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Research Paper

The inhibitory effect of newly synthesized ethnyl-derivatives on the activity of cytochrome P_{450} isoenzymes in rat liver microsomes *in-vitro*

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Abstract. The inhibitory effect of six newly synthesized ethnyl-derivatives on the activity of cytochrome P_{450} isoenzymes were investigated in rat liver microsomes. 6 µM concentration of each compound against different concentrations of the substrates (1, 2, 4, 8 and 16 µM) was used: 7-ethoxy resorufin "EROD" in β-Naphthoflavone "β-NF"- induced CYP 1A isoenzyme and 7-pentoxy resorufin "PROD" in phenobarbital "PB"- induced CYP 2B were used. It was found that all the compounds inhibited the activity of CYP 1A at the high concentration. CYP 2B was inhibited by the same compounds except for the first one. The binding experiments was carried out to delineate the exact nature of binding between these compounds and CYP- isoenzymes (CYP 2B; 1A; 3A; 2E1 and 2D1) in PB, β-NF, DEX, pyrazol-induced and control rat liver microsomes respectively. It was found that, only compounds III and IV at high concentration "80 µM" revealed an interaction with the active site of CYP_{450}. A type I binding for compound IV was obtained. In contrast, compound III showed a type II or reverse type I binding. The enzyme kinetics "V_{max} and K_m" of the different inhibitors were investigated. The inhibitory effect of the compounds III and IV at different concentrations (10, 20, 40 and 80 µM) in untreated rat liver microsomes on the Dex-O-demethylation was studied. It was found that neither compound III nor IV had no effect except the concentration of 80 µM 0f compound IV which exert a complete inhibition, as well as, the effect of these compounds at the concentration on of 6 µM against different concentrations of erythromycin (2.5, 5,10,15, 20 and 25 m) indicative CYP 3A1 activity on the dealkylation reaction in Dex-induced rat liver microsomes. Also, the results revealed that compounds III, IV, V, VI and VII inhibited the activity of CYP 2E1. It was concluded that these derivatives proved to be variable potential inhibition on CYP_{450} isoformes in rat liver microsomes.

Keywords: Cytochrome P_{450}, ethnyl-derivatives, P_{450} inhibitor, microsomes, cytotoxicity.

INTRODUCTION

Cytochrome P_{450} (CYP) enzymes are found in microorganisms, plants, and mammals. P_{450} enzymes primarily catalyze the chemically challenging reaction of C-H bond oxidation but other activities are known, including dealkylation, desaturation, C-C bond formation, dehydration, decarboxylation and skeletal rearrangements (Ortiz, 2005). By virtue of these varied activities, P_{450} enzymes are involved in biological process as diverse as carbon source assimilation, biosynthesis, and biodegradation of endogenons compounds, antibiotics synthesis, xenobiotic and drug metabolism, bioprotection, and plant insect interactions (Ortiz, 2005).

Drug-drug interactions have become an important issue in health care. It is now realized that many drug-drug interactions can be explained by alternation in the metabolic enzymes that are present in the liver and other extra-hepatic tissues. Many of the major pharmacokinetics interactions between drugs are due to

hepatic cytochrome P_{450} enzymes being affected by previous administration of other drugs. After co-administration, some drugs act as potent enzyme inducers, whereas others are inhibitors (Zakia, 2008).

P_{450} enzymes are involved in biological processes as diverse as carbon source assimilation, biosynthesis and biodegradation of endogenons compounds, xenobiotic and drug metabolism, bioprotection and plant insect interactions. CYP has multiple isozymes with different substrate specificities and the broad ranges of substrates for the hepatic microsomal mixed-function oxidase MFO system is based on the existence of these multiple isozymes. These isozymes have been shown to be the hemoprotein products of the CYP supergene family (Fukushima et al., 2008; Newwsome et al., 2013).

Reactive metabolites are a common product of phase (I) oxidative reactions mediated by cytochrome P_{450} (CYP) - dependent mixed function oxygenases, although also examples of other phase (I) "e.g. flavin-mono oxygenases; FMOs" and phase (II) drug metabolizing reactions have been described (Zhou et al., 2005). The adverse reactions via different inter-related process such as formation of free radicals, oxidation of thiols and covalent binding with endogenous macromolecules, resulted in the oxidation of cellular components or inhibition of normal cellular functions (Vignati et al., 2005; Wang et al., 2009).

The oxidative pathways were mediated by cytochrome P_{450} monooxygenases, the use of P_{450} inhibitors and inducers showed that CYP 1A1, CYP 2B, and CYP 2E1 are involved (Gradolatto et al., 2004).

