IN VITRO PROTEIN BINDING AND TISSUE DISTRIBUTION OF D(+) USNIC ACID

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SUMMARY

In vitro protein binding of D(+) usnic acid in rabbit plasma and purified bovine serum albumin was investigated by equilibrium dialysis. The drug was highly protein bound, approximately 99.2%, and the extent of protein binding remained constant at usnic acid concentrations in the range of 1-5 μg/ml. The extent of binding, however, tended to decrease at low albumin concentrations and higher drug concentrations; Scatchard plot analysis indicated the existence of two classes of binding sites with association constants of 34.3×10^{-6} and 1.43×10^{-6} M respectively. Tissue distribution studies of usnic acid were undertaken in rats following i.p. administration. Usnic acid was well distributed into well perfused organs. The tissue/plasma ratio in lungs was high, which could be advantageous in a therapeutic agent for pulmonary tuberculosis.

KEY WORDS

usnic acid, protein binding, tissue distribution

INTRODUCTION

D(+) Usnic acid [2.6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H96H)-dibenzofuran-dione] is a lichen antibiotic obtained from several lichen species, including *Usnea*, *Cladonia*, etc. It is obtained as yellow coloured needles or orthorhombic crystals and has three forms: D(+), L(-) and racemic, with m.p. 199, 201 and 191°C, respectively. The specific rotation of D(+) usnic acid is $[\alpha]_D^{20}$ + 495 and that of L(-) usnic acid is $[\alpha]_D^{20}$ + 452 in chloroform /1/. Usnic acid is a monobasic acid. It has three pKa values, viz., 4.4, 8.8 and 10.7, corresponding to three hydroxy groups at the 3, 9 and 7 positions, respectively /2/. The structure of usnic acid is shown in Fig. 1. Stoll *et al.* /3/ reported that among lichen products usnic acid in particular has a high antitubercular activity. The comparative antitubercular activity (*in vitro*) of the D(+) and L(-) forms of usnic acid was investigated by Shibata *et al.* /4/ and they were reported to be equally potent. Marked antitubercular activity was observed by Marshak and Kushner /5/ when usnic acid was given in combination with a low dose of streptomycin. It has been effectively used against tuberculosis in humans /6,7/, and it has also potent antitumour activity /8,9/. In a recently conducted study, Krishna /10/ reported that usnic acid (+,- and the Na salt of the + form) in BSA solution inhibited the growth of *M. tuberculosis* and *M. lufu in vitro* at a concentration of 8 μg/ml. In the USSR, a usnic acid preparation is available with the trade name 'Binan' and is used for its antibacterial action /11/; in China it is available as 'Sun-Lo' and is used for its expectorant action /3/. Usnic acid complexed with triethanolamine is used in Germany for the production of deodorants /12/.

Pharmacokinetic studies on usnic acid were conducted in rabbits /13,14/. The plasma levels remained above 8 μg/ml (MIC) for 12 hours

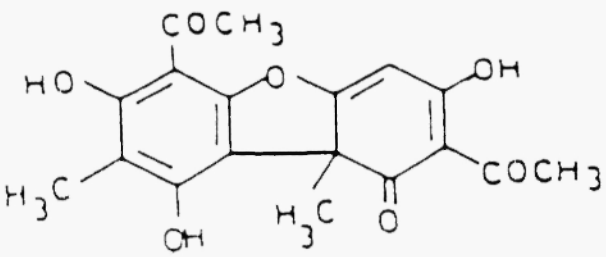

Fig. 1: Usnic acid.

after intravenous administration of usnic acid (5 mg/kg) and 48 hours after oral administration (20 mg/kg). The oral availability reported was 77.8% and t_{max} > 12 hours on administration as the soluble sodium salt. The half life of usnic acid following oral administration was longer than that following i.v. administration (18.9 and 11.4, respectively).

Drug-protein binding has significant influence on distribution of drugs. Plasma proteins exert a 'buffer and transport function' in the distribution process. Only the free, unbound fraction of drug can leave the circulatory system and diffuse into tissues. Hydrophobic drugs such as usnic acid are often found to be considerably bound to plasma proteins and erythrocytes. So far, no data are available on the protein binding of usnic acid and its tissue distribution. We studied *in vitro* protein binding (with bovine serum albumin) of usnic acid and its tissue distribution in rats.

