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# High Performance Liquid Chromatographic and Biological Studies of 2-[(4-Methyl-2-nitrophenyl)azo]-3-oxo-N-phenylbutanamide (Yellow Pigment)

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A simple reverse phase high performance liquid chromatographic (HPLC) method was developed for the quantitative determination of 4-methyl-2-nitroaniline (MNA) a potential reduction product of 2-[(4-methyl-2-nitrophenyl)azo]-3-oxo-N-phenylbutanamide (pigment yellow 1, PY1). Quantifications were carried out by integrations of the peak areas using internal standardization method with N-(2,4-dichlorobenzoyl)-aniline (DCBZA) as an internal standard. The proposed dye (PY1) and its potential reduction product, 4-methyl-2-nitroaniline (MNA) and the internal standard were separated by a reverse phase HPLC system. The dye was then incubated with NADH to investigate its possible reduction into the corresponding primary amine (MNA). However, no reduction was observed. The proposed compound was also incubated with rat microsomal preparations fortified with NADPH. In both experiments, compounds in the incubates were extracted into dichloromethane (DCM) and finally evaporated under nitrogene. No metabolic reduction to MNA was observed as well. This indicates the *in vitro* stability of PY1 and hence safety of the dye in terms of potential toxicity from this reduction product.

**Key Words: Azo dyes, *In vitro* metabolism, Printing inks, Azo reduction, 2-[(4-Methyl-2-nitrophenyl)azo]-3-oxo-N-phenylbutanamide, HPLC.**

## INTRODUCTION

Many azo compounds are used as colouring agents in a variety of products such as drugs and cosmetics, textile, paper, food *etc*.[1,2]. A number of azo compounds are also used in the manufacure of printing inks[2,3]. In the present study, a prototype compound derived from this class, 2-[(4-methyl-2-nitrophenyl)azo]-3-oxo-N-phenyl-butanamide (pigment yellow 1, PY1), was selected for studying the qualitative and quantitative determination of its reduction product 4-methyl-2-nitroaniline (MNA) by HPLC. Pigment yellow 1 is a commercially available pigment which is used in the formulation of inks requiring fulltone lightfastness and alkali resistance[3]. It was also planned to establish whether the reduction into MNA would occur with NADH

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and also with hepatic rat microsomal preparations fortified with NADPH using PY1 as a substrate (Fig. 1).

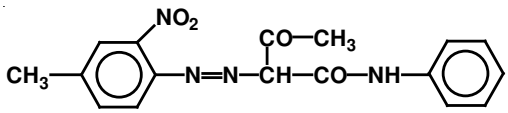


Fig. 1. Structure of 2-[(4-methyl-2-nitrophenyl)azo]-3-oxo-N-phenylbutanamide (pigment yellow 1, PY1)

It was previously reported that nicotinamide adenine dinucleotide (NADH), an important co-factor for the metabolism of organic compounds, non-enzymatically reduced a variety of azo dyes to generate the corresponding amines[4]. This reaction was reported to be pH-dependent and increased with decreasing pH[4]. In the present study, it is aimed to establish whether this previously observed reductive reaction would occur in the case of PY1 in the presence of NADH. The literature search revealed that studies on *in vitro* metabolism of azo compounds are quite rare[5,6]. Another aspect of the study was therefore to investigate if any metabolic reduction occured in the presence of rat microsomal preparations fortified with NADPH. This is very important because the azo reduction in the organism often results in the formation of the corresponding primary amines with potential toxicity[7]. It was previously reported that phenylazo-2-naphthol and the corresponding metabolic reduction products were of genotoxic or carcinogenic effects[8-11].

