THE *IN VIVO* METABOLISM OF 5-(4-NITROPHENYL)-4-PHENYL-2,4-DIHYDRO-3H-1,2,4-TRIAZOLE-3-THIONE IN RATS

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SUMMARY

It is known that substituted 1,2,4-triazole-3-thione derivatives have several biological activities, such as antimicrobial, diuretic and antidepressant activities. In our previous studies, the antifungal activity of 5-(4-aminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione was found to be active against *Candida tropicalis* K1022. The aim of this study was to investigate the *in vivo* metabolic pathway of 5-(4-nitrophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione which was selected as a model compound for this study. The substrate and its potential metabolites, i.e. the acetylation and nitro reductive products, were synthesized and then separated using HPLC on a reverse phase system. In the *in vivo* metabolism study, a 4 mg dose was administered i.p. to male Wistar rats. Blood samples were collected at 0, 2, 4, 8, 12, 24 and 56 hours after administration and were passed through a Sep-Pak cartridge. The acetylated metabolite [5-(4-acetylaminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione], the amine metabolite [5-(4-aminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione] and an unknown metabolite were detected.

KEY WORDS

1,2,4-triazoline-3-thione, *in vivo* metabolism, rat

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INTRODUCTION

4,5-Disubstituted-1,2,4-triazole-3-thiones have been reported to have a wide variety of biological activites, such as antidepressant, antimicrobial, antitubercular, analgesic, anti-inflamatory, diuretic, hypoglycemic and antithyroid /1-8/. In our previous studies, we investigated their antibacterial and antifungal activities and found that they were active /9,10/. The purpose of this study was to determine the biotransformation of 1,2,4-triazoline-3-thione administered i.p. to rats. First, the sodium salt of the substrate and its potential metabolites were synthesized and we then investigated the *in vivo* metabolism of 5-(4-nitrophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione.

MATERIALS AND METHODS

Chemicals

Sodium 5-(4-nitrophenyl)-4-phenyl-1,2,4-triazole-3-thiolate [**I**], 5-(4-nitrophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione [**II**] and 5-(4-aminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione [**III**] were synthesized in our laboratory according to previously described methods /9-13/. Only the acetylated metabolite (5-(4-acetylaminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione [**IV**]) was an original compound.

5-(4-acetylaminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione [IV]

Compound **IV** was prepared by heating appropriate thiosemicarbazides with 2N sodium hydroxide for 1 hour. The precipitate formed after cooling was acidified with hydrochloric acid. It was washed with ethanol; m.p. >305°C; yield 28%.

Compound **IV**: Anal.: found: C 62.27; H 4.75; N 18.03. $C_{10}H_{14}N_4OS$ requires: C 61.92; H 4.55; N 18.05.

UV λ max nm (ε): 204 (25047.7), 265.8 (11763.4), 371 (9901.1).

^{1}H-NMR: δ (ppm) 2.02 (3H, s, CH_3-CO-NH-), 7.19 (2H, d, protons of ortho position at acetylaminophenyl group), 7.30-7.69 (7H, m, protons of phenyl ring in 4 position of triazole ring and protons of ortho position at acetylaminophenyl group), 10.04 (1H, s, CH_3-CO-NH-), 13.77 (1H, s, NH proton signal of triazole ring).

HPLC apparatus and conditions

All measurements were performed with HPLC apparatus consisting of a Waters Model 510 pump, a Waters Model 481 UV detector, a Rheodyne Model 7725 injector and an integrator (Unicam 4880 Chromatography Data Handling System) was used for data collection.

A reversed-phase μBondapak C_{18} column (300 mm x 3.9 mm i.d.; Waters Assoc. Milford, MA, USA) was used for the analysis. The mobile phase consisted of methanol-water (50:50, v/v). The mixture was passed through a 0.45 μm Millipore filter before use. The solvent flow-rate was 0.7 ml/min. The mobile phase was degassed in an ultrasonic bath (Bransonic 221) prior to use. The UV detector was set at 260 nm. HPLC-grade methanol was obtained from Merck.

Thin layer chromatography

TLC was performed on silica gel GF 254 developing with benzene: acetone:acetic acid (69:30:1), UV light at 254 nm being used for visualisation.

