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Short Communication

High-performance liquid chromatographic determination of usnic acid in plasma

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ABSTRACT

A high-performance liquid chromatographic method for the determination of usnic acid in human plasma using diclofenac sodium as internal standard is described. Plasma proteins were precipitated with methanol. A 250 mm × 4 mm I.D. Nucleosil. C_{18} (5 μm) column with a mobile phase consisting of methanol–phosphate buffer (pH 7.4) (70:30, v/v) was used. Chromatography was performed at ambient temperature with flow-rate of 1 ml min^{-1} and ultravioilet detection at 280 nm. Each analysis required no longer than 7 min. Quantification was achieved by measurement of the peak-height ratio and the absolute recovery varied from 93.8 to 97.3%. The limit of quantitation of usnic acid in plasma was 0.25 μg ml^{-1}. The intra-day relative standard deviation (R.S.D.) ranged from 1.24 to 4.53% and the inter-day R.S.D. from 2.23 to 8.25% at three different concentrations. The method was applied to the determination of plasma levels of usnic acid after intravenous and oral administration to study its disposition in a healthy male rabbit.

INTRODUCTION

Usnic acid (Fig. 1) is a lichen antibiotic obtained from various species of lichens such as *Usnea, Cladonia, Cetraria, Ramalina, Parmelia* and *Lechalona* [1]. It is effective agains both Gram-positive and Gram-negative microorganisms [2], and is known to be a potent anti-tuberculosis [3–8], anti-tumour [9] and enzyme-inhibiting agent [10–12]. Usnic acid has been found to be effective against *Mycobacterium lufu* (a model organism for leprosy) *in vitro* [13]. From the available reports, usnic acid appears to be a promising chemotherapeutic agent for human tuberculosis, possibly leprosy and certain tumours. Spectrophotometric [14], gas chromatographic [15] and polarographic [16] determinations of usnic acid in pharmaceuticals and galenical preparations have been reported. However, there are no reports of its determination in biological fluids for the study of its disposition and metabolism.

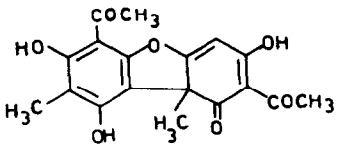

Fig. 1. Structure of usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2*H*,9b*H*)dibenzofurandione).

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EXPERIMENTAL

Chemicals and reagents

d-Usnic acid was purchased from Fluka (Buchs, Switzerland), diclofenac sodium was a kind gift from TTK Pharma (Madras, India), methanol of high-performance liquid chromatographic (HPLC) grade was purchased from Qualigens Fine Chemicals (Bombay, India) and potassium dihydrogenphosphate (analytical reagent grade) was obtained from Glaxo (Bombay, India).

Preparation of standards

A stock solution of 1 mg/ml usnic acid was prepared in ethyl acetate–methanol (30:70, v/v) and of 1 mg/ml internal standard (diclofenac sodium) in doubly distilled water and stored at 4°C. Appropriate dilutions of these solutions were made with methanol to produce working standard solutions containing 50 μg/ml usnic acid and 20 μg/ml internal standard.

Extraction procedure

Volumes of 0, 10, 50, 100, 250 and 500 μl of usnic acid working standard solutions were placed in screw-capped test-tubes, diluted to 500 μl with methanol, then 0.5 ml of blank plasma was added, thus providing calibration standards of 0, 1, 5, 10, 25 and 50 μg/ml. A 125-μl volume of internal standard equivalent to 2.5 μg of diclofenac sodium was added to the mixture, which was shaken on a vortex mixer for 1 min, then 0.5 ml of phosphate buffer (pH 5.8) and 2 ml of methanol were added. The tubes were capped and vortex-mixed for 5 min and then centrifuged at 3000 *g* for 5 min. The supernatant layer was separated.

Plasma samples obtained from rabbit after intravenous and oral administration of usnic acid were extracted by adding 0.5 ml of plasma sample to tubes containing 0.5 ml of methanol. The samples were then treated in the same way as the standards.

Chromatography

A Shimadzu HPLC system equipped with a solvent-delivery system (Model LC-6A), UV–VIS spectrophotometric detector (Model SPD-6AV) and a data processor (Model C-R4A) was used. A Nucleosil C_{18} column (250 mm × 4 mm I.D., 5 μm particle size, supplied by Macherey–Nagel, Düren, Germany) was used. The mobile phase was methanol–phosphate buffer (pH 7.4) (70:30, v/v).

Aliquots of 20 μl of the supernatant of the sample extracts were injected into the HPLC system and eluted with the mobile phase at a flow-rate of 1.0 ml/min. The eluates were monitored at 280 nm with the detector range setting fixed at 0.005 a.u.f.s.

RESULTS AND DISCUSSION

Quantification

Peak-height ratios of usnic acid to the internal standard were measured. A representative calibration graph of usnic acid to diclofenac sodium peak-height ratio *versus* usnic acid concentration in the range 0–50 μg/ml resulted in the regression equation $y = 0.1442x + 0.1547$ ($r = 0.998$). Calibration graphs were constructed on different days over two weeks to determine the variability of the slopes and intercepts. The results showed very little day-to-day variation.