## EXPERIMENTAL

The proposed dye, PY1, was kindly provided from DYO Company, Izmir, Turkey. 4-Methyl-2-nitroaniline (MNA) was purchased from Aldrich. N-(2,4-dichlorobenzoyl)-aniline (DCBZA) was previously synthesised in our laboratory and its melting point was uncorrected[12]. Glucose-6-phosphate dehydrogenase (G6PD) was purchased from Boehringer Manheim Corporation Ltd. UK, glucose-6-phosphate disodium salt (G-6-P) from British Drug House (Dorset, UK), nicotinamide adenine dinucleotide reduced form (NADH) and nicotinamide adenine dinucleotide phosphate disodium salt (NADP) from Sigma Ltd. UK. All solvents and other chemicals were obtained from Merck.

**High-performance liquid chromatography (HPLC):** An Agilent 1100 quaternary pump was used for solvent delivery (Agilent Technologies, Germany). The injector was a Rheodyne Model 7725i with a 20 µL injection loop. Separation of the components was achieved by 4.6 mm × 150 mm long (5 µm) Zorbax Eclipse XDB-C8 (Agilent) column maintained at 25 ºC. Signals were monitored by Diode-Array detector The compounds were eluted with a mobile phase of 67:33, v/v acetonitrile:water at a flow rate of 1 mL/min. They were detected by their absorbance at 220 nm. The retention times of the compounds under these conditions were 7.73 min for PY1 and 2.59 min for MNA and 3.39 min for the internal standard (DCBZA) (Fig. 2a).

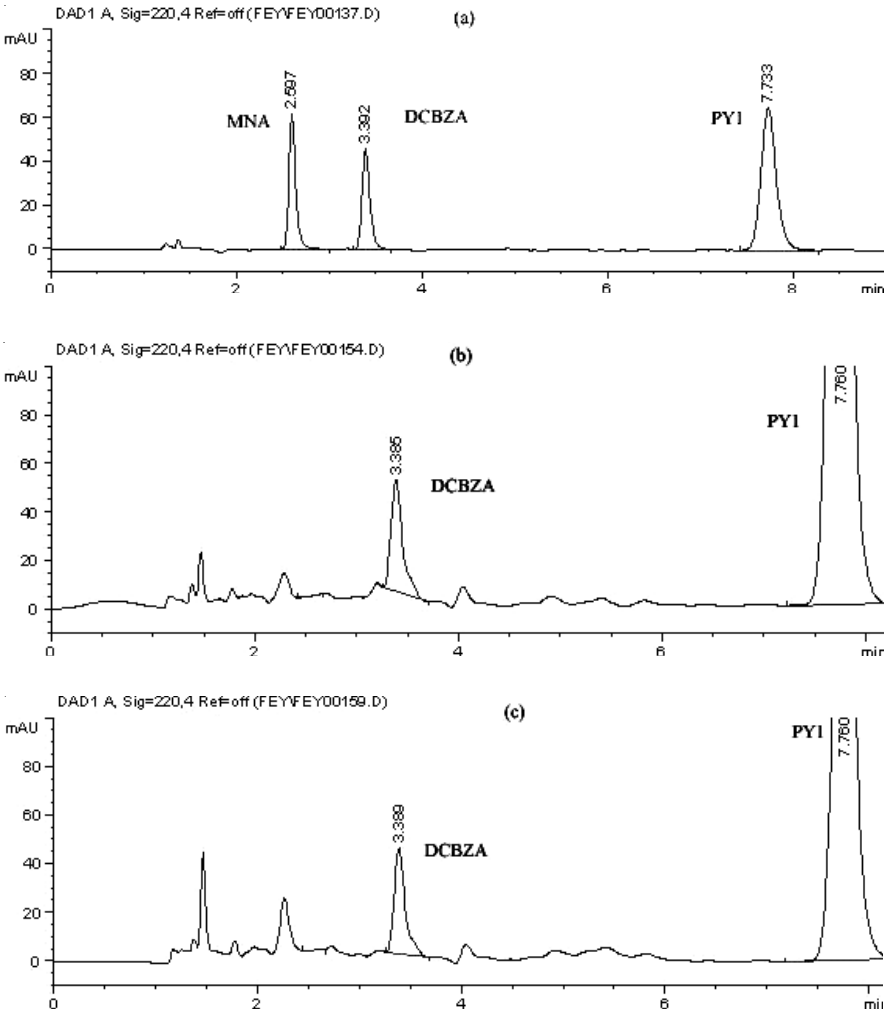


Fig. 2. (a) HPLC chromatogram from authentic standards; (b) from NADH experiment with PY1 as a substrate at pH 7.4; (c) from NADH experiment with PY1 as a substrate at pH 3.5

