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High-performance liquid chromatographic assay for the separation and characterization of metabolites of the potential cytostatic drug oracine

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Abstract

Oracine (**I**), a potential cytostatic drug, is enzymically converted to a number of metabolites whose formation has been studied in vitro and in vivo. The metabolites were separated by reversed-phase HPLC and characterized by UV spectra. Preparative TLC served for the isolation of the individual metabolites to allow their identification. Two metabolites were identified by Fourier transform NMR as 11-dihydrooracine (**II**) and a phenolic product (**III**). Two further metabolites (**IV**, **V**) were characterized. Some minor, presumably 11-dihydro metabolites and an 11-oxo metabolite produced in vitro and in vivo were revealed.

Keywords: Oracine

1. Introduction

As part of the preclinical tests the biotransformation of the potential cytostatic oracine {6-[2-(2-hydroxyethyl)aminoethyl]-5,11-dioxo-5,6-dihydro-11*H*-indeno[1,2-c]isoquinoline hydrochloride, Fig. 1, **I**} [1] has been studied in male rats in vitro and in vivo. The base of oracine and its hydrochloride are directly cytotoxic against the cells of the Yoshida, Ehrlich and Gardner tumours and they have strong inhibition effect on DNA synthesis in tumour cells in vitro. The mechanism of its action is probably based on the intercalation effect [2].

We started from in vitro experiments (incubation with the microsomal fraction and with isolated hepatocytes) which allow the characterization of

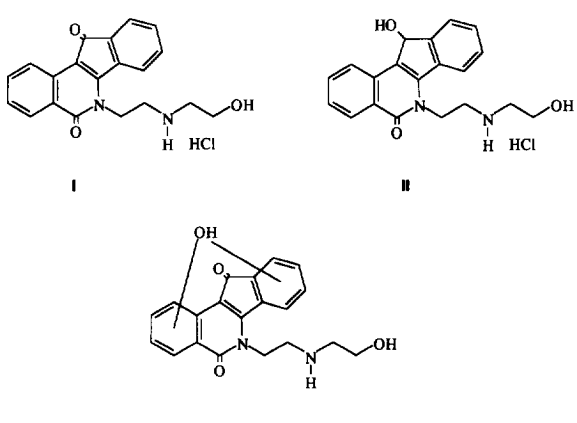

Fig. 1. Chemical structures of oracine (**I**), 11-dihydrooracine (**II**) and phenolic hydroxyoracine (**III**).

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metabolites produced under well controlled conditions in samples containing limited amounts of interfering substances. These findings facilitated the orientation in the spectrum of metabolites shown in the subsequent in vitro studies. The in vitro metabolites are likely to represent products of the first phase xenobiotic metabolism, i.e. of the oxidation and reduction reactions or possibly their combination.

An HPLC method for the separation and characterization of the oracine metabolites has been worked out, some of the individual metabolites were isolated and purified by preparative TLC and prepared for identification by means of NMR.

2. Experimental

2.1. Chemicals

Sodium hexanesulphonate (approximately 98%) and chloroform were obtained from Sigma-Aldrich (Prague, Czech Republic), acetonitrile and methanol were HPLC grade and obtained from J.T. Baker (Prague, Czech Republic), *n*-butylamine and triethylamine were HPLC grade and obtained from Merck (Darmstadt, Germany), 26% aqueous ammonia, ethyl acetate, cyclohexane, phosphoric acid, $FeCl_3$ and $K_3Fe(CN)_6$ were analytical reagent quality and obtained from Lachema (Brno, Czech Republic), NADPH was obtained from Boehringer (Mannheim, Germany).

Chromatographic plates Silufol UV 254 were obtained from Kavalier (Sázava, Czech Republic) and layers prepared in our laboratory from Kieselgel 60H from Merck. Ethyl acetate for extractions and methanol from Lachema for dissolution of the residues were purified and distilled.

Oracine (Fig. 1, **I**) and its reduced product {'11-dihydrooracine', *R,S*-6-[2-(2-hydroxyethyl)aminoethyl]-5-oxo-11-hydroxy-5,6-dihydro-11*H*-indeno-[1,2-*c*]isoquinoline hydrochloride (Fig. 1, **II**)} were obtained from the Research Institute of Pharmacy and Biochemistry in Prague (Czech Republic) and from the Research Institute of Organic Syntheses in Pardubice-Rybitví (Czech Republic) [1,3].