TABLE I

PRECISION OF DETERMINATION OF USNIC ACID IN PLASMA

Concentration (μg/ml)	Intra-day (n = 6)			Inter-day (n = 6)		
	Mean found (μg/ml)	S.D. (μg/ml)	R.S.D. (%)	Mean found (μg/ml)	S.D. (μg/ml)	R.S.D. (%)
1.0	1.012	0.046	4.53	1.017	0.048	4.71
10.0	10.00	0.26	2.61	9.915	0.82	8.25
50.0	49.02	0.61	1.24	48.88	1.09	2.23

TABLE II

ABSOLUTE RECOVERY AND ACCURACY OF DETERMINATION OF USNIC ACID IN PLASMA

Concentration (μg/ml)	Absolute recovery (mean ± S.D., n = 3) (%)	Accuracy (mean ± S.D., n = 6) (%)	Range (n = 6) (μg/ml)
1.0	93.43 ± 2.36	101.77 ± 4.29	0.97–1.09
10.0	97.28 ± 1.08	99.15 ± 8.18	9.15–11.26
50.0	98.3 ± 1.24	98.03 ± 1.21	48.28–49.94

Limit of quantitation

The limit of quantitation was attained with plasma samples containing 0.25 μg/ml usnic acid.

Precision

The precision of the assay was determined by assaying plasma samples containing usnic acid at three different concentrations (1, 10 and 50 μg/ml). The intra-day relative standard deviation (R.S.D.) was 1.24–4.53%. The inter-day R.S.D. varied from 2.23 to 8.25% (Table I).

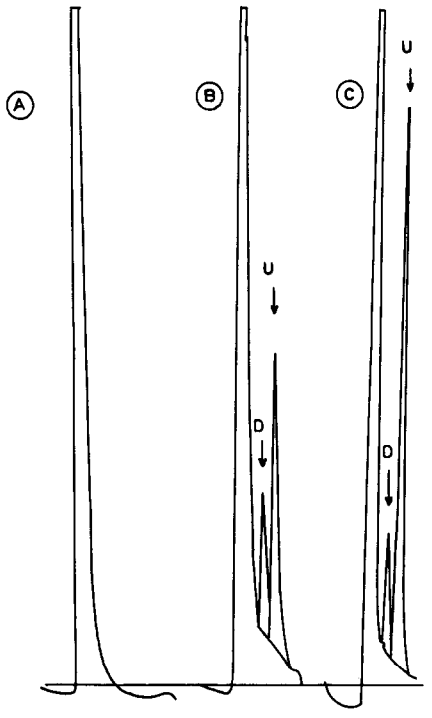

Fig. 2. Chromatograms of usnic acid (U) in plasma with diclofenac sodium (D) as internal standard. (A) Blank plasma; (B) standard (2.0 μg/ml) in plasma; (C) plasma sample 8 h after intravenous administration of usnic acid at a dose of 5 mg/kg body weight.

Recovery

The extraction recovery of usnic acid was assessed at concentrations of 1, 10 and 50 μg/ml. Plasma samples (in triplicate) containing usnic acid and internal standard were extracted and injected. Three samples of the same amount of compound in methanol were directly injected and the peak heights were measured. Absolute recovery was calculated by comparing the peak heights for direct injection of pure usnic acid with those for plasma samples containing the same amount of usnic acid. The absolute recoveries of usnic acid ranged from *ca.* 93 to 98% (Table II).

The accuracy of the method was calculated by comparing the concentrations measured for usnic acid-supplemented plasma with the actual added concentrations. The results are given in Table II.

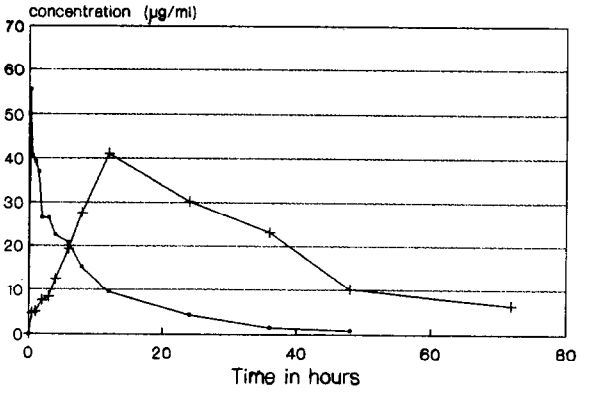

Fig. 3. Plasma concentration profiles after administration of (●) 5 mg/kg intravenous and (+) 20 mg/kg oral usnic acid to a rabbit.

Chromatograms corresponding to blank plasma, blank plasma spiked with usnic acid and internal standard and plasma samples obtained after intravenous administration of 5 mg/kg body weight usnic acid to a healthy male rabbit are shown in Fig. 2. No endogenous interfering peaks were visible in blank plasma. The two peaks were well separated with retention times of 5.0 and 4.4 min for usnic acid and the internal standard, respectively. The capacity and selectivity factors are 1.17 and 1.3, respectively.

Typical plasma concentration–time profiles obtained with this assay after administration of 5 mg/kg body weight (intravenously) and 20 mg/kg body weight (orally) usnic acid to the rabbit is shown in Fig. 3.

In conclusion, the HPLC method presented here is simple, selective and sufficiently sensitive to determine plasma concentrations of usnic acid.

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