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High-pressure liquid chromatographic analysis for identification of in vitro and in vivo metabolites of 4-phenethyl-5-[4-(1-(2-hydroxyethyl)-3,5-dimethyl-4-pyrazolylazo)phenyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione in rats

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Abstract

All azo colorants whose metabolism can liberate a carcinogenic arylamine, are suspected of having carcinogenic potential. Therefore, a new azo compound 4-phenethyl-5-[4-(1-(2-hydroxyethyl)-3,5-dimethyl-4-pyrazolylazo)phenyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione (substrate) was prepared to investigate its in vitro and in vivo biotransformation in rats by HPLC. Chromatographic separation of substrate and its metabolites was performed using a Chromasil C_{18} column. The mobile phase consisted of acetonitrile and water in a linear gradient system. From the biotransformation of this compound, the reduction metabolite 4-(2-phenethyl)-5-(4-aminophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione was identified by comparing it to reference standard by HPLC-DAD. In the in vivo study, identification of the unknown peak which was the *N*-acetylation metabolite was confirmed by LC–MS spectrometry. Besides this, the azo compound was reduced to its corresponding amine in intestinal and cytosolic parts. In addition, oxidation of the methyl group and the phenyl ring, and reduction of azo group to hydrazo were identified in the cytosolic part using LC–MS.

Keywords: Azo; Metabolism; HPLC

1. Introduction

Since the early 1900s, azo compounds, synthesized by the diazotization of aromatic amines, are the largest chemical class of dyes and have been widely used in textile dyeing, color paper printing, and food and leather industries. In addition, they are highly economically feasible in terms of synthesis, firmness and variety of color compared to natural dyes [1,2]. Azo compounds or their metabolic intermediates (e.g. aromatic amines) represent a potential human health risk as some of them have been shown to be potentially mutagenic and carcinogenic [3,4].

Many arylazo compounds are metabolized by biological systems to their corresponding primary amines probably via a hydrazo intermediate. The reduction is catalyzed by mammalian liver microsomal and cytosolic enzymes, intestinal bacteria and other microorganisms [5–8]. Generally, the current in vivo and in vitro metabolism study relates to azo compounds having an aromatic ring such as phenyl, naphthyl and their substituted derivatives [9–11]. When an azo compound is ingested by a mammal, it is reduced by anaerobic intestinal microorganism, and possibly by azo reductase synthesized in the intestinal wall or in the liver, to free aromatic amines. Many aromatic amines (naphtilamin, amino benzene) are carcinogenic and mutagenic, and some widely used bis-azo dyes are reduced to benzidine and its derivatives. However, there is some research on commercially used azo compounds containing a heterocylic ring [12,13]. Tartrazine and polar yellow contain a pyrazole moiety as heterocylic ring and the substrate has a similar structure to these colorants, which are widely used in food and textiles. Therefore, 4-[(1-hydroxyethyl)-3, 5-dimethyl-4-pyrazolylazo)phenyl]-5-

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phenethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (substrate) was synthesized and selected as a model compound to investigate in order to clarify the in vivo and in vitro metabolic pathway of azo compound bearing heterocylic ring as triazole and pyrazole. The substrate was firstly synthesized in our laboratory and the structure was evaluated by spectroscopic methods and elementary analysis. The substrate and its metabolites were determined by HPLC-DAD and LC–MS.

2. Experimental

2.1. Chemicals

The acetonitrile used was HPLC grade, supplied by Merck. Water was distilled and purified through a Milli-Q reagent Water system (Millipore Corp., Bedford, MA). Glucose-6-phosphate dehydrogenase was purchased from Merck. Nicotinamideadenine dinucleotide phosphate monosodium salt and glucose-6-phosphate disodium salt were obtained from Sigma. Potassium dihydrogenorthophosphate and disodium hydrogen phosphate hydrate were both purchased from B.D.H. $MgCl_2{\cdot}6H_2O$ was obtained from FSA Laboratory, UK. The substrate, 4-[(1-hydroxyethyl)-3,5-dimethyl-4′-pyrazolylazo)phenyl]-5-phenethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione was original, the synthesis method and its structure determination are given above. Its various metabolites (M_1, M_2, M_3, M_4) were synthesized in our laboratory (Fig. 1) [14–17].