Animals

Male Wistar rats (250-300 g) were used throughout this study. Rats were housed in humidity- and temperature-controlled rooms and allowed standard food and water. Rats were fasted overnight before administration of compound **I**. Compound **I** was dissolved in isotonic saline (2 mg/ml) and 2 ml were administered to rats intraperitoneally.

Blood samples (0.3±0.2 ml) were collected from the ophthalmic veins of the rats by sterile capillary tube under ether anesthesia. Serial blood samples were drawn predose and 2, 4, 8, 12, 24, and 56 h after administration of compound **I**. Plasma was prepared by centrifugation of blood.

Preparation of standards

Stock standard solutions of all compounds were seperately prepared in the mobile phase (methanol-water) to give a concentration of 3±1 μg/ml. Spiked plasma standards were then prepared by adding 20 μl of the stock standard solution of each compound. The standard solutions (10 μl) were injected onto the HPLC column.

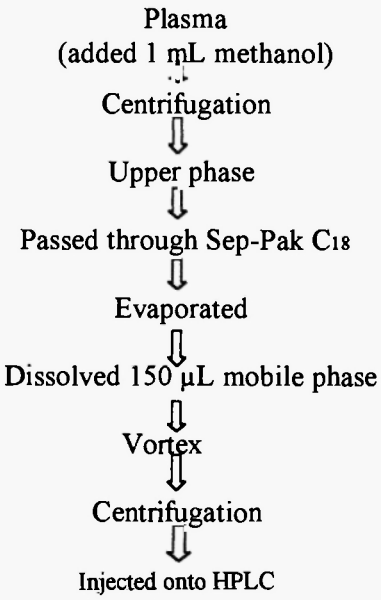
Preparation of biological samples

Method 1: Methanol (1 ml) was added to plasma to precipitate plasma proteins, and after centrifugation the upper phase was removed. This phase was passed through a Sep-Pak C_{18} cartridge. Before using the cartridges, they were washed with distilled water (1 ml) and methanol (1 ml). The filtered solution was evaporated and the residue was dissolved in the mobile phase (150 µl). After shaking with a vortex mixer, the aliquot was centrifuged and analyzed by HPLC (**Scheme 1**).

Method 2: Distilled water (1 ml) was added to the precipitated plasma proteins. The proteins were denatured by heating at 50-60°C

Scheme 1

Sample preparation

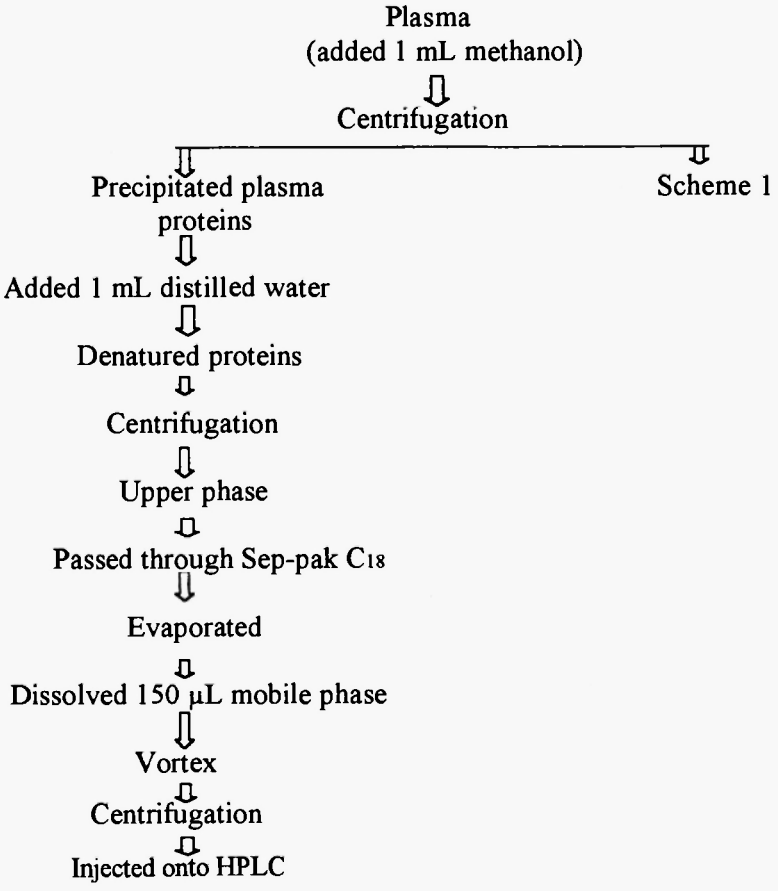


for 3 hours. The samples were centrifuged and passed through Sep-Pak cartridges. The rest of the procedure was as described above (**Scheme 2**).