2.2. Animals and biological materials

Male rats of the Wistar type (300 – 350 g) from Velaz (Lysá nad Labem, Czech Republic) were kept for fifteen days in the animal house of our Faculty. They were fed with standard diet, fasted 12 h before the experiment, killed by decapitation in ether anaesthesia. In the in vivo experiments the animals were kept in metabolic cages. Urine and faeces were collected following a single (150 mg/kg) or repeated (150 mg/kg on each of three consecutive days) oral administration of oracine.

The liver homogenate was prepared in the ratio of 1:3 (w/v) in the 0.2 *M* Na phosphate buffer (pH 7.4) in the Potter and Elvehjem homogenizer. Microsomal fraction was obtained by fractional ultracentrifugation [4]. Hepatocytes were isolated by a slight modification of the method of Berry and Friend [5]. Urine and faeces were kept at −18°C after collection. Faeces were ground in distilled water, solids were removed by filtration.

2.3. Incubation and extraction

Microsomal suspension (1 ml in 0.2 *M* Na phosphate buffer pH 7.4, i.e. an aliquot corresponding to 1 g of the original wet liver tissue) was incubated with 4 μmol substrate and 6 μmol coenzyme NADPH in the overall buffer volume of 3 ml. Incubation at 37°C under aeration lasted 45 min.

A 20-ml volume of isolated hepatocytes ($3.3 \cdot 10^6$/ml) were shaken in the Krebs and Henseleit buffer with the substrate (0.4 μmol/ml) for 50 min at 37°C under introduction of air.

All incubations were terminated by adding an aqueous solution of 5% ammonia to pH 10.3–10.5 and extracted twice with the double of their volumes of ethyl acetate (96–99% oracine and metabolites being recovered after double extraction in control experiments). Combined extracts were evaporated to dryness and the dry residue was dissolved in 400 μl methanol.

Samples of the urine and faeces were alkalized with 5% aqueous ammonia to pH 10.3–10.5 and twice extracted with double of their volume of ethyl acetate, combined extracts were evaporated to dry-

ness, the residue dissolved in methanol (1 ml for the three-day collections).

2.4. Chromatographic separation and detection of the metabolites

2.4.1. Analytical TLC

TLC on Silufol 254 nm plates, first saturated with ammonia vapour was used for the separation of the metabolites and the non-metabolized substrate. The methanolic solution of the dry residues of the extracts from the incubates (10 μl, corresponding to 0.1 μmol of the original substrate) or from the excretions (10 μl) was spotted. The mobile phase chloroform–methanol–26% ammonia (80:10:1, v/v) was used for ascendent development. We presume that coloured (orange) compounds **I**, **III**, **IV**, **V**, U_5 quenching the fluorescence excited by 254 nm (absorbing with a maximum near 280 nm) have retained the 11-oxo group of **I** and we shall designate them accordingly. Compounds exhibiting a bluish fluorescence when excited with 366 nm like the **II** standard are designated as 11-dihydro compounds. Some substances were detected with a 15% $FeCl_3$–1% $K_3Fe(CN)_6$ (1:1, v/v) reagent.

2.4.2. HPLC

The HPLC system consisted of a Spectra Series P200 gradient pump from Spectra-Physics Analytical (Fremont, CA, USA), a Rheodyne Model 7125 injector with a 20-μl sample loop from Rheodyne (Cotati, CA, USA) and a 5-μm Spherisorb ODS2 cartridge (250×4 mm I.D.) with a 5-μm guard column LiChrospher 100 RP-18 (4×4 mm I.D.) from Hewlett-Packard (Amsterdam, Netherlands). A 5-μm CGC Sepharon SGX C_{18} column (150×3 mm I.D.) from Tessek (Prague, Czech Republic) was also tested. The column temperature was ambient and the flow-rate was 1.8 ml/min. Detection was carried out with multichannel UV-Vis detector PU4021 from Pye Unicam (Cambridge, UK), operated at 280 nm and 341 nm. Analytical runs were processed by Apex version 3.0 software from DataApex (Prague, Czech Republic) on 486/66 PC from Autocont (Hradec Králové, Czech Republic). The mobile phase was prepared by mixing buffer (10^{-2} m*M* sodium 1-hexanesulphonate and 10^{-4} m*M* triethylamine; H_3PO_4 to pH 3.27) with acetonitrile (75:25, v/v).