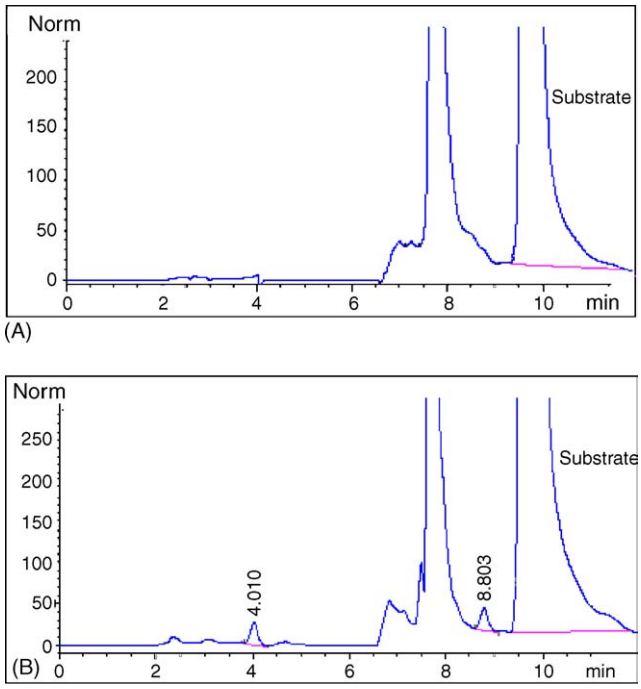

Fig. 2. Typical chromatograms of (A) control with boiled rat cytosole, (B) following extraction of rat cytosole incubation mixture with substrate. M_1: 8.803 min and M_7: 4.010 min. Mobile phase acetonitrile–water with linear gradient system at a flow rate 0.8 ml/min, diode array detection at 254 nm.

2.1.1. 4-[(1-Hydroxyethyl)-3,5-dimethyl-4-pyrazolylazo)-phenyl]-5-phenethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (substrate)

Intermadiate product 1-[4-(1-(2-hydroxyethyl)-3,5-dimethyl-4-pyrazolylazo)benzoyl]-4-phenethylthiosemicarbazide was prepared in four steps from ethyl 4-aminobenzoat following the reported procedures [18,19]. To the intermediate compound (0.005 mol) was added 2N NaOH (15 ml) and the mixture refluxed for 4 h. It was cooled and acidified with HCl. The cyclization product was filtered and recrystallized from ethanol. Elemental analysis was performed on a Perkin Elmer 240C. ^{1}H NMR spectrum was recorded on a Bruker AVANC-DPX 400 spectrometer in DMSO. Analytical calculations were performed for $C_{23}H_{25}N_7OS$, 61.72/61.79 (C), 21.90/21.57 (H), 5.63/5.84 (N); ^{1}H NMR (400 MHz) (DMSO-d_6/TMS) δ ppm; 2.42 (3H,

