fasted 12 h prior to the experiment, and sacrificed by decapitation under ether anaesthesia to obtain the respective tissues. Dogs (*Canis familiaris* var. Beagle) and mini-pigs (*Sus scrofa* var. alba Götingen mini-pig) were obtained from the breeding station of The Military Research Institute (Hradec Králové, Czech Republic). The animals were sacrificed by bleeding to death under total anaesthesia, their liver removed and used to prepare liver homogenates.

The liver homogenate was prepared in the ratio of 1:3 (w/v) in a 0.1 *M* sodium phosphate buffer pH 7.4 in the Potter and Elvehjem homogenizer. The microsomal fraction was obtained by fractional ultracentrifugation of the liver homogenate in 0.1 *M* sodium phosphate buffer (pH 7.4) [7]. The microsomes were resuspended in the same buffer and stored at −24°C.

2.2. Incubation and extraction

Standard incubation mixtures were prepared in Eppendorf microtubes. Microsomal suspension (0.1 ml in 0.1 *M* sodium phosphate buffer pH 7.4, i.e., an aliquot corresponding to 0.1 g of the original wet liver tissue) was incubated with 0.4 μmol of substrate and 0.6 μmol of coenzyme NADPH in a total buffer volume of 0.3 ml. Incubation at 37°C under aeration was carried out for 30 min.

All incubations were terminated by adding 5% aqueous ammonia solution to pH 10.8–11.0 and extracted three times with double their volume of distilled ethyl acetate. The combined extracts were evaporated to dryness under vacuum. The dry sam-

ples were dissolved in methanol prior to their HPLC injection.

2.3. Chemicals and reagents

Oracin, *rac*-11-dihydrooracin and its pure enantiomers were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic) and from the Research Institute for Organic Syntheses (Pardubice, Czech Republic). Coenzyme NADPH was purchased from Boehringer Mannheim (Prague, Czech Republic). Acetonitrile (MeCN) and methanol (both HPLC grade) were obtained from Merck (Prague, Czech Republic) and sodium perchlorate monohydrate and perchloric acid (''puriss. p.a.'' grade) from Fluka (Prague, Czech Republic). All other chemicals were of the highest purity commercially available.

2.4. High-performance liquid chromatography

Liquid chromatography was carried out using a Spectra Series P200 gradient pump from Spectra-Physics (Fremont, CA, USA), a HP 1100 Series autosampler and a HP 1100 Series thermostatted column compartment from Hewlett-Packard (Waldbronn, Germany) and a multichannel UV–Vis detection system PU4021 from Pye Unicam (Cambridge, UK). Enantiomers of reduced oracin were detected using a detection wavelength of 340 nm. The fluorescence detection system was a FS 970 (Spectra-Physics) with excitation wavelength 340 nm and emission wavelength 418 nm. All experiments were carried out at 25°C. Data from chromatographic runs were processed using a chromatography station for Windows CSW (version 1.6) software from DataApex (Prague, Czech Republic) on a 486/66 PC from AutoCont (Hradec Králové, Czech Republic). Most work was performed using a 25×0.46 cm I.D. Chiralcel OD-R column (Daicel, Tokyo, Japan). Preliminary tests were also made using a 25×0.46 cm I.D. column packed with Cyclobond I, Cyclobond I acetylated, Cyclobond I RSP, Cyclobond II, Cyclobond III and Cyclobond III acetylated columns (Astec, Advanced Separation Technologies, Whippany, NJ, USA), a 15×0.4 cm I.D. Chiral-AGP column (ChromTech, Norsborg, Sweden) and a 15×0.46 cm I.D. Ultron ES-OVM column (Shinwa, Kyoto, Japan).

3. Results and discussion

3.1. Selection of a suitable CSP

Several types of chiral columns were tested to find the one with optimum interactions between the stationary phase and compounds studied.