Various inhibitors of P_{450} were proposed for use drugs (Zakia, 2008). Many of them are characterized by selectivity and high affinity for P_{450} (Matsunaga et al., 2004). It is now realized that many drug-drug interactions can be explained by alterations in the metabolic enzymes that are present in the liver and other extra-hepatic tissues. Many of the major pharmacokinetic interactions between drugs are due to hepatic cytochrome P_{450} ($P_{450\ or}$ CYP) enzymes being affected by previous administration of other drugs. After co administration, some drugs act as potent enzyme inducers, whereas others are inhibitors. However, reports of enzymes inhibition are very much more common. Understanding these mechanisms of enzyme inhibition or induction is extremely important in order to give appropriate multiple-drug therapies. The cytochrome P_{450} (or CYP) isoenzymes are a group of heme-containig enzymes embedded primarily in the lipid bilayer of the endoplasmic reticulum (ER) of hepatocytes, it takes part in the metabolism of many drugs, steroids and carcinogens (Zakia, 2008).

Arecaidine but-2-ynyl esterTosylate "Compound I" and Arecaidine propargyl ester Tosylate “compound II" are piperidine derivatives or acetylene derivatives. The piperidine ring is often used as a template for biologically active compounds (Zeng et al., 1995). Recently, a series of newly synthesized piperidine derivatives were also shown to possess strong antioxidant properties measured as inhibition of lipid peroxidation, hydroxyl radical scavenging activity, and interaction with the stable free radical α, α diphenyl-β-picryl hydrazyl "DPPH" (Alexidis et al., 1995). Acetylene 4-propyl benzene "Compound III" and Acetylene 4-cthyl amine benzene "Compound IV" are acetylene derivatives. 9-ethynyl phenanthrene "Compound V" and 2(1–propynyl phenanthrene "Compound VI" are phenanthrene derivatives Compound VIII has a piperidine ring and pyrimidine ring as well as thiol group and flouro amine cyclohexane group.

The purpose of this study was to investigate the inhibitory properties of different compounds against different cytochrome P_{450} isoforms such as CYP_{450} 1A1; 2B1; 3A1; 2E and 2D1 by using differentially induced rat liver microsomes pre-treated by β-NF" naphthoflavone"; PB" Phenobarbital"; Dex; pyrazol and untreated microsomal fraction respectively. Additionally, the aim of this study was to acquire more information concerning hepatic metabolism of these newly synthesized compounds in rats.

MATERIALS AND METHODS

Materials

Seven drugs were used and recently synthesized at Department of Medicinal chemistry. School of Pharmacy, Aristotelian, University of Thessaloniki (Alexidis et al., 1995). The first compound was arecaidine but -2-ynyl ester tosylate with M.W: 365.44; compound II arecaidine propargyl ester Tosylate, M.W: 351.42; compound III phenyl acetylene propane M.W: 144.217; compound IV phcnyl acetylene 4-ethyl amine, M.W: I45-205; compound V 9-ethynyl phenanthrene, M.W: 2029; compound VI "2-(l-propynyl phenanthrene, M.W: 216.3 and compound VII flouro amino cyclohexane thio pipridine pyrimidene "which is a newly synthetic compound and obtained from Synthetic Chemistry Department, Vrije Universteit. 7-ethoxyresorufm, 7-pentoxy resorufm, and dexamethasone "DEX" were obtained from Sigma Chemical Co, and pyrazol were purchased from Merck. Erythromycin was purchased from the Vrije Universteit Academical Hospital, Amsterdam, Netherlands. ρ-nitrophenol was obtained from Baker Chemicals, Deventer, Netherlands. β-Naphthoflavone " β-NF" phenobarbital "PB" and all other chemicals and solvents were used in analytical grade.

Animals

Pretreatments and isolation of the microsomes

120 male Wister rats weighed 180 to 220 g were

purchased from Harlan CPB (Zeist, Netherlands) and were housed in humidity 50% and temperature controlled rooms at 22°C with a 12 h lighting cycle. Food and water were provided *ad libitum*. The rats were pretreated with β-NF (60 mg/kg, i.p.) once a day for 2 days, with PB (90 mg/kg, i.p.) once a day for 2 days, with pyrazol (250 mg/kg, i.p) once a day for 2 days or with DEX (100 mg/kg, i.p.) once a day for 3 days. Rat liver microsomes were prepared 24 h after the last treatment by differential centrifugation (Lake, 1987; Lundgren et al., 1987) and stored at-80°C until use. Protein concentrations were determined by the method of Appiah-Opong et al. (2007) using bovine serum albumin "BSA" as a standard.

7-mcthoxy; 7-ethoxy; and 7-pentoxy resorufin-O-dealkylase activity (CYP_{450} 1A2, 1A1. and 2B1):

The formation of resorufen after 0-dealkylation of 7'-mcthoxy, and 7-ethoxy resorufm in "B-NF pretreated microsomes" and 7-pentoxyresorufm in "PB pretreated microsomes" which are specific metabolic probes for CYP_{450} 1A2, 1A1 and 2B1, respectively (Burke et al., 1994) were measured according to Burke and Mayer (1974). In a fluorometer cuvette 2 ml, 0.1 M phosphate buffer pH 7.8, 25 µl microsomal suspension (2.2 mg/ml of protein), 25 µl test compound solution "6 µM" dissolved in "buffer or methanol" and 50 µl of the corresponding alkoxyresorufin "1, 2, 4, 8, and 16 µM" were added. A fluorescence of baseline at a 530/586 excitation/emission wavelength was recorded with a Perkin Elmer Model 3000 fluorescence spectrophotometer and a 100 µl aliquot of NADPH (5 mM in buffer) was added to start the dealkylation reaction resorufin formation which was followed for 2 min. The baseline was calibrated by adding l0 µl resorufin (10 µM in ethanol). All measurements were performed in duplicate.