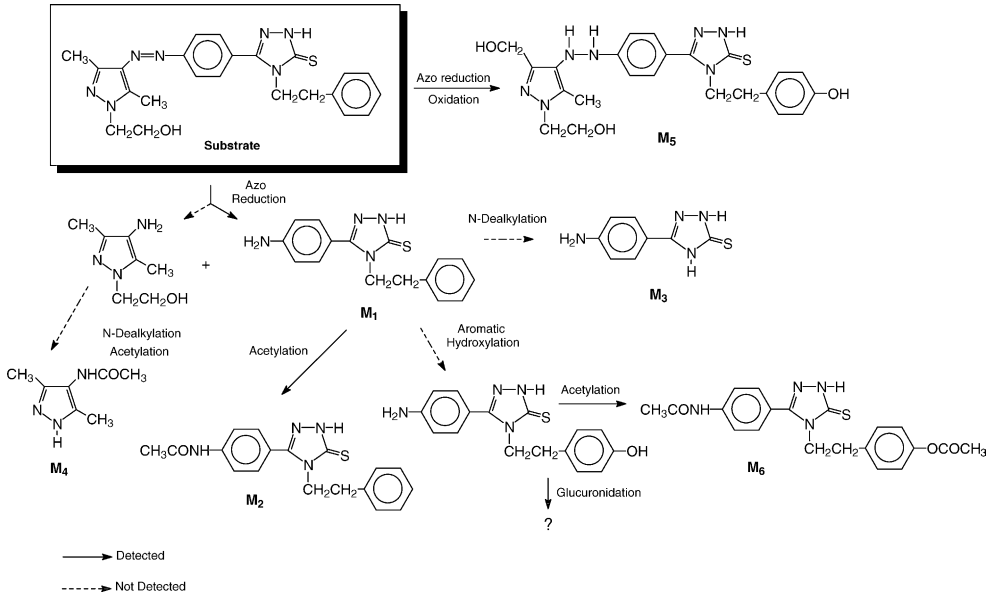

Fig. 1. Suggested metabolic pathways of 4-[(1-hydroxyethyl)-3,5-dimethyl-4′-pyrazolylazo)phenyl]-5-phenethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione.

s, pyrazole C_5 methyl), 2.62 (3H, s, pyrazole C_3 methyl), 2.99 (2H, t, $-CH_2\underline{CH_2}C_6H_5$), 3.76 (2H, q, $-CH_2\underline{CH_2}OH$), 4.12 (2H, t, $-\underline{CH_2}CH_2C_6H_5$), 4.28 (2H, t, $-\underline{CH_2}CH_2OH$), 4.94 (1H, t, $-CH_2CH_2O\underline{H}$), 7.00–7.23 (5H, m, $-CH_2CH_2\underline{C_6H_5}$), 7.58 (2H, d, *m*-position of phenyl to azo); 7.81 (2H, d, *o*-position of phenyl to azo), 14.01 (1H, s, NH proton signal of triazole ring).

2.2. Preparation of microsomal, cytosolic and intestinal fractions

Rat liver microsomes were prepared using the calcium chloride precipitation method as described by Schenkman and Cinti [20]. The cytosolic and intestinal fractions were isolated from untreated Albino Wistar rats (200–250 g) using the same method. Aliquots of microsomal, cytosolic and intestinal fractions were stored at −80 °C until use.

2.3. Incubation of substrate with microsomal, cytosolic and intestinal fractions

Incubations were carried out in a shaking water-bath at 37 °C using a standard co-factor solution consisting of NADP (2 μmol), G-6-P (10 μmol), G-6-P dehydrogenase suspension (1 unit) and aqueous $MgCl_2$ (50%, w/w) in phosphate buffer (0.2 M, pH 7.4, 2 ml). Co-factors were pre-incubated for 5 min to generate NADPH, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (0.5 μmol) in metanol (50 μl). The incubation was continued for 30 min, ter-