MATERIALS AND METHODS

Protein binding studies

D(+) Usnic acid was obtained from Fluka Chemie AG, Buchs, Switzerland. Protein binding determinations were carried out by equilibrium dialysis. The dialysis membrane, having a molecular weight cut off of 12,000-14,000, was obtained from V.P. Chest Institute, New Delhi. The membrane was pretreated by sequential washing in distilled water (twice for 2 h each), in 30% isopropanol (45 min) and stored in distilled water at 40°C for 2 days. The membrane was allowed to equilibrate at room temperature for about 1 h prior to use. Dialysis was carried out in teflon microdialysis cells consisting of two chambers (1.5 ml each) separated by the membrane. Experiments were conducted using bovine serum albumin (BSA) (Cohn's fraction V, Sigma Chemicals, St. Louis, MO) in phosphate buffer pH 7.4 and using rabbit plasma. After mixing and equilibration at room temperature for 30 min, spiked plasma and albumin solutions (1.5 ml) containing various concentrations of drug were dialysed against 1.5 ml phosphate buffer, pH 7.4, for 12 h at 37°C and 15 rpm. Volume shifts into the chamber containing the plasma/BSA solution were determined by measuring fluid remaining in the solutions after dialysis. All determinations were done in triplicate. Usnic acid content in plasma and buffer was determined using HPLC /15/.

Determination of extent of protein binding

The extent of protein binding in plasma and albumin solution (> 6.5 g/l) was studied over a range of eight concentrations of usnic acid (1, 2, 3, 5, 10, 20, 30, 50 μg/ml).

Usnic acid and albumin concentration dependent study and binding parameters

The extent of protein binding at different usnic acid concentrations (5, 10, 20, 40, 60 μg/ml) and at different albumin concentrations (1.625, 3.25, 4,875, 6.5, 11.375, 14.625, 17.875, 21.125, 24.375 g/l) were evaluated to investigate the effect of usnic acid and albumin concentration on the extent of binding. Initial estimates of the number of classes of binding sites were obtained using a Scatchard plot /16,17/ and then fitted to the equation

$$r = \sum_{i=1}^{m} \frac{n_i k_i Df}{1+k_i Df}$$

where r = ratio between the two molar concentrations of bound drug and albumin;
n = no. of classes of binding sites;
i and k_i = no. of binding sites respectively, and
Df = concentration of drug (moles/litre)

Tissue distribution studies

These studies were undertaken in male albino Wistar rats weighing 160-240 g obtained from the National Institution of Nutrition (Hyderabad, India). The animals were housed in separate cages and were fed with standard diet and water *ad libitum*. The solution for injection was prepared by dissolving usnic acid in water and adjusting the pH to 8.5 with sodium bicarbonate. A single dose of 25 mg/kg body weight was given intraperitoneally. 3,6,12 and 24 h after drug administration, rats were lightly anaesthetised with ether and blood samples were collected from the retro-orbital venus plexus into heparinised tubes. Blood samples were centrifuged at 2600 *g* for 5 min and the plasma was separated and stored at -20°C until analysis. Whole blood samples were also stored at -20°C until analysis. Rats were killed by cervical dislocation and lungs, kidney, liver, spleen, testes,

heart, fat tissue and brain were quickly removed. The tissues were washed with isotonic saline and aliquots were blotted with filter paper, weighed and stored at -20°C until analysis. Plasma and blood samples were analysed for usnic acid using HPLC /15/. Intestines were thoroughly rinsed with isotonic saline and the washings were stored at -20°C. The usnic acid level in the intestinal lumen was also estimated by the same method.

Tissue determinations

Tissues of each organ were homogenised in a tissue homogeniser (Tempo Instruments Co., Bombay, India) in phosphate buffer, pH 5.8 /12/ and the final volume of homogenate was noted. Usnic acid was extracted from aliquots of homogenates by mixing with corresponding amounts of phosphate buffer, pH 5.8, and methanol. The samples were centrifuged at 10,000 *g* for 10 min and the supernatant was separated. Usnic acid was estimated by HPLC /15/.