**Preparation of stock solutions and calibration samples for quantitative determination of MNA:** The stock solutions of PY1 (80 μg/mL), MNA (20 μg/mL) and the internal standard (DCBZA) (100 μg/mL) were freshly prepared in acetonitrile. The concentration ranges were 1.65-39.6 nmol/mL for MNA and 6-84 mmol/mL for PY1 at 220 nm. The eight dilutions were made from these stocks to their volumes with acetonitrile. Each volume has contained 22.6 mmol/mL of internal standard. Then, injections from each volume were made into HPLC in 20 μL. The calibration graphs were plotted using the internal standardization method by integrations of the peak area ratios for both PY1 and MNA.

**The reaction of PY1 with NADH:** For the reactions of PY1 with NADH, the azo dye (1 μmol in 150 μL acetonitrile/tube) and NADH (1 μmol) were mixed either in a 0.2 M pH 7.4 phosphate buffer or in a pH 3.5 acetate buffer (1 mL). They were incubated in a shaking water bath at 37 ºC for 0.5 h. Controls were run simultaneously with a substrate and buffer solutions (without NADH). The reaction was stopped by extraction with dichloromethane (2 mL × 5mL). The extracts were evaporated to dryness at 20 ºC using a stream of $N_2$. Dry incubates were reconstituted in 200 μL of acetonitrile for HPLC analysis, mixed with vortex and centrifuged at 10000 rpm and the supernatant was injected into HPLC in 20 μL.

***In vitro* hepatic microsomal metabolism of PY1:** Hepatic washed male wistar rat microsomes were prepared at 0 ºC using the calcium chloride precipitation method[13]. Incubations were carried out in a shaking water bath at 37 ºC for 0.5 h using a standard co-factor solution at pH 7.4. Co-factor generating solutions consisting of NADP (1 μmol), glucose-6-phosphate (5 μmol), glucose-6-phospate dehydrogenase (1/2 unit), magnesium chloride (50 %, w/w) (10 μmol) prepared in phosphate buffer (1 mL, 0.2 M, pH 7.4) were preincubated for 5 min before addition of microsomes (1 mL) equivalent to 0.5 g original liver and substrate (1 μmol in 150 μL acetonitrile/flask). Controls which were run simultaneously with normal incubations comprised (1) substrate, cofactors and denaturated microsomal preparation (previously heated in boiling water for 10-15 min), (2) substrate, buffer solution (pH 7.4) (without cofactors) and microsomal preparation. Metabolic reactions were stopped by extraction with dichloromethane (2 mL × 5 mL) The metabolic extracts were evaporated to dryness at 20 ºC using a stream of $N_2$. Dry incubates were reconstituted in 200 μL of acetonitrile for HPLC analysis.

## RESULTS AND DISCUSSION

Reverse-phase HPLC systems and mobile phases including buffer solutions with the gradient elution were mostly used for the analysis of azo dyes[13-20]. In the present study, PY1, its potential reduction product (MNA) and the internal standard (DCBZA) were separated using a simple isocratic reverse-phase HPLC system with a mobile phase without any buffer solution. The compounds were eluted in reasonable retention times (Fig. 2a). The present quantitative HPLC method allows the rapid and reliable determination of both MNA and PY1. The calibration curves were linear over the ranges 1.65-39.6 nmol/mL ($y = 0.0272x - 0.0146$; $R^2 = 0.9980$) for MNA and 6-84 nmol/mL ($y = 0.0364x - 0.1027$; $R^2 = 0.9915$) for PY1.