2.4.3. Preparative TLC

Major metabolites were separated by TLC on 500 μm thick layers prepared in the laboratory. The suspension of 75 g Kieselgel 60H from Merck in 110 ml methanol and 110 ml triethylamine was spread on glass plates using the CAMAG (Mutlenz, Switzerland) applicator.

Methanolic solutions of the extracts of faeces and urine (obtained after repeated oral administration of oracine, five days collection) were deposited in band form. They were then developed ascendently with chloroform–methanol–26% ammonia (80:10:1, v/v), the separated bands of the metabolites were scraped off and extracted with methanol. The separation and elution of the metabolites was repeated to obtain chromatographically pure metabolites (single HPLC peak).

3. Results and discussion

3.1. Substances studied

Fig. 2 schematically represents the results of the TLC analysis of the extracts from in vitro incubations and from the excretions after in vivo administration of oracine. Spots (coloured and absorbing at

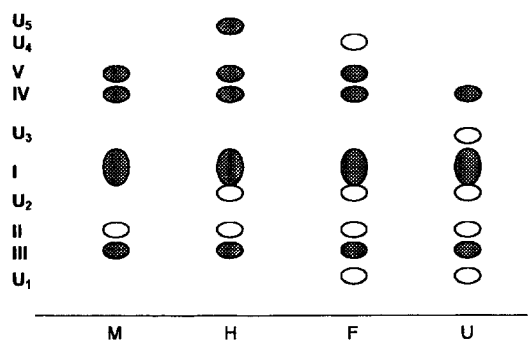

Fig. 2. Schematized chromatogram of the metabolites of oracine (I) produced on incubation with the rat liver microsomal fraction (M), isolated hepatocytes (H), in the faeces (F) and in the urine (U). Open areas, fluorescent spots; dotted areas, absorbing spots. For designation of the spots (left) see text.

254 nm or fluorescent under 366 nm) not present in the appropriate blanks are shown. It is assumed that substances with 11-oxo group are coloured and substances devoid of it (such as 11-dihydro compounds) are not.

Naturally, metabolites which are neither coloured and absorbing nor fluorescent or are below the detection threshold are not included. Neither are those which would not be extracted in ethyl acetate at pH 10.3–10.5[1] or would be insoluble in methanol. It cannot yet be stated with certainty that no spot contains more than one compound: yet testing by HPLC and by TLC in additional solvents including two-dimensional arrangement make this possibility unlikely.

Substances which were identified or at least characterized are represented by Roman numerals, other spots are indicated by **U** and an Arabic numeral in the order of increasing R_F value. Compound **I** is remaining oracine (Fig. 1). Compound **II** has been identified as a secondary alcohol, 11-dihydrooracine, mainly by NMR and by comparison with a racemic standard obtained by chemical synthesis [3]. It is found in all samples from in vitro and in vivo experiments.

Compounds **III** and **IV** react with $FeCl_3$–ferricyanide. They retain the 11-oxo group (colour and UV spectrum). Compound **III** was detected in all materials under study. Its spot has a more reddish hue which can, after fading, be revived by exposing the chromatogram to ammonia vapour. It is a hydroxy-oracine; the position of the phenolic hydroxyl has been determined by means of NMR (not reported here for patent considerations).

Compound **IV** was also demonstrated in all materials under study; its formation in microsomal incubates requires oxygen and NADPH. Preparative TLC did not give enough material for NMR studies. Treatment of **I** with oxidants gave too small yields which only allowed to compare the retention and spectra of the products with other 11-oxo compounds, but were not sufficient for NMR and MS studies. If **IV** were a positional isomer of **III**, an intramolecular hydrogen bond would have to be considered to account for its retention behaviour.

Compound **V** was found in the extracts of aerobic microsomal and hepatocyte incubates and in the faeces. It is an orange metabolite (11-oxo; see also Fig. 4) negative in the $FeCl_3$–ferricyanide test. Its retention behaviour (high R_F value and prolonged retention time) is remarkable. The possibility of changes in the side chain will be investigated.