P-nitrophenol hydroxylation ″CYP 450 2E1″:

Aromatic hydroxylation of p-nitrophenol ″PNP″ in pyrazol microsomes, which is catalyzed by CYP_{450} 2E1, was estimated by the formation of p-nitrocatcchol in pyrazol pretreated microsomes (Koop, 1986). Incubation mixtures (1 ml) consisted of 40 µl p-nitrophenol (100 mM in H_2O), microsomal protein (1mg/ml) the test compound by concentration of 6 uM dissolved in buffer and 0.1 M phosphate buffer pH 6.8. After 3 min, pre incubation at 37°C, the reaction was initiated by the addition of 0.1 ml NADPH (10 mM in buffer). Trichloroacetic acid (TCA, 0.5 ml, 0.5 M) was added after 10 min to stop the reaction. After centrifugation, 1 ml of the clear supernatant was mixed with 0.1 ml NaOH (10 N) and the p-nitrocatechol formed was measured spectrophotometrically at 513 nm. All measurements were done in duplicate.

Erythromycin N-demethylation "CYP 450 3A1":

Erythromycin N-demethylation, which in rat liver is catalyzed primarily by CYP_{450} 3A1, was assessed spectrophotometrically by measuring the production of formaldehyde (HCHO) in DEX pretreated rat liver microsomes, according to Wrighton et al. (1985). The incubation mixture (1 ml final volume) contained 0.1 ml $MgCl_2$ (150 mM in buffer), 0.1 ml erythromycin (10 mM in bitter) with different concentrations (2.5, 5, 10, 15, 20 and 25 mM), 0.1 ml microsomal protein, the appropriate amount of test compound at a single concentration of 6 µM in buffer (50 mM phosphate , PH 7.25). The mixture was pre-incubated at 37°C for 3 min, and the reaction was started with 0.1 ml NADPH (10 mM in 1% $NaHC0_3$). After 10 min, the reaction was terminated by the addition of 0.5 ml 12.5% TCA. After centrifugation, 1 ml of clear supernatant was mixed with 1 ml Nash reagent heated at 50°C for 30 min and measured spectrophotometrically at 412 nm. A standard HCHO solution (1 µM /ml) was used for the calibration curve. All measurements were done in duplicate.

Cytochrome P_{450} binding spectra:

An amino DW-2 UV/VIS spectrophotometer was used to record binding spectra of selected compounds by concentration of 80 mM using liner microsomes from rats pretreated with B-NF, PB, Pyrazol or DEX. For the measurements, Tandem cuvettes were used and the protein concentrations were adjusted to 2 mg/ml. A 50 mM potassium phosphate buffer, with 0.1 M. EDTA pH 7.4 was used and all substrates were tested at a final concentration of 1 mM. The incubations were carried out at 25°C and the binding spectra were recorded from 350 to 500 mm (Alexidis et al., 1996).

Analysis of the Dextrometrophan “Dex-m” metabolites by HPLC

The Dex-metabolites were performed against compound III and IV by the concentrations of 10, 20, 40 and 80 M in control microsomal fraction for CYP 2D1 according to Howie et al. (1977). On an HPLC system consisting of a Gilson 305 pump, a separations 759 A detector, set at 254 nm , a Shimadzu C-RIB chromatopack recorder and two C18 Columns”l00x3mm chromatopack” in series. As mobile phase, a 1% aqueous acetic acid/ methanol/ethyl acetate (90:15:0.1) solution was used, and the flow rate was adjusted at 0.4 ml/min. All samples were deproteinized by adding 0.1 ml 12.5% TCA before injection. For the analysis, 20 µl of the supernatant were used.

RESULTS

Inhibition of various P_{450}

The ability of the test compound I to VII, synthesized and

purified recently to inhibit the different P_{450} isoenzymes was tested using liver microsomes from differentially pretreated rats. Figure 1 illustrates the effect of compound III and IV by the concentrations of 0, 10, 20, 40 and 80 µM on Dex-O-dealkylation reaction in control microsomal fraction of rat liver by CYP_{450} 2D1. It is clear that either compound III or IV by the low concentrations "10 and 20 µM "has no inhibitory effect, while the other concentrations "40 and 80 µM" of the two compounds have an inhibitory effect (panel. a,b)

As indicated in panel (c), that the compounds I, II, III, IV, V, VI and VII by the concentration of 6 µM against different concentration different of erythromycin concentrations 2.5, 5, 10, 15, 20 and 25 mM" in Dex-induced rat liver microsomes for 0-dealkylation of the isoenzyme CYP_{450} 3A1 activity, revealed an variability effects. Compounds I, II and III have no inhibitory effect on the enzyme activity, while the compounds IV, V and VI exerted a markedly inhibitory effect on CYP_{450} 3A1 activity. In contrast, compound VII showed inhibitory effect by the low concentrations "10 and 15 mM", while the high concentrations "20 and 25 mM" have no inhibitory effect.