Method 3: After the blood samples were centrifuged, they were seperated into plasma and blood cells. The plasma was prepared according to methods 1 and 2. Methanol (2 ml) and distilled water (1 ml) were added to the blood cells and the mixture was heated at 70°C

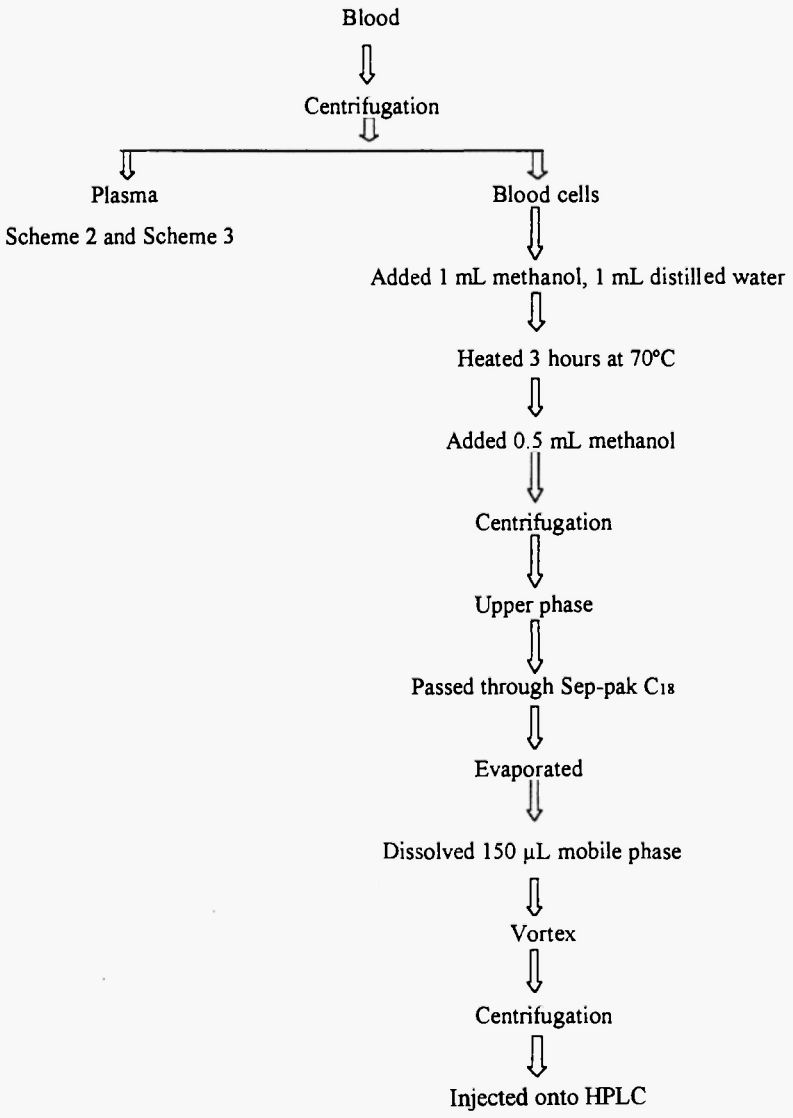
Scheme 2

Sample preparation



for 3 hours. After denaturation, methanol (0.5 ml) was added and the mixture was centrifuged. The upper phase was separated. The rest of the procedure was the same as in methods 1 and 2. This method is shown in **Scheme 3**.

Scheme 3



RESULTS AND DISCUSSION

Synthesis and characterization

1-(p-Acetylaminophenyl)-4-phenyl thiosemicarbazide was cyclized by heating under reflux for 4 hours with 2N sodium hydroxide, and the acyl group (acetyl or benzoyl) was hydrolyzed /9,14/. Although the acetylated derivative of 5-(4-aminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione [**III**] was expected to be synthesized by reaction with acetic anhydride, the diacetylated derivative was obtained by Rollas *et al.* /9/. However, the monoacetylated derivative was required in our investigation. For this reason, the cyclization of 1-(p-acetylaminophenyl)-4-phenyl thiosemicarbazide instead of the acetylation of 5-(4-aminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione was used to prepare the acetylated metabolite which was refluxed for 1 hour in 2N sodium hydroxide with a low yield (28%).