Among the compounds demonstrated in the faeces and urine products of extrahepatic biotransformation enzymes, even of those of the intestinal bacteria, may be also involved. Metabolites labelled U_1 (faeces, urine), U_2 (hepatocyte incubations, faeces, urine), U_3 (urine) and U_4 (faeces) are reduction products (colourless, fluorescent) detected in low amounts which do not allow their isolation and identification by means of NMR. Metabolite U_5 is another minor metabolite (11-oxo) detected in hepatocyte incubations. In looking for the nature of these minor metabolites we shall also try incubations of already established metabolites [6].

3.2. HPLC

Several combinations of buffer (with different pH), methanol and acetonitrile were evaluated as possible mobile phases. First mobile phases tested consisted of *n*-butylamine, water and methanol or acetonitrile. pH was adjusted with phosphoric acid. Although such systems separated **I** and **II**, the separation of other products was poor. Ion-pair chromatography was selected for the next stage of our work. Sodium hexanesulphonate was used as the counter-ion. pH value was optimized to 3.27 in order to support the ionization of the base of the analyte and thus the ion-pair formation. This kind of chromatography was found as the most suitable. The pH of the mobile phase was a very important factor influencing retention. A slight increase of pH above the optimum prolonged the analysis time without improving the separation. Chromatographic behaviour was tested using two types of columns. The Tessek Separon SGX C_{18} columns did not separate well. The Hewlett-Packard Spherisorb ODS2 columns separated well and they were found stable after a number of injections in the order of thousands. Only **I** and **II** were available for the initial optimi-

[1]To check this point the aqueous layer remaining after ethyl acetate extraction was evaporated to dryness, the residue extracted with methanol and subjected to TLC. No metabolite spots were detected.

zation experiments. Direct synthesis of hypothetical metabolites proved very difficult and the isolation of metabolites by preparative TLC was the main research strategy. The positions of the peaks of compounds **III–V** were determined by co-chromatography with substances obtained by preparative TLC.

Fig. 3 shows column chromatograms, recorded at 280 nm, of metabolites in the extracts of incubates