In addition, Figure 2 (panel a) revealed the effect of all compounds by single dose 6 µM on erythromycin-O-dealkylation "2.5, 5, 10, 15, 20, 25 µM" in DEX-induce a rat liver microsomes for 3A1. A line weaver Burk plot of ethoxyresorufin "EROD" by concentrations of 1,2,4,8 and 16 µM in β-NF-induced rat liver microsomes for the activity of CYP_{450} 1A1/1A2 isoenzymes, as indicated in panel (b), the inhibitory effect of compounds I, II, III , IV,V and VI by the concentration of 6 µM on EROD (ethoxyresorufin- O-dealkylation) in β-NF-induced rat liver microsomes for isoezymes CYP 1Al. Different concentrations of the substrate were used (1, 2, 4, 8 and 16 µM). The results revealed that the compounds III and IV have a competitive inhibition with β-NF pretreated microsomal fraction against all the concentrations of the substrate. As indicated in panel (C), compounds I and II exerted an inhibitory effect against the high concentration of ER "ethoxyresorufin" (8 and 16 µM). Figure 3 illustrates the effect of different concentration of pentoxyresorufin 0-dealkylation "PROD". 1, 2, 4, 8 and 16 µM, in PB-induced rat liver microsomes for isoenzymes CYP 2B. It has been found that at the high concentration of the substrate "PR", the activity of the enzymes was increased under the effect of compound I only (panel a). Panel (b) revealed the effect of the compounds I, II, III, IV, V and VI by 6 µM concentration against different concentration of the substrate "PR" (1, 2, 4, 8 and 16 µM) in PB-induced rat liver microsomes for CYP_{450} 2B. It was appeared that compounds II, III and IV, have an inhibitory effect on the activity of isoenzymes, while the other compounds have no inhibitory effect. Panel (c) revealed the standard curve of p-nitrocatechol in different concentration (1, 10, 20, 30, 40, 50 and 60 µM) for hydroxylation reaction in pyrazol-pretreated rat liver microsomes for CYP_{450} 2E1. Panel (d) showed the effect of test compounds (I, II, III, IV, V, VI and VII) by the concentration of 6 µM on p-nitrophenol hydroxylation in pyrazol-pretreated rat liver microsomes for CYP_{450} 2E1 isoenzymes. The compiled data revealed that the compounds III, IV, V, VI and VII have inhibitory effect on the activity of CYP_{450} 2E1, while compounds I and II did not display any effects. The most potent inhibitor was compound VI, then V and, III and IV, while compound VII was less potent.

Substrate binding to cytochrome P450

Binding spectra were recorded for a number of compounds, selected on the basis of the results from the CYP_{450} inhibition studies. For compounds III and IV, double binding spectra were recorded using for differentially pre-treated rat liver microsomes. Compound III revealed a measurable binding to CYP_{450} 2B1/2B2 in PB-pretreated rat liver microsomes. The binding type was binding type II or reverse type I (Figure 4, panel a). Compound IV double binding spectra were recorded using four differentially pretreated rat liver microsomes (PB, β-NF, Dex, pyrazol induced rat liver microsomes). Compound IV exerted a typical type I spectrum, with a minimum at 420 nm and a maximum at 389 nm (Figure 5, panels a, b, c) for PB; B-NF; Dex; Phyrazol-induced rat liver microsomes respectively was observed.

The binding experiment resulted that a type II binding or reverse type I spectrum was recorded for compound III by using PB-pretreated rat liver microsomes for CYP_{450} 2B1. These results indicated a shift of the haem-iron spin equilibrium from low spin to high spin suggesting that the compound III was bound to the haemoprotein moiety which identified as a haem containing enzyme of cytochrome P_{450}. Additionally no P_{450} binding spectra could be recorded any more with compound III in any other differentially pretreated microsomes used. A type I spectrum was recorded for the compound IV using four differentially pretreated rat liver microsomes (PB, β-NF, Dex, Pyrazol-induced), suggesting that a direct interaction of compound IV with the haem-containing enzyme "a haemprotein" of CYP_{450} 2B1, 1A1, 3A1 and 2E1.