RESULTS AND DISCUSSION

Protein binding studies

Results obtained for the extent of usnic acid binding to rabbit plasma proteins and bovine serum albumin solution are presented in Table 1. Usnic acid was highly bound to plasma protein (99.2%) and only a small fraction was present in free form, the extent of binding remained constant over the drug concentration range 1 to 50 μg/ml and it was not influenced by the concentration of usnic acid in the range 5-50 μg/ml at higher concentration (6.5 g/l) of albumin. The binding was however reduced to 72% at higher concentrations of usnic acid (40-50 μg/ml) at low concentration of albumin (3.25 g/l). As the extent of binding is the same in plasma and in BSA solution, it can be concluded that albumin was likely the major protein responsible for the binding and the plasma constituents (such as bile salts) did not affect the binding. It is clear from Fig. 2 that the percentage binding of usnic acid increased with increase in concentration of albumin up to 6.5 g/l indicating albumin concentration dependent binding; however, it remained constant (99%) beyond this concentration.

Fig. 3 shows the Scatchard plot of the protein binding profile of usnic acid with BSA. The curve obtained from the plot may be

TABLE 1

Percent usnic acid bound to BSA (*in vitro*) and rabbit plasma proteins

Conc. of usnic acid (μg/ml)	Mean percent bound		
	BSA Conc (g/l)		Rabbit Plasma
	3.25	6.5	
1.0	-	99.4	99.0
2.5	-	98.7	99.4
5.0	99.9	99.3	99.6
10.0	99.7	99.7	99.0
20.0	99.5	99.0	98.3
30.0	99.0	99.3	99.8
40.0	82.6	99.3	99.2
50.0	72.4	99.0	99.4

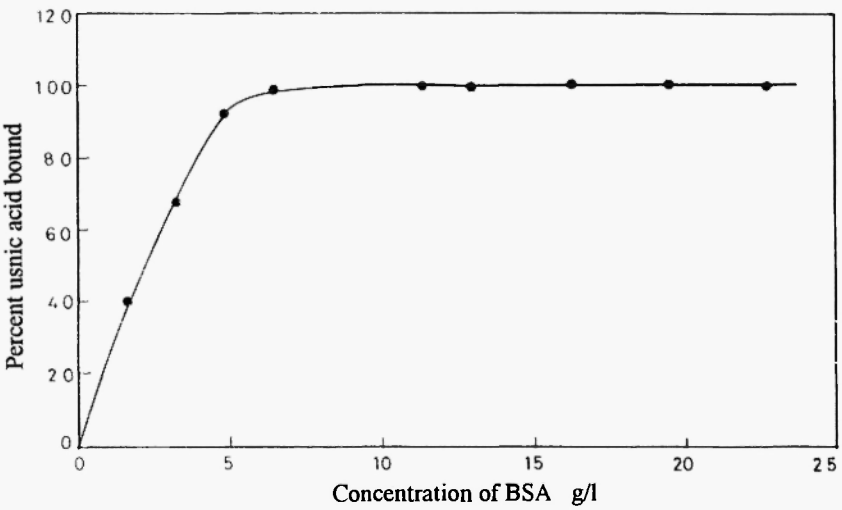

Fig. 2: Percent usnic acid bound to BSA at usnic acid concentration 50 μg/ml.

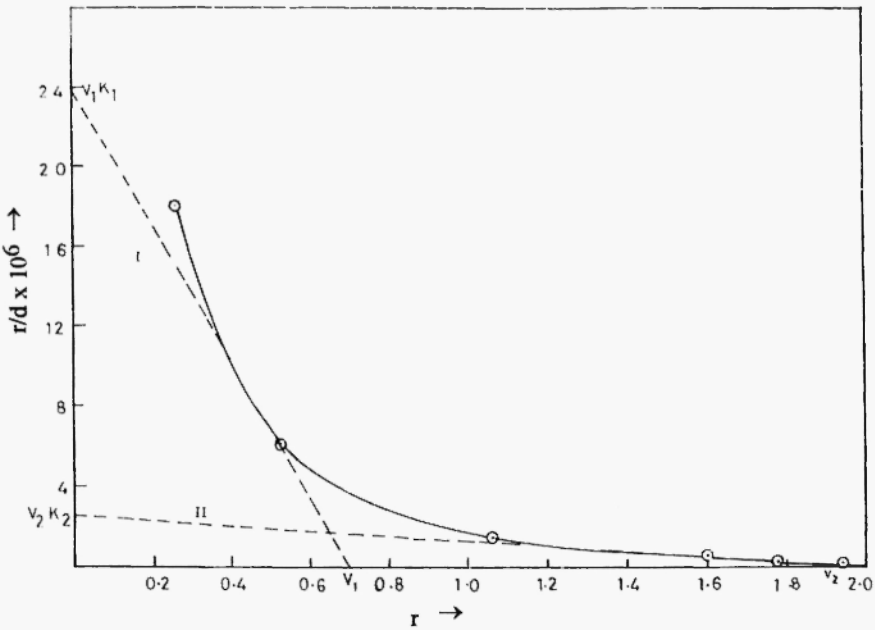

Fig. 3: Scatchard plot of the binding of usnic acid with BSA.