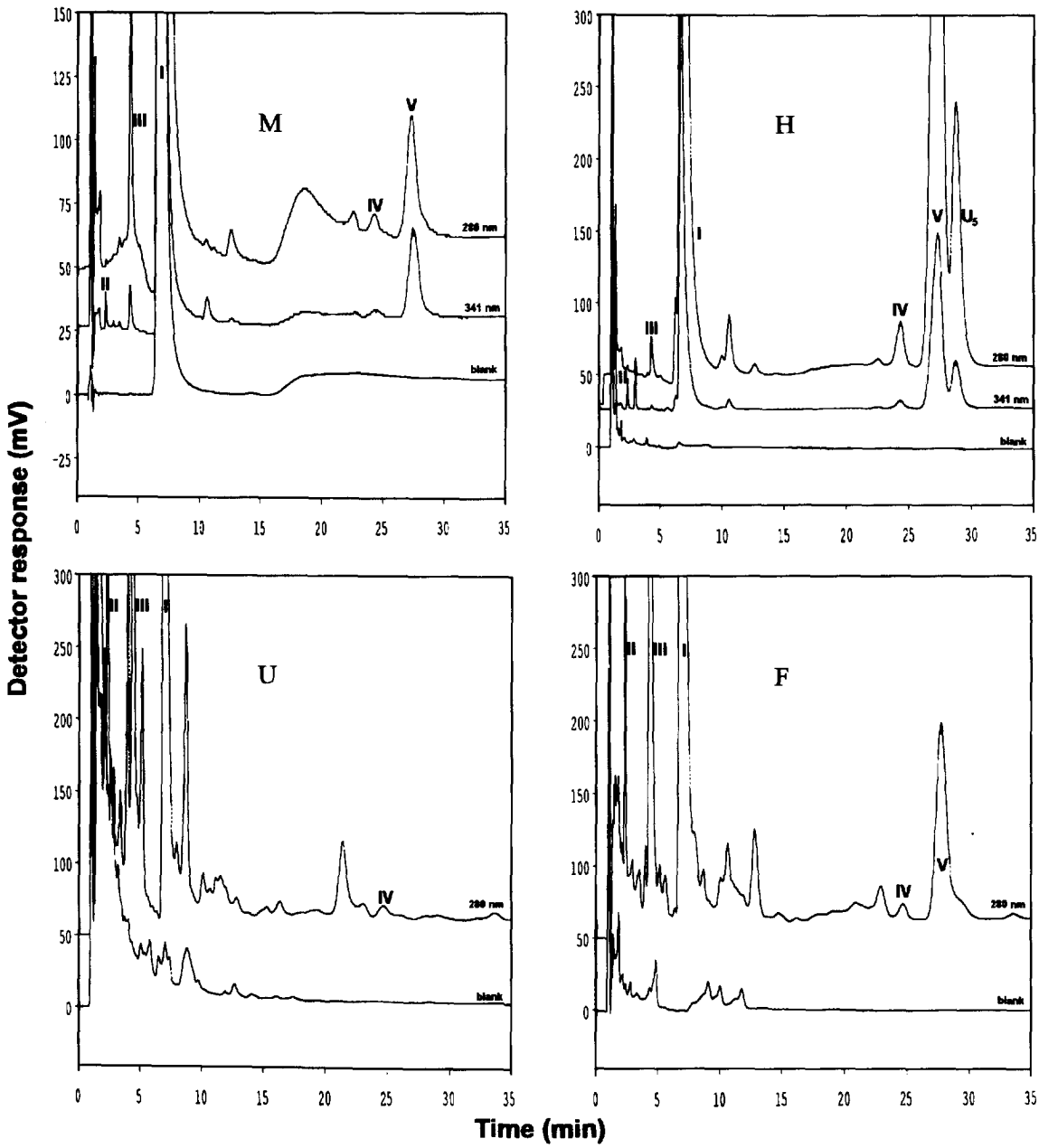

Fig. 3. HPLC chromatograms of the extracts from the incubates of I with the microsomal fraction (M) and hepatocytes (H) and from the urine (U) and faeces (F). Reading at 280 nm for experimental samples and blanks and, in case of M and H experimental samples, also at 341 nm.

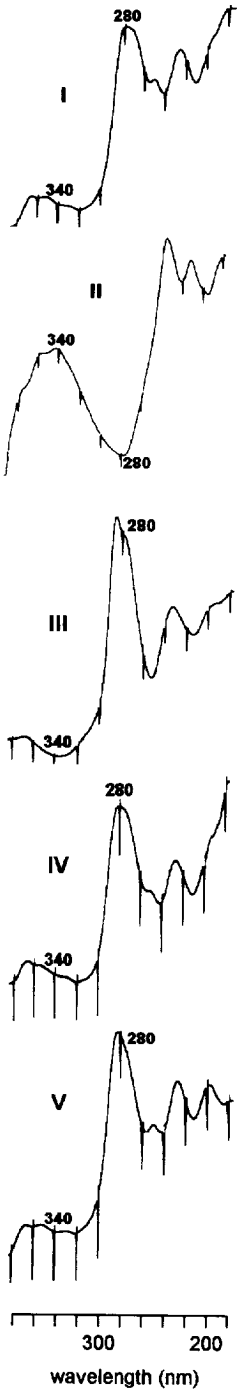

Fig. 4. The diode array detector UV readings in the positions of peaks of compounds I–V. For II–V the readings were taken at the peak maximum, for I at the ascending limb.

with the microsomal rat liver fraction and with the isolated hepatocytes, as well as in the extracts from urine and faeces following a single oral administration of oracine (150 mg/kg). The chromatograms were also recorded at 341 nm (not shown). Faeces and urine collected within 3 days after the administration were extracted for this analysis. The peaks **I–V** of Fig. 3 (U, F) were assessed under the assumption that **III–V** have an absorbance coefficient (280 nm) identical with that of **I**. Their amounts were summed up giving an approximate preliminary estimate of the yields of the administered drug, excreted in the form of **I–V**: 4% in the faeces, 2.5% in the urine.

Reduced oracine (**III**) has an asymmetric centre. We succeeded in separating the synthetically prepared racemate in its enantiomers chromatographically and determining their proportions in the enzymically produced metabolite [7,8].

Comparison of the UV absorption spectra recorded by the diode-array detector (Fig. 4) shows the similarity between the 11-oxo compounds **I** and **III–V**, which markedly differ from **II**. The resolution of compounds **III** and **IV** by HPLC was better than that by our TLC method.

Samples of the minor metabolites were not available and they may represent, in addition to the constituents of the biological matrix, the unlabelled peaks in the chromatograms Fig. 3.

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