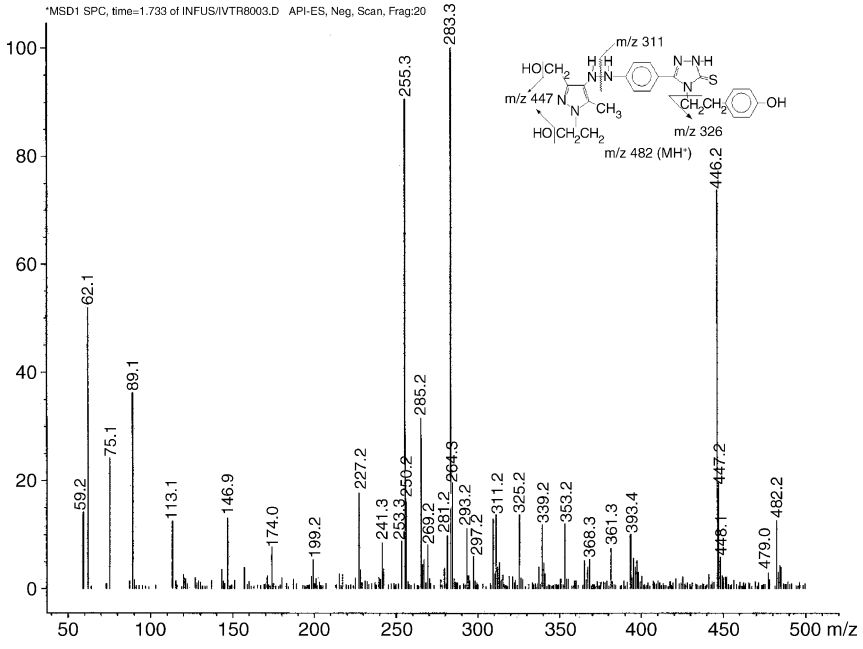

Fig. 3. Electrospray mass spectrum of M_5 obtained from collected fractions with retention time at 4.010 min.

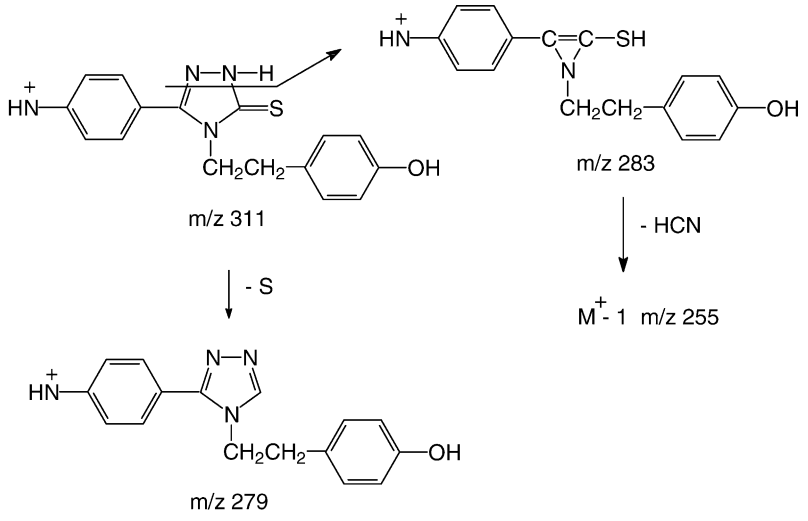

Fig. 4. The mass fragmentation of 1,2,4-triazole-3-thione.

minated and extracted with dichloromethane [21]. The organic extracts were evaporated. The residues were reconstituted in 200 μl of mobile phase for HPLC. The same method, described as above, was used for incubation with cytosolic and intestinal fractions.

2.4. In vivo metabolism study

The experiments were reviewed and approved by the Marmara University School of Medicine Animal Care and Use Committee. All procedures in this study were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (USA) and Declaration of Helsinki. Adult male Wistar rats weighing 250–300 g were used in this research and were housed in humidity and temperature-controlled rooms and allowed standard food and water ad libitum. The substrate (250 mg/kg) was prepared in 5% gum arabica and given orally. Two hundred microliters of blood was collected from the ophtalmic veins of the rats by sterile capillary tube under ether anesthesia and blood samples were drawn 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, 24, 48 and 72 h following the injection.

2.5. Preparation of blood samples

Two hundred microliters of blood was drawn from rats and centrifuged to separate into plasma and blood cells.

2.5.1. Preparation of plasma samples

Plasma samples were prepared according to refs. [22,23]. One milliliter of acetonitrile–methanol (50:50, v/v) was added to 100 μl of plasma to precipitate plasma proteins, and plasma was centrifuged at 10.000 rpm for 15 min at 4 °C. The liquid phase was transferred to another tube and evaporated under nitrogen. The residue was dissolved in 50 μl mobile phase and injected onto HPLC.

2.5.2. Denaturation of plasma proteins

One milliliter of distilled water was added to the lower phase (the precipitated plasma proteins) and this part was heated to 50–60 °C for 3 h to denature proteins. One milliliter of acetonitrile–methanol (50:50, v/v) was added and the mixture centrifuged at 10.000 rpm for 15 min. The liquid phase was transferred to another tube and evaporated under the nitrogen. The residue was dissolved in 50 μl mobile phase and injected onto the HPLC.