This original compound was elucidated using elemental analysis, UV, and ^{1}H-NMR spectroscopic methods.

The UV spectra of compound **IV** showed absorption maxima at 205 and 267 nm in ethanol, and due to the phenyl group, it showed a maximum absorption at 371 nm.

When NMR spectral data for compound **III** and **IV** were compared, the NH_2 protons of compound **III** were observed by a widespread peak at 3.76-5.00 ppm, but in compound **IV**, the singlet for the methyl protons of the acetyl group was determined at 2.02 ppm and the amide NH proton at 10.04 ppm. In addition to this, there were peaks at 7.30-7.69 ppm that were assigned to the protons of the phenyl ring and the protons at the meta position of the acetylaminophenyl group; protons at the ortho position were defined at 7.19 ppm. The NMR spectra of metabolite **IV** showed the expected NH proton signal of the triazole ring as a singlet at 13.77 ppm /14-16/. These NMR data and elemental analysis proved that metabolite **IV** was synthesized.

Discussion of biotransformation

The metabolism of 5-(4-nitrophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione [**II**] was studied *in vivo* in rats. We synthesized some of the potential metabolites. The biotransformation pathway of compound **II** is shown in Figure 1. Substrate II does not dissolve in isotonic saline and therefore its sodium salt [**I**] was synthesized and