Following incubation and extraction of PY1, the HPLC results showed that NADH did not reduce this azo dye in both buffer solutions (Figs. 2b and c).

This was contrary to the previous experiments on sulphonic acid-substituted phenyl-azo-2-naphthol derivatives which were reduced to the corresponding amines by NADH[4]. Following *in vitro* microsomal metabolism of PY1, no reduction into MNA was observed as well (Fig. 3b). In addition, no enzymatic hydrolysis of PY1

from the aromatic amide moiety was observed with HPLC since no aniline with a retention time of 2.12 (Fig. 3a) was observed by HPLC (Fig. 3b). Similarly, no other peaks were seen in control tubes (Figs. 3c and 3d).

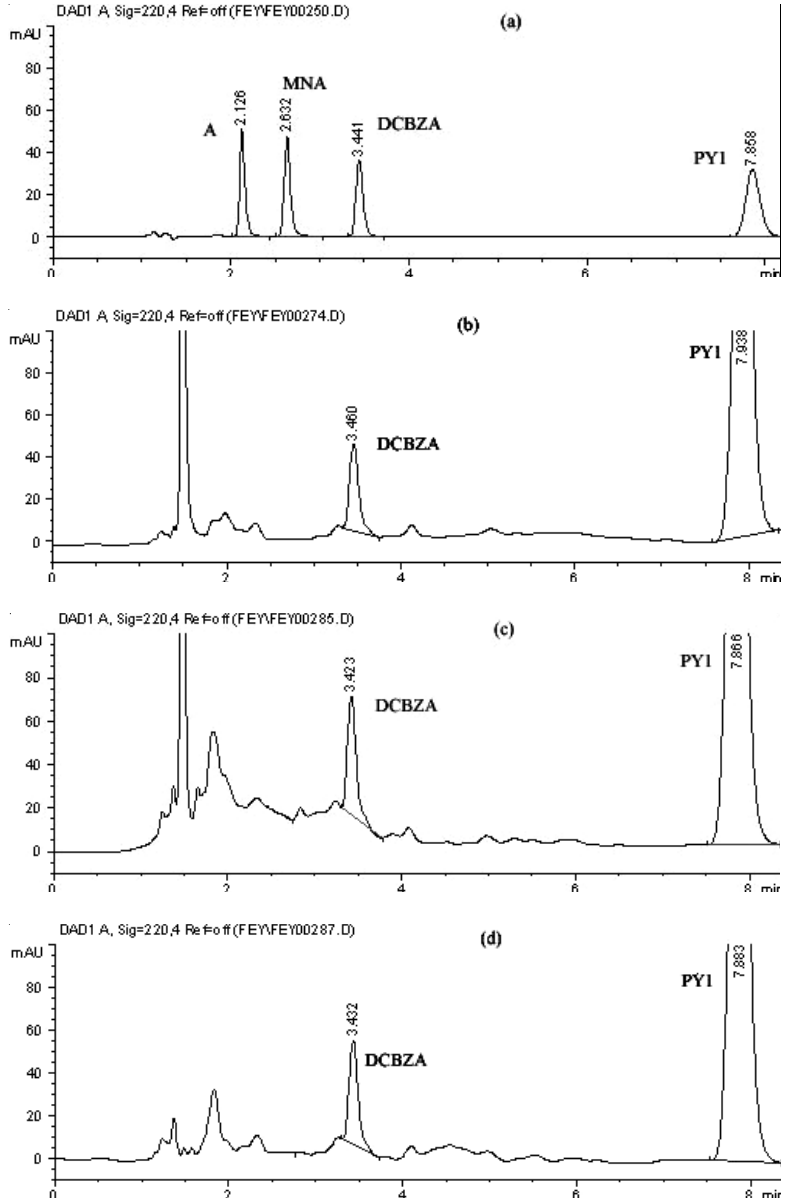


Fig. 3. A reversed phase HPLC chromatogram obtained (a) from reference standards (b) following extraction of male rat microsomal incubation mixture with PY1 (c) from control tube with boiled microsomes (d) from control tube without cofactors (A = aniline, see text for HPLC conditions and abbreviations)