Cyclobond I (β-cyclodextrin bonded), Cyclobond II (γ-cyclodextrin bonded) and Cyclobond III (α-cyclodextrin bonded) have 7, 8 and 6 glucopyranose units, respectively, which are arranged in the shape of a hollow truncated cone. These Cyclobond materials have been used as a basis for making several derivatives. In this study, six types of cyclodextrins were used: Cyclobond I, Cyclobond I Acetylated, Cyclobond II, Cyclobond III, Cyclobond III Acetylated and Cyclobond I RSP. None of the stationary cyclodextrin phases under test proved suitable for the separation of the enantiomers. Molecules of the analytes do not seem to fit into the glucose cone or to differ in their fit.

Additional types of CSP were tested: immobilized proteins, namely immobilized α_1-acid glycoprotein (orosomucoid) designated as Chiral-AGP column and a more recent immobilized ovomucoid designated as Ultron ES-OVM. No separation of the stereoisomers of **II** was achieved on the Chiral-AGP column, whereas at least partial separation was achieved using the ovomucoid column.

Cellulose derivatives used as the stationary phase for liquid chromatography exhibit, in general, excellent possibilities for chiral resolution. Chiralcel OD-R type, a cellulose tris (3,5-dimethylphenyl carbamate) on silica was examined and this column proved suitable for the separation of reduced oracin, with both enantiomers being well separated ($R_{1,2}$= 2.51, k'_1=4.40, k'_2=6.30 and α=1.43), as shown in Fig. 3. The aqueous buffered eluent containing MeCN was optimised to give good resolution, by systematically varying the solvent ratio and the pH.

3.2. Stereospecificity of microsomal reductases

Interspecies differences in biotransformation ac-

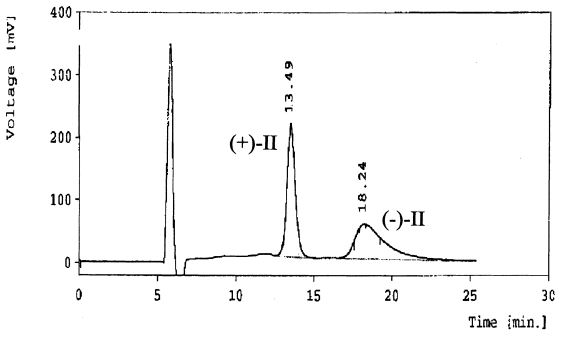

Fig. 3. HPLC chromatogram of separation of (+) and (−) enantiomers of 11-dihydrooracin. Chiralcel OD-R column, 0.1 *M* $NaClO_4$ (pH 4.6)–MeCN (145:55, v/v) at a flow-rate 0.5 ml/min and wavelength 340 nm (UV–Vis detection).

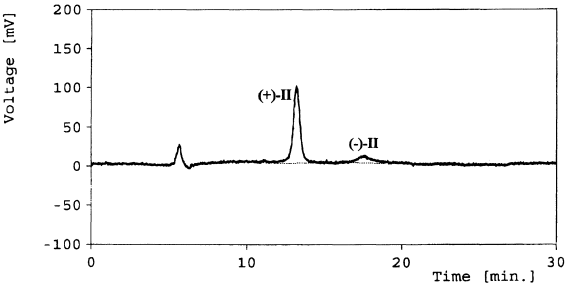

Fig. 4. HPLC chromatogram with fluorescence detection for separation of (+) and (−) enantiomers of 11-dihydrooracin in mouse. Chiralcel OD-R column, 0.1 *M* $NaClO_4$ (pH 4.6)–MeCN (145:55, v/v) at a flow-rate 0.5 ml/min and excitation and emission wavelengths 340 nm and 418 nm, respectively.

tivity and stereospecificity of microsomal reductases were examined using the following laboratory animals: rat, mouse, guinea pig, rabbit, dog and mini-pig.