regarded as the sum of two or more straight lines each representing a different class of binding site /19/; in the present case one binding site with $V_1 = 0.7$ is of high affinity and has an association constant $k_1 = 34.3 \times 10^{-6}$ M, and a second class of binding sites ($V_2 = 2$) of low affinity with an association constant $k_2 = 1.43 \times 10^{-6}$ M. The latter class suggests electrostatic interaction between usnic acid and protein due to hydrogen bonds or Van der Waals forces.

Tissue distribution studies

The time course of tissue usnic acid concentration is shown in Figures 4-6. The tissue/plasma concentration ratios of usnic acid are given in Table 2. The results indicate that tissues can be grouped into three based on the distribution pattern of usnic acid. The first group consist of lung, liver and blood in which the ratio of usnic acid concentration in tissue to plasma is 1.0, the second group consists of spleen, heart and kidney in which the ratio is 0.3-0.65, and the third group consists of fat tissue, brain and testes in which the ratio is 0.1-0.15.

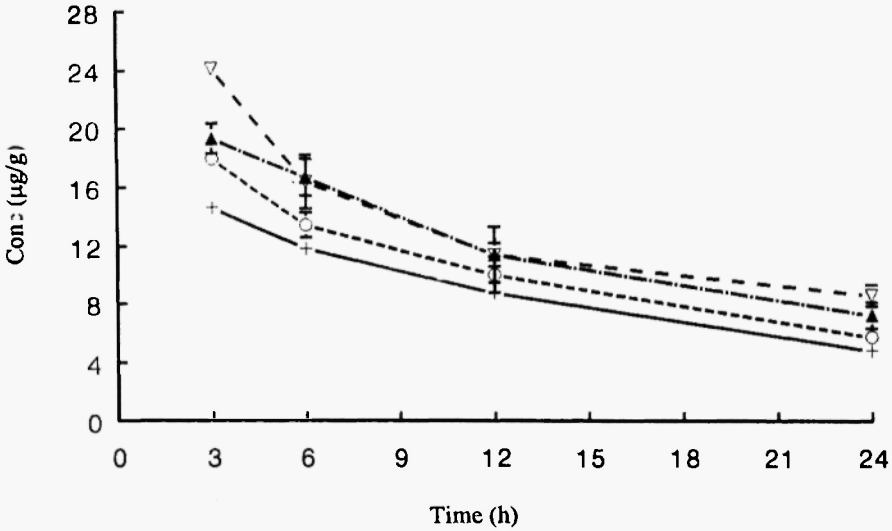

Fig. 4: Tissue distribution of usnic acid: - + - Plasma, - O - Blood, - ▲ - Liver, -∇ - Lung.

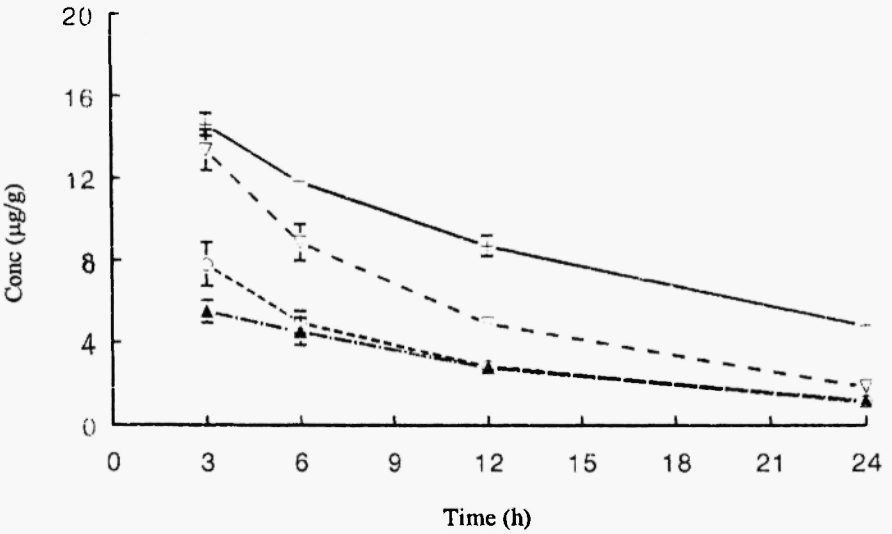

Fig. 5: Tissue distribution of usnic acid: - + - Plasma, - O - Heart, - ▲ - Kidney, -∇ - Spleen.