2.5.3. Denaturation of red blood cells

Red blood cell samples were prepared using the same procedure as in the preparation of denaturated plasma proteins. After evaporation, the residue was dissolved in 50 μl of mobile phase and a 20 μl aliquot was injected onto the HPLC system.

The samples prepared from blood for each rats at constant time were separately injected onto HPLC. The blank samples of plasma, denaturated plasma proteins and red blood cells were prepared for comparison with samples at 1, 2, 3, 4, 5, 6, 7, 8, 12, 24, 48 and 72 h. In addition, the metabolites were determined by comparing the retention time of peaks obtained with standard solutions.

2.6. Standards

Stock standard solution of M_1, M_2, M_3, M_4 and substrate were separately prepared in acetonitrile (1 mg/ml), 20 μl of the stock standard solution of each compound was transferred to a tube and diluted to 1 ml with mobile phase to give a final concentration of 20 μg/ml. Aliquots of 20 μl of the standard solutions were injected onto the HPLC column. The retention times were observed at 8.812, 8.051, 4.674, 5.733, and 9.683 min for M_1, M_2, M_3, M_4 and substrate, respectively.

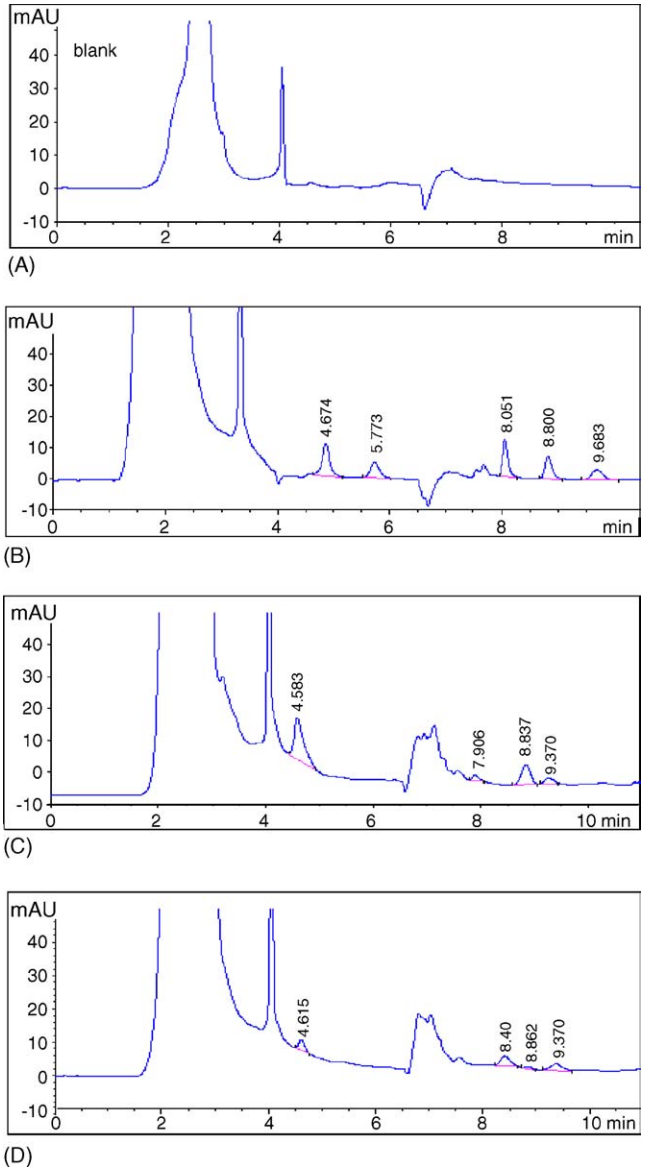

Fig. 5. (A) Blank and (B) spiked sample. M_3: 4.674 min, M_4: 5.773 min, M_2: 8.051 min, M_1: 8.800 min and substrate: 9.683 min. (C) Plasma sample at 3 h M_5: 4.583 min, M_2: 7.906 min, M_1: 8.837 min and Substrate: 9.370 min. (D) Plasma sample at 5 h, M_5: 4.615 min, M_6: 8.400 min, M_1: 8.862 min, substrate: 9.370 min. Mobile phase acetonitrile–water with linear gradient system at a flow rate 0.8 ml/min, diode array detection at 254 nm.