Metabolism of the test compounds in liver microsomes

In-vitro metabolism of the test compounds using rat liver microsomes was monitored by HPLC. As shown in Figure 6 (panel a), the spectrum of two concentrations (10 and 20 µM) of compounds III and IV was observed. These chromatograms exerted that no effect was appeared on the isoenzymes of CYP 2D1 in control microsomal fraction panels (b,c) illustrated the effect of high concentration (40 to 80 µM) of the compounds III and IV

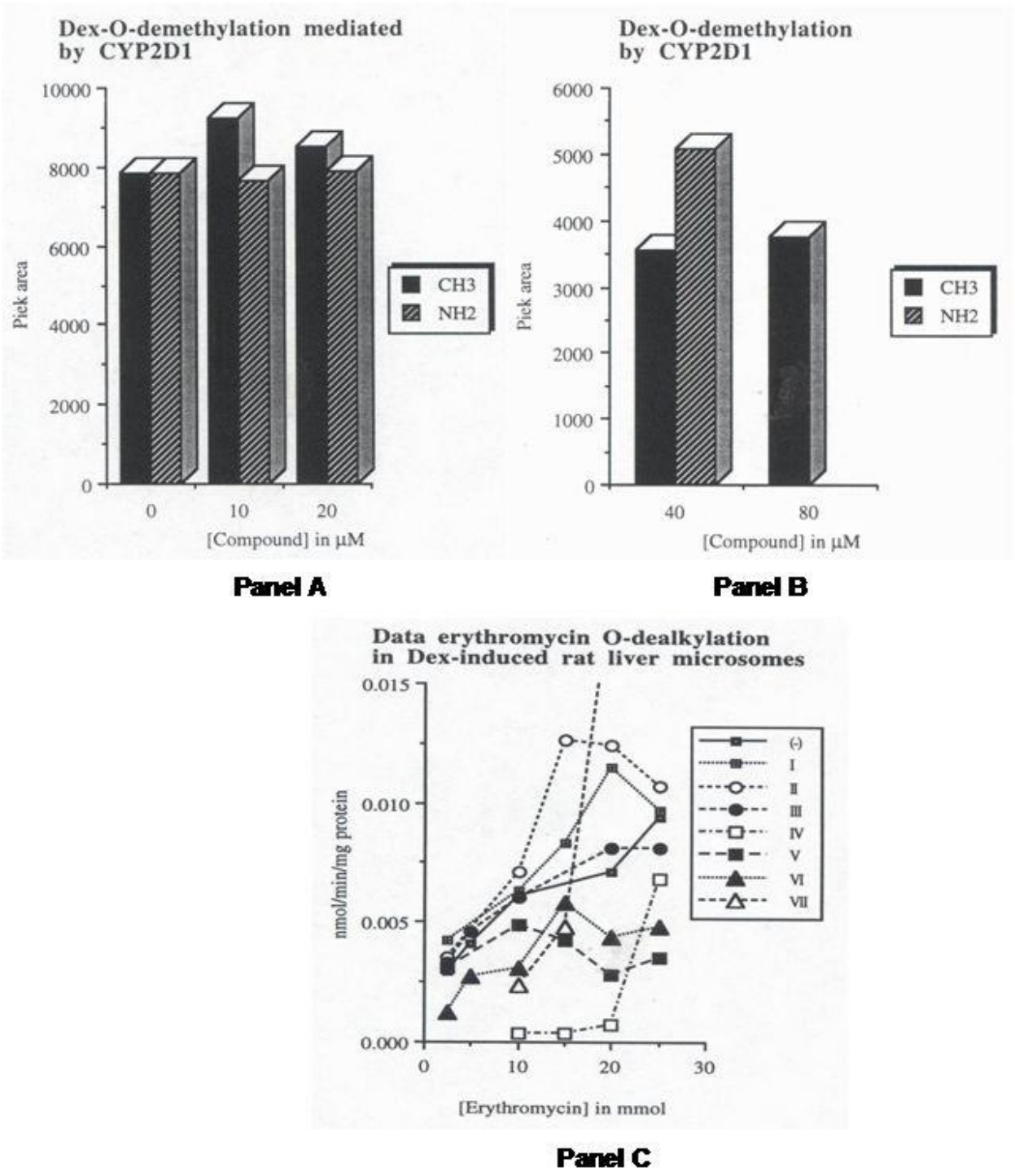
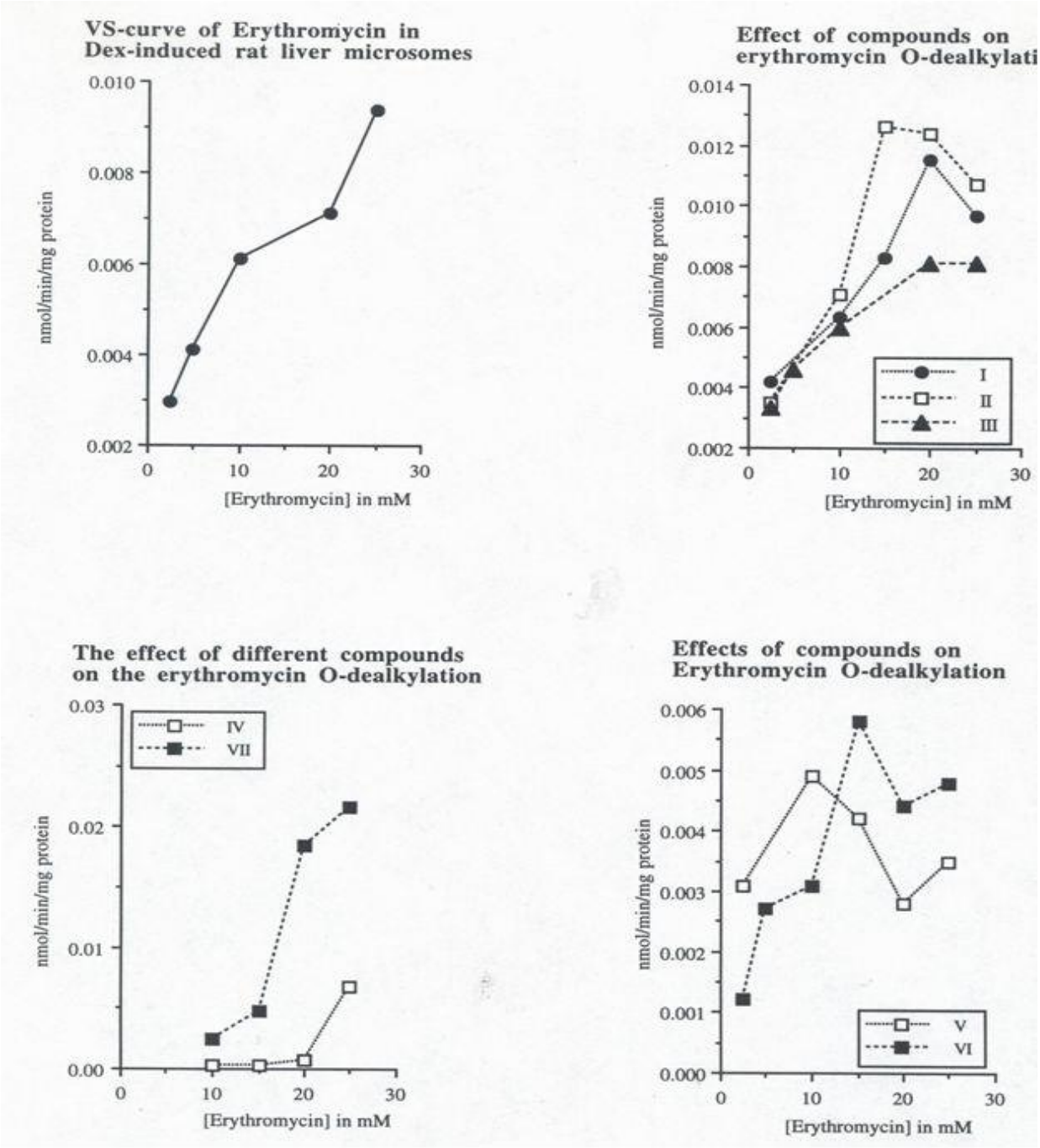