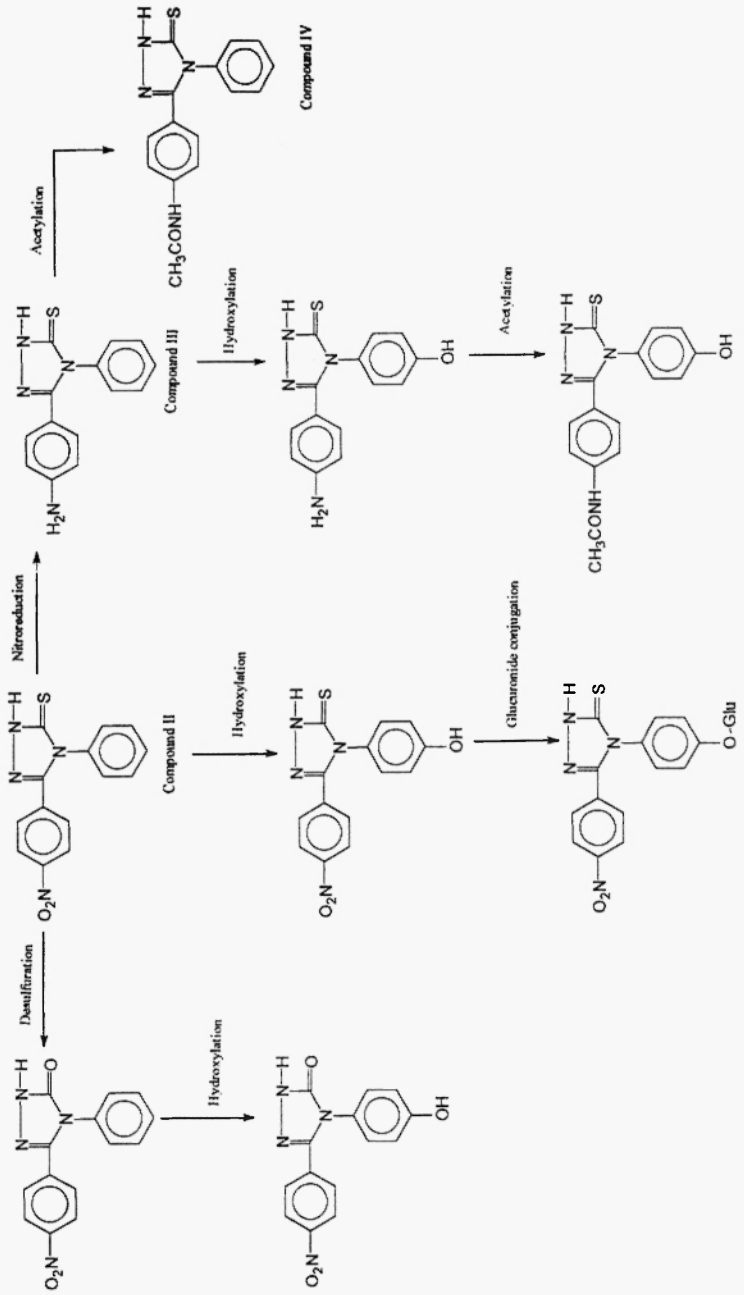


Fig 1: The proposed metabolic pathway of 5-(4-nitrophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione

injected into rats. After administration of substrate, blood samples were drawn at 0, 2, 4, 8, 12, 24 and 56 hours.

Previous investigations have shown that samples prepared according to Scheme 1 were only observed at plasma peaks. These results show that the substrate and its metabolites could be bound to plasma proteins. For this reason, the plasma proteins were denatured by heating (the substrate and its metabolites are resistant to heat) and blood samples were prepared as shown in Schemes 2 and 3.

The retention times for compounds **II**, **III** and **IV** were 12.8, 6.3 and 7.9 min, respectively. All of them were well separated and easily determined.

When the plasma sample at 8 hours, which included denatured blood proteins (Scheme 3), was compared to blank plasma, the acetylated metabolite was detected at the retention time of 7.95 min (the retention time of the reference standard was 7.9 min) (Fig. 2).

In the one parallel study, blood samples were drawn at 2 and 3 hours. HPLC analysis of the drawn blood samples at 2 and 3 hours, which were prepared according to scheme 2, showed an unknown polar metabolite with retention time of between 2.917 and 2.717 min (Fig. 3). It was thought that it could have been a metabolite hydroxylated on the phenyl group at the 4 position of the triazole ring. This will be investigated in another study.

Reference standards at constant concentrations were added to the blank plasma in order to compare the retention times of the substrate and the metabolites in plasma with the plasma taken from rats. When the blank plasma and spiked plasma were injected in 20 μl aliquots onto the HPLC column, the retention times were found to be approximately the same as the retention times of the reference standards (Fig. 4).

In the TLC study, 2, 4, 8, 12, 24 and 56 hour blood samples were pooled. The substrate and potential metabolite, 0 hour blood sample and the pooled blood sample were examined by TLC using a mobile phase of benzene:acetone:acetic acid (69:30:1, v/v/v). In this chromatogram, the amine metabolite and an unknown nonpolar metabolite were detected.

In this investigation, none of rats died. The most important result was that the nitro group of the substrate was reduced to the amine metabolite and was then acetylated. Generally the substrate was metabolized into more polar compounds.