2.7. Instrumentation and chromatographic method

The chromatographic system used to develop this technique was a Hewlett Packard 1100 featuring a column oven at 22 °C (G1316 A), a quaternary pump (G 1311 A), a manual injector (G 1328 B) and DAD detector (G 13115 B) which was set at 254, 310 and 350 nm. Data acquisition was performed using a chromatography software package (Agilent Chemstation version 9.01(1206)).

Chromatographic separation of substrate and its metabolites was performed using Chromasil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size), made of stainless steel. The optimal composition of the mobile phase was achieved by different mixtures of pure acetonitrile and water. The acetonitrile was filtered through a 0.45 μm GH-membrane filter. The mobile phase consisted of elution at 0.8 ml/min with a 3 min linear gradient from 23% acetonitrile/77% water to 70% acetonitrile/30% water, followed by 9 min 70% acetonitrile/30% water and then the composition was returned to the initial conditions (12 min); also the column was equilibrated for 15 min before the next injection. The volume injected into the chromatographic system was 20 μl.

2.8. LC–MS

The unknown metabolite's peaks (M_5, M_6, O or S-glucuronide metabolite, with constant retention times) were collected owing to the lack of synthetic standards, and the confirmation of these metabolites was elucidated by mass spectrometry. The mass spectrometry was performed using Agilent 1100 MSD spectrometer in the electrospray mode by Turkish Scientific and Technical Research Council (TUBİTAK) laboratory.

3. Results and discussion

The in vivo and in vitro metabolism pathway of 4-[(1-hydroxyethyl)-3,5-dimethyl-4′-pyrazolylazo)phenyl]-5-phenethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (substrate) was investigated. The substrate and suggested metabolites are shown in Fig. 2.

3.1. In vitro metabolism

The substrate was incubated with microsomal, cytosolic and intestinal fractions of rats. Substrate was observed, but no metabolites were detected in the microsomal part.

After incubation with the cytosolic fraction, a peak was detected at the same HPLC retention time of 8.803 min as the synthetic standard of M_1, which arises naturally from reduction of azo group (Fig. 2). In the chromatogram, a peak obtained from collected fractions M_5 with the retention time of 4.010 min produced API-ES mass spectrum with a molecular ion at *m*/*z* 482 ($M^+ + 1$) and principal fragment ions at *m*/*z* 447, 446, 326, 311, 283, 255 as shown in Figs. 3 and 4. These data confirmed that oxidative hydroxylation of both the methyl group at position three of pyrazole and the phenyl ring, and reduction of the azo group to hydrazo had occurred. It was reported that, methyl group oxidation was observed at the third position of 3,5-dimethylpyrazole [24]. In addition, the diagnostic ions proved that oxidative hydroxylation took place at the methyl group rather than the sulphenic acid in the triazole ring. This is more likely since it was previously confirmed that the 1,2,4-triazoline-3-thione type of substrate did not produce any sulphenic acid metabolites [25]. Also the presence of M_1 in cytosolic fraction was confirmed by UV analysis. In addition to the cytosolic frag-