Figure 1. Effect of phenyl acetylene propane "III"; phenyl acetylene ethyl amine "IV by four concentrations (10 and 20 µM), Panel A and (40 and 80 µM), Panel B. Also, using all compounds "I, II, III, IV, V, VII" by single concentration "6 µM" against different concentrations "2.5, 5, 10, 15, 20, 25 mmole" on P_{450} 3A1 in DEX-induced rat liver microsomes by erythromycin O-deakylation "Panel C".

on the activity of CYP_{450} 2D1 in O-dealkylation reaction of control rat liver microsomes. These chromatograms revealed that the most potent inhibitor was compound IV by concentration 80 µM on CYP_{450} 2D1.

Additionally, we examined the inhibitory activity of all compounds in this study by using HPLC experiment. The illustrative chromatograms in panels b and c revealed that, the most potent inhibitor was compound IV by concentration



Panel A

Figure 2. Panel A: Effect of compounds "I, II, III, IV, V, VI and VII" by single concentration (6 µM) on erythromycin O-dealkylation "2.5, 5. 10, 15, 20, 25 mM" in dex-induced rat liver microsomes for CYP 3A1. Panel B and C: Line- weaver-Burk Polt for cytochrome P450-mediated EROD activity in microsomes in β-NF induced rats by different concentrations "1, 2, 4, 8, 16 µM" for CYP_{450} 1A1 and 1A2 activity "Panel B". The inhibitory effect of the compounds by 6µM on ethoyresorufin (ER) in β-NF induced rat liver CYP 1A1 AND 1A2 by different concentration of the substrate "1, 2, 4, 8 µM" "Panel C".

of 80 µM on CYP_{450} 2D isoenzymes in control rat liver microsomes for O-dealkylation reaction. The concentration "10 and 20 µM" of compounds III and IV exerted no inhibitory effect on CYP_{450} 2D1.