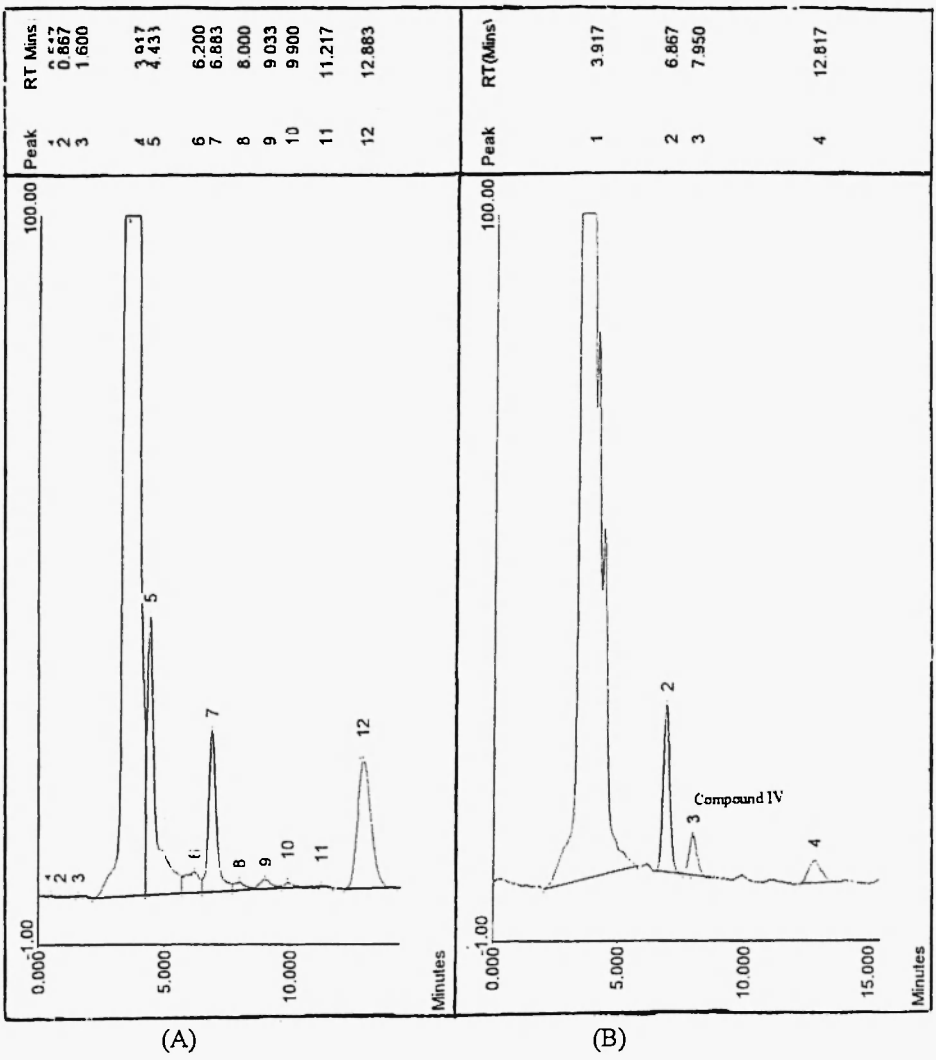


Fig. 2: Chromatograms of the denatured blood cells of (**A**) blank plasma, (**B**) plasma sample containing compound II 8 hours following intraperitoneal administration. Chromatographic conditions: mobile phase, methanol-water (50:50, v/v); flow-rate, 0.7 ml/min; injection volume, 20 µl; UV detection at 260 nm.