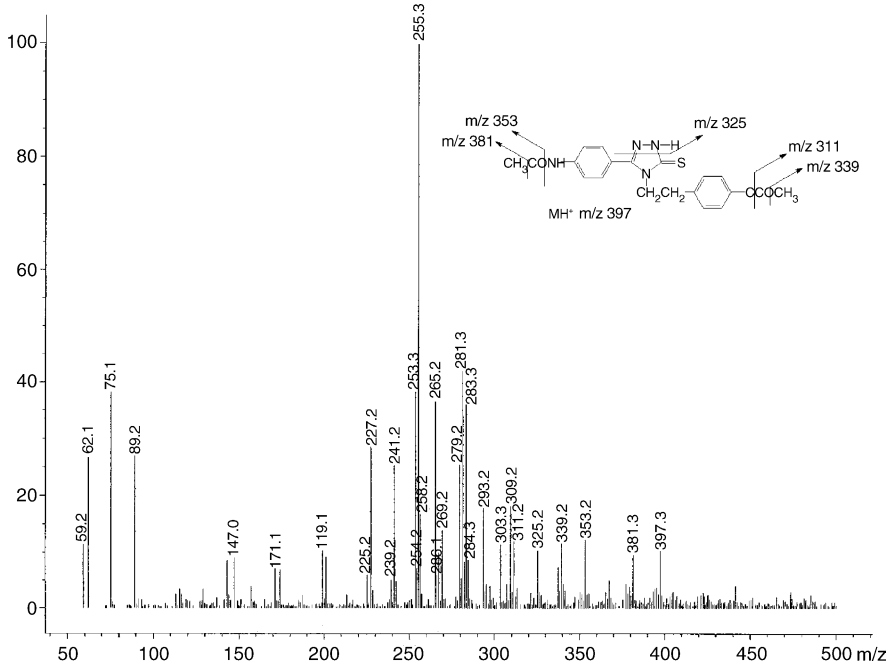

Fig. 6. Electrospray mass spectrum of M_6 obtained from collected fractions with retention time at 8.400 min.

mentation, substrate and azo reduction metabolite (M_1) were identified from the intestinal part.

3.2. In vivo metabolism

The substrate was dissolved in 5% gum arabica and rats were administered a single oral dose of 250 mg/kg. Blood was collected at 1, 2, 3, 4, 5, 6, 7, 8, 12, 24, 48 and 72 h after dosing. Blank and 20 μg/ml of each standard were spiked with blank plasma as shown in Fig. 5.

It was found that the substrate was converted to its reduction metabolites M_1 and M_4 but only M_1, 4-(2-phenethyl)-5-(4-aminophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione, was detected in plasma between 1 and 8 h (except 7 h) after administration, and also the substrate was identified in plasma at 1–6 h intervals. Furthermore, the substrate was not observed at 7, 8, 12, 24, 48 or 72 h. Acetylated triazole amine, M_2 was eluted at the same retention time as the synthetic standard with at 7.906 and 7.947 min with a blood collection time at 3 and 4 h, respectively (Fig. 5).

The unknown peaks detected at 2, 3, 4, 5, 6 and 7 h with the retention time 4.583 ± 0.021 min were analyzed by Mass spectrometry. Molecular ion was observed at m/z 489.0 and diagnostic ions ($M^+ - 1$), at m/z 311 (loss of 177 fragment from molecular ion), 283 and 255. It was supposed that these peaks could be O-glucuronide or S-glucuronide of substrate according to the literature [26].

It was thought that the peak with a retention time of 8.400 min was the diacetylated derivative M_6 at 5 h (Fig. 5). The presence of M_6 was confirmed by mass spectrometry, which identified a molecular ion at m/z 397 (MH^+) and fragments at m/z 381, 353, 339, 325 and 311 as shown in Fig. 6. The other fragments at m/z 283, 279 and 255 were illustrated as in Fig. 4.

In this study, the reason for the denaturation of plasma proteins and blood cells was that some metabolites could be bound as part of a red blood cell and protein cake, especially substituted 1,2,4-triazole-3-thione whose metabolites had been previously detected in these denaturated parts [15]. It was thought that the azo reduction metabolite (M_1) could be found in these parts, so the denaturated plasma protein and red blood cell samples were prepared with this in mind. Plasma proteins were obtained from the centrifuged plasma and were denatured by heating and then injected onto HPLC. Nevertheless no substrate or metabolites were detected. In addition, denatured red blood cell samples were analyzed but no peaks were found, when compared to blank denaturated red blood cells.

4. Conclusion

In a conclusion, this work clarified the metabolism of 4-[(1-hydroxyethyl)-3,5-dimethyl-4′-pyrazolylazo)phenyl]-5-phenethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione in vitro and in vivo. It was shown that the azo compound in rats was reduced into corresponding amine derivatives. In addition, oxidation of the methyl group and the phenyl ring, and reduction of the azo group to hydrazo were identified in cytosolic part using LC–MS.

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