A UV–Vis detection system PU 4021 from Pye Unicam was used to develop a suitable method for the separation of the enantiomers of **II** (used as standards). Since the actual reducing activity of the smooth endoplasmic reticulum enzymes is relatively low, it was necessary to find a different, more sensitive method to detect both stereoisomers of **II** in our experimental samples (microsomal fraction). Thus, fluorescence detection was used to quantify enantiomers of **II** in all biological samples. Since the peaks of the excitation spectrum bands are close to the peaks of the absorption spectrum of the molecule, the same excitation wavelength 340 nm was selected as that used for UV detection. The optimum emission wavelength was determined, based on the spectrum of 11-dihydrooracin measured using a spectrofluorimeter (LS50B from Perkin-Elmer). The highest intensity was observed at 418 nm. By comparison with UV detection, fluorescence detection increased detection sensitivity by two orders of magnitude, with minimum detection limits for (+)-11-dihydrooracin and (−)-11-dihydrooracin injected being reduced down to 10 ng and 20 ng, respectively. Such low detection limits were adequate for determining the stereospecificity of the respective microsomal reductases.

All chromatographic runs were performed using the following conditions: Chiralcel OD-R column thermostatted at 25°C, 50-μl of sample injected and fluorescence detection (with excitation and emission wavelengths at 340 nm and 418 nm, respectively). As the mobile phase, an optimised mixture of 0.1 *M* $NaClO_4$ (pH 4.6 adjusted with $HClO_4$)–MeCN (145:55, v/v) was used, with flow-rate 0.5 ml/min in all runs (Fig. 4).

All measurements were carried out on three animals of each species. Average values (±S.D.) of the yield of the enantiomers of **II** at the microsomal level are given in Table 1.

The stereospecificity of the biotransformation enzymes was determined by quantitative determination of the (+) or (−) enantiomers of **II** formed. Higher stereospecificity of the reductases was detected in rat and guinea pig. While rat biotransformation enzymes preferentially generated (+)-**II** (90%), guinea pig enzymes preferentially gave rise to (−)-**II** (86%).

Table 1
Yields of the enantiomers of **II** in different laboratory animals at the microsomal level

Species	Yield (±S.D.)	
	(+)-**II** (ng)	(−)-**II** (ng)
Mouse	1054±103	257±36
Rat	1989±147	238±36
Guinea pig	404±55	2414±157
Rabbit	2857±254	5002±391
Dog	2226±195	1347±71
Mini-pig	1390±109	343±28

These data represent the biotransformation stereospecificity of reductive enzymes at the microsomal level. Results are shown as mean±S.D. (n=3).

Table 2
Total yield of **II** [sum of (+) and (−) enantiomers] produced by microsomal reductases in different animal species

Species	Yield (±S.D.)	
	Metabolite **II** (ng/0.1 g wet liver tissue)	Metabolite **II** (ng/1 mg protein)
Mouse	1311±165	676±81
Rat	2227±174	1392±102
Guinea pig	2817±167	1583±93
Rabbit	7859±234	5614±165
Dog	3572±195	1881±102
Mini-pig	1733±109	1171±73

These data represent the mean of triplicate determinations±S.D. and show the differences in activity of microsomal reductases studied with respect to oracin.

Mouse and mini-pig enzymes also displayed significant stereospecificity, with less than 20% of (−)-**II** generated by the enzymes of either species. Although dog and rabbit microsomal enzymes did not manifest any clear-cut stereospecific effect, higher formation of (+)-**II** was detected in dog (62%) and of (−)-**II** in rabbit (64%).

These data also allow for comparison to be made of the total activity of microsomal reductases in different animal species. The individual enantiomers of **II** were quantified using the respective calibration curves, with subsequent correction for the protein content as determined according to Markwell et al. [8] (Table 2).

Total reductase activity expressed per unit of protein content displayed significant interspecies variation for the species examined. The activity of rabbit microsomal reductases was nearly an order of magnitude higher in comparison with that of rat. Reducing activity in individual species was found to decrease in the following order: rabbit, guinea pig, rat, dog, mini-pig and mouse.

4. Conclusions

The present data show that, based on the respective enantiomers patterns of **II** (and, thus, interspecies differences), the species examined can be classified in three basic groups. In the first group which comprises rat, mouse and mini-pig, the biotransformation pathway of **I** preferentially generates (+)-**II**. For the second group including rabbit and dog, the enzymes generate both stereoisomers of **II** in significant quantities. The third group is represented by guinea pig with its microsomal reductases preferentially generating (−)-**II**. These results also show that the highest activity of microsomal reducing enzymes was found in rabbit which is in accordance with published data [9].

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