It is well known that the degree to which an azo group is reduced depends on the electron density around the -N=N- bond. In present case, the carbonyl groups on the aliphatic carbon atom or the nitro group on the aromatic ring may stabilize the azo bond because of its electronic effects rather than the steric hinderance on the structure[21]. It may be concluded that the observed biochemical stability of PY1 may eliminate the risks of potential toxicity from reduction products. The present HPLC procedure can be used in a variety of applications with PY1 in fields such as industrial process development/quailty control, forensics and toxicology. Experiments are under way in our laboratory to determine the chemical and biochemical fate of different kind of azo structures in the organism to help for their safer usage in the environment.

## REFERENCES

1. J.C.W. Maynard, in ed.: J.A. Kent, Handbook of Industrial Chemistry, Van Nostcand Reinhold, New York, pp. 809-61 (1983).
2. M. Clayton, (Ed. R.H. Leach, C. Armstrong, J.F. Brown, M.J. Mackenzie, L. Randall and H.G Smith) The Printing Ink Manual, VNR International, Berkshire, England, edn. 4, Ch. 4, pp. 109-281 (1988).
3. NPIRI Raw Materials Data Handbook, National Printing Ink Research Institute, Pigments, Vol. 4, pp. 3-19 (1983).
4. S. Nam and V. Renganathan, *NADH Chemo.*, **40**, 351 (2000).
5. M.H. Koh and J.W. Gorrod, *Drug Metabol. Drug Interact.*, **7**, 253 (1989).
6. B. Kaymakcioglu, E. Oruc, S. Rollas and M. Ulgen, *Drug Metabol. Drug Interact.*, **15**, 107 (1999).
7. W.G. Levine, *Drug Metabol. Rev.*, **23**, 253 (1991).
8. K.T. Chung, *Mutation Res.*, **114**, 269 (1983).
9. P. Moller and H. Wallin, *Rev. Mutation Res.*, **462**, 13 (2000).
10. S. Tsuda, N. Matsusaka, H. Madarame, S. Ueno, N. Susa, K. Ishida, N, Sekihashi, K. Kawamura and Y.F. Sasaki, *Mutation Res.*, **465**, 11 (2000).
11. L. Sztandera, A. Garg, S. Hayik, K.L. Bhat and C.W. Bock, *Dyes Pig.*, **59**, 117 (2003).
12. M. Ulgen and J.W. Gorrod, *J. Facul. Pharm. Ist.*, **30**, 1 (1994).
13. J.B. Schenkman and D.L. Cinti, *Methods Enzymol.*, **52**, 83 (1978).
14. A. Shan, D. Harbin and C.W. Jameson, *J. Chromatogr. Sci.*, **26**, 439 (1988).
15. M. Chen, D. Moir, F.M. Benoit and C. Kubwabo, *J. Chromatogr.*, **825**, 37 (1998).
16. J.A. Zlotnick and F.P. Smith, *J. Chromatogr.*, **733**, 265 (1999).
17. M. Perez-Urquiza, M.D. Prat and J.L. Beltran, *J. Chromatogr. A*, **871**, 227 (2000).
18. E. Seclaman, A. Sallo, F. Elenes, C. Craamareanu, C. Wikete, S. Timofei and Z. Simon, *Dyes Pig.*, **55**, 69 (2002).
19. A. Rehorek, K. Urbig, R. Meurer, C. Schafer, A. Plum and G. Braun, *J. Chromatogr. A*, **949**, 263 (2002).
20. C. Baiocchi, M.C. Brussino, E. Pramauro, A.B. Prevot, L. Palmisano and G. Marci, *Int. J. Mass Spect.*, **214**, 247 (2002).
21. A. Pielesz, I. Baranowska, A. Rybak and A. Wlochowicz, *Ecoto. Environ. Safety*, **53**, 42 (2002).

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