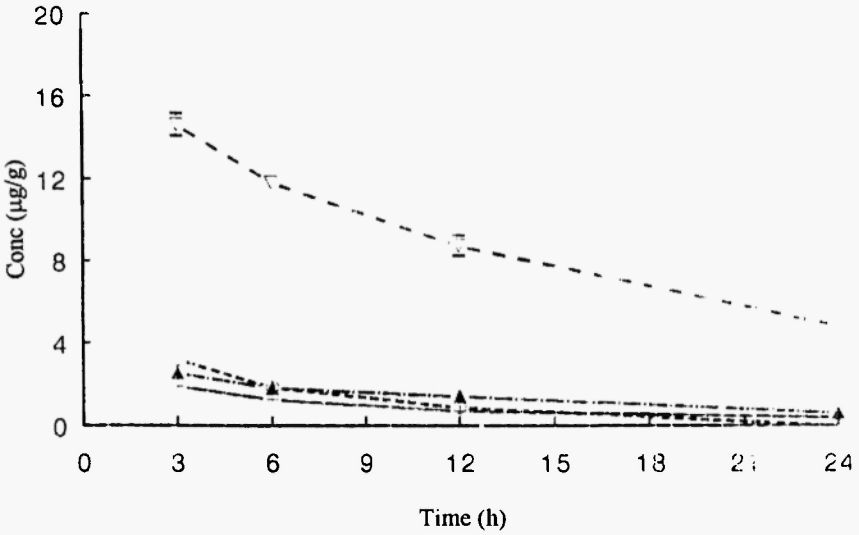

Fig. 6: Tissue distribution of usnic acid. - + - Testes, - O - Brain, - ▲ - Fat, -▽ - Plasma.

The concentration of usnic acid in lung was higher than in any other tissue till 6 h. After 6 h the concentration of usnic acid in lungs and liver remained more or less constant but still higher than in other organs. The high concentration of usnic acid in lungs and liver is consistent with the high perfusion rates of these organs /20/. The high concentrations of usnic acid in lungs could be advantageous in its therapeutic utility in the treatment of pulmonary tuberculosis. However, usnic acid may have limited use in CNS infections, as the concentrations in brain are very low. The ratio of usnic acid in blood to plasma over one indicates the binding of usnic acid to cells in blood. The high concentrations of usnic acid in the intestinal lumen indicate that usnic acid is excreted into the intestines, probably through the bile, either in free form or as conjugate or both. Usnic acid contains three -OH groups indicating glucuroinidation as one of the possible metabolic pathways. The glucuronide(s) of usnic acid could have been excreted via bile into the intestines, where they are probably hydrolysed by intestinal esterases to the parent molecule, resulting in enterohepatic recycling.

In summary, usnic acid is extensively bound to plasma albumin and has two classes of binding sites, a single high affinity binding site and

TABLE 2

Time course of mean tissue/plasma ratios of usnic acid (n=3)

Tissue	3h	6h	12h	24h
Blood	1.227	1.134	1.145	1.192
Liver	1.110	1.410	1.303	1.503
Lung	1.648	1.385	1.339	1.777
Heart	0.535	0.420	0.327	0.239
Kidney	0.337	0.382	0.322	0.247
Spleen	0.915	0.752	0.563	0.390
Testes	0.131	0.108	0.083	0.085
Brain	0.221	0.159	0.104	0.064
Fat tissue	0.176	0.155	0.162	0.130
Intestinal lumen contents	1.227	1.222	1.267	0.986

two low affinity binding sites. The characterisation of binding sites involving interaction studies using other drugs such as warfarin are in progress. Usnic acid is well distributed into different tissues. Its levels are particularly high in lungs and liver, and a considerable amount is bound to blood cells.

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