DISCUSSION

In this investigation six newly synthetically compounds were tested as far as their inhibitory potency and selectivity

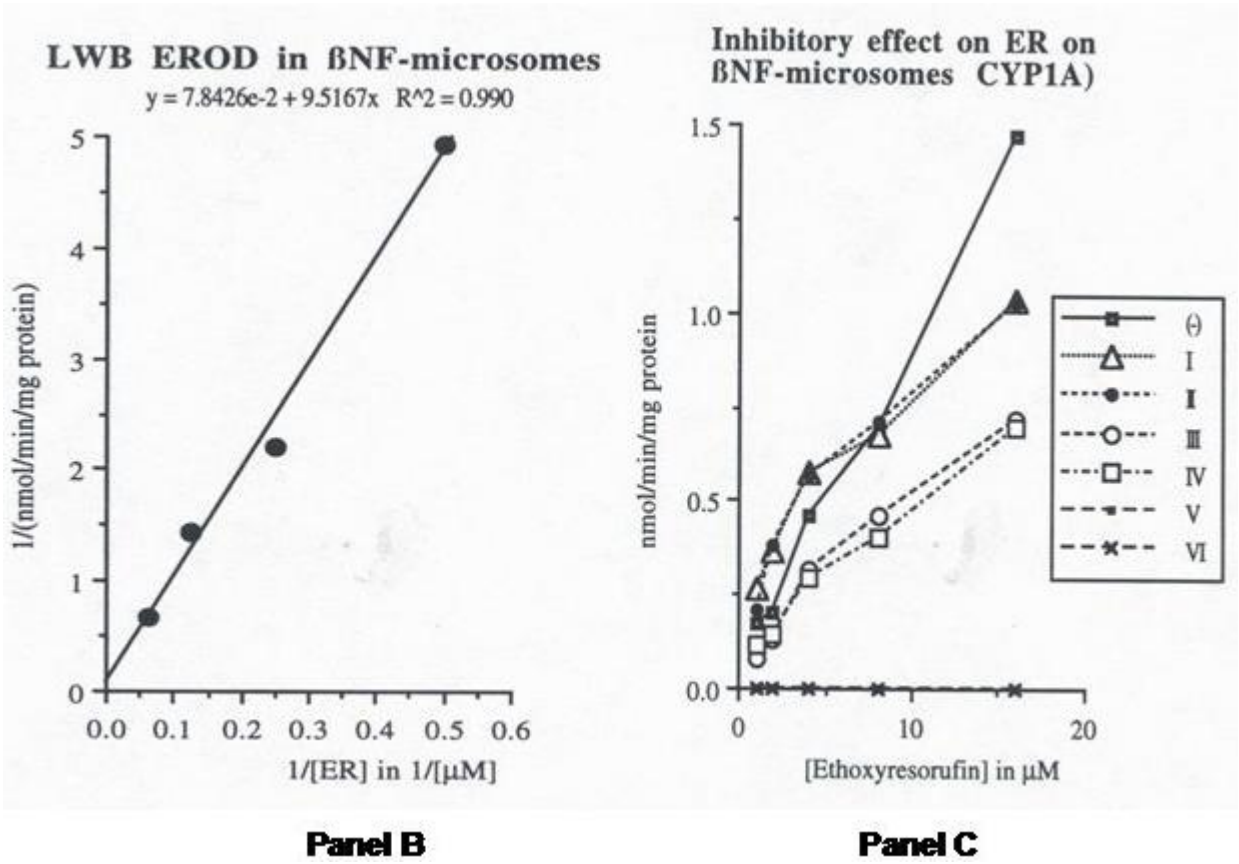


Figure 2. Contd.

towards five different CYP_{450}-isforms occurring in differentially induced rat liver microsomes. The aim of the present study was to determine the inhibitory effect of different compounds on cytochrome P_{450} iso-enzymes "CYP_{450}" activity in liver microsomes prepared from untreated rats and pretreated with the enzyme inducers such as "phenobarbital "PB", β-naphthoflavone "β-NF", pyrazol "PYR" and Dexamethaone "DEX". In addition, more information concerning hepatic metabolism of these newly synthesized compounds on rat liver microsomes was acquired.

Cytochrome P_{450} constitute a haemoprotein super family that catalyses the metabolism of a wide variety of xenobiotics and endogenous compounds to more polar metabolites. The P_{450}-dependent metabolism has also been demonstrated to be responsible for the toxicity of various drugs and other xenobiotics, and the importance of bioactivation by cytochrome P_{450} is well established (Fukushima et al., 2008; Alexidis et al., 1996). The biotransformation of xenobiotics to reactive intermediates or toxic metabolites is considered to be a major cause of adverse drug reactions observed in the clinic. As a result of this, both regulatory authorities as well as pharmaceutical industries are currently paying significant attention to address metabolism-mediated toxicity (Vagnati et al., 2005). Many reactive metabolites are unstable and therefore direct detection is very difficult. However, many of these unstable and highly reactive metabolites are able to bind covalently to proteins. Several methods are available to detect or quantify covalent binding of drugs and their metabolites to macro-molecules, including radiochemical and immunological methods.

More recently, *in-vitro* systems based on cell-lines expressing CYP isoforms have been described as a useful tool to detect cytotoxicity related to the production of reactive or cytotoxic metabolites. In these models the metabolic activity of transfected cells is in general very low and there is a significant risk that he amount of toxic metabolite formed is too low to cause any measurable toxic effect (Vagnati et al., 2005).