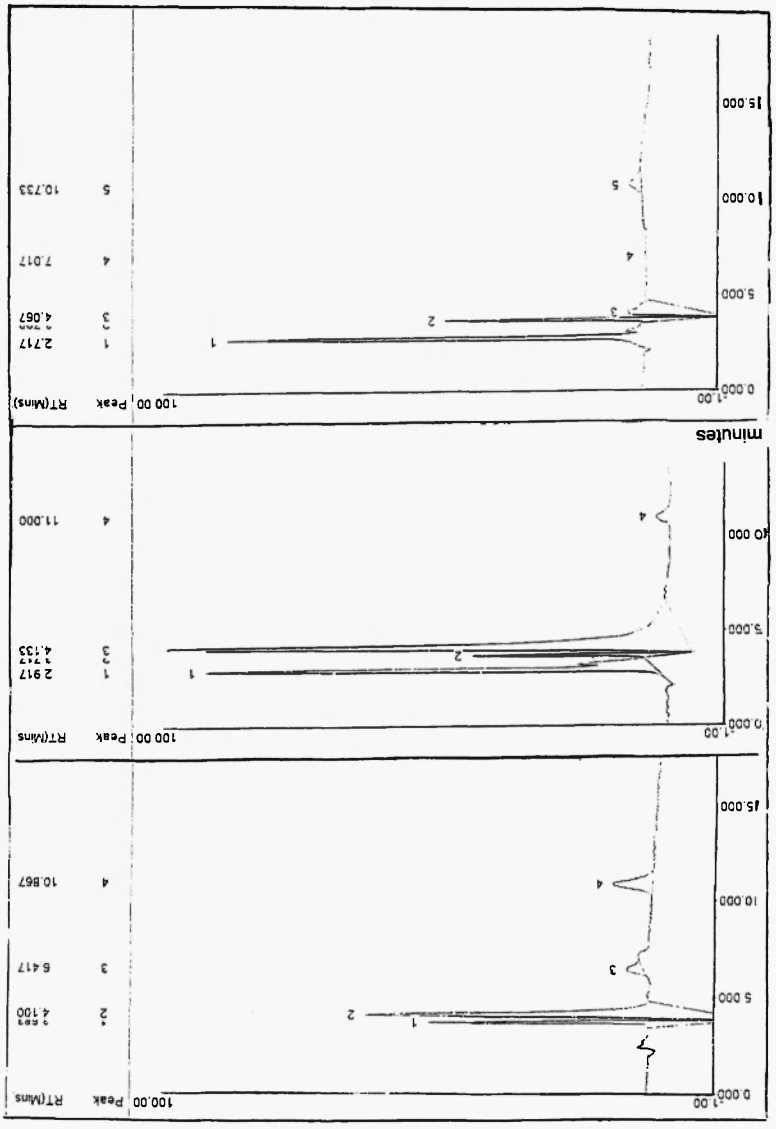
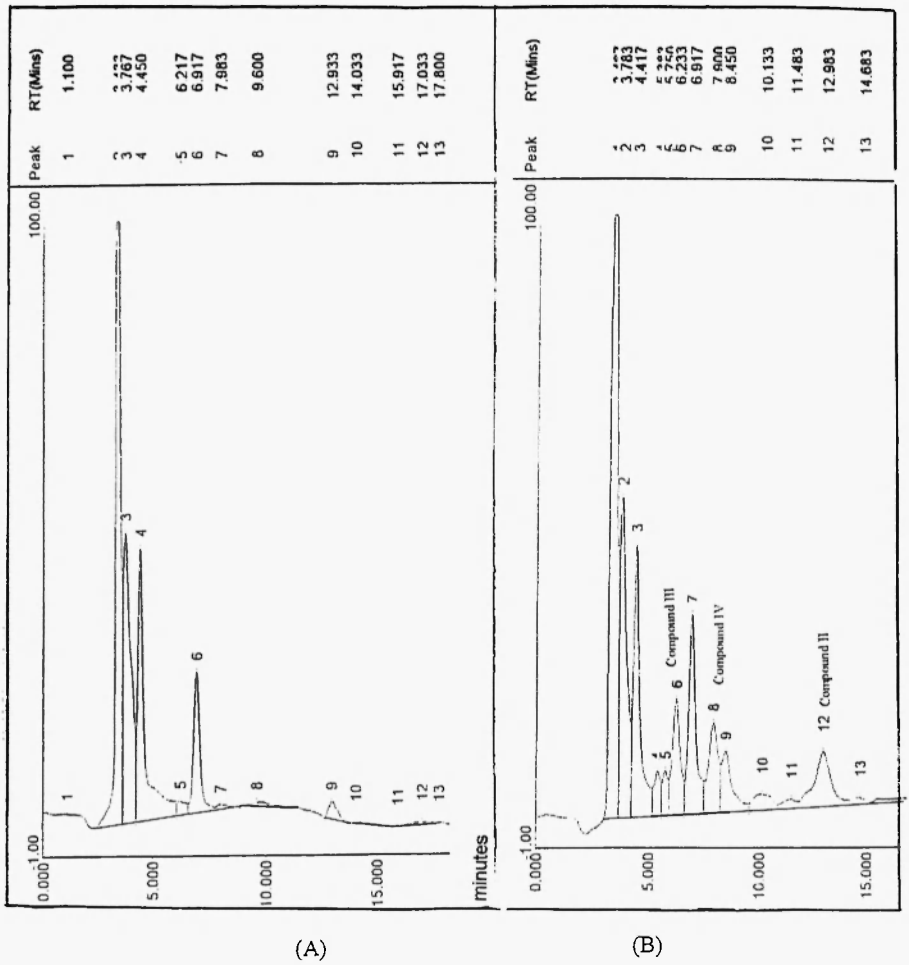


Fig. 3: Chromatograms of the denatured proteins of (**A**) blank plasma, (**B**) sample at 2 hours, (**C**) sample at 3 hours. Chromatographic conditions: mobile phase, methanol-water (50:50, v/v); flow-rate, 0.7 ml/min; injection volume, 20 µl; UV detection at 260 nm.



(A) (B)

Fig. 4: Chromatograms of (**A**) denatured blank plasma, (**B**) denatured plasma samples spiked with known quantities of reference standards. Chromatographic conditions: mobile phase, methanol-water (50:50, v/v); flow-rate, 0.7 ml/min; injection volume, 20 µl; UV detection at 260 nm.

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