Our results indicated that, the compound III and IV by lower concentration" 10 and 20 µM" have no inhibitory effect on Dex-O-dealkylation reaction specific for CYP_{450} 2D1, while the other concentrations (40 and 80 µM) revealed an inhibitory effect (Figure 1A and B). This finding may be due to the amino group "—NH_2" of compound IV and the methyl group "-CH_3" of the compound III. Also, it had been documented that the amino group is very most potent to CYP_{450} 2D isoenzyme (Ortiz, 2005). Also, the inhibitory effect was concentration-dependent.

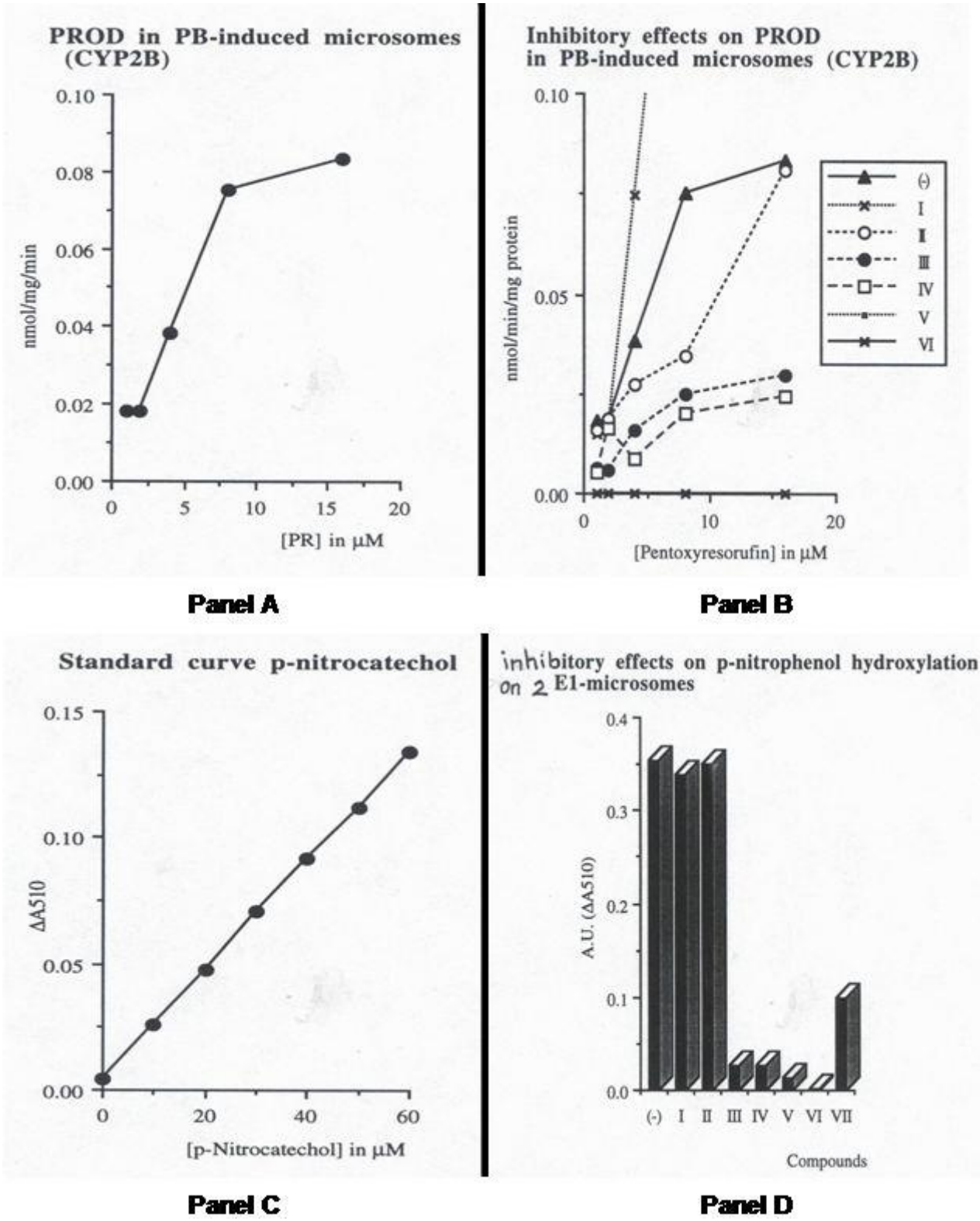


Figure 3. Effect of different concentration of Pentoxyresorufin "PROD" [1, 2, 4, 8, 16 µM] in PB-induced microsomes for CYP_{450} 2B "Panel A", the inhibitory effect of Compounds I, II, III, IV, V and VI by single concentration "6µM" on PROD in PB-induced microsomes for CYP 2B1 and 2B2 "Panel B", the standard curve of ρ-nitrocatechol by different concentrations [1, 10, 20, 30, 40, 50, 60 µM] in pyrazol-pretreated microsomes for hydroxylation activity by CYP 2E1 "Panel C", and the inhibitory of all compounds by single concentration "6 µM" in pyrazol- pretreated microsomes on ρ-notrophenol hydroxylation for CYP450 2E1 "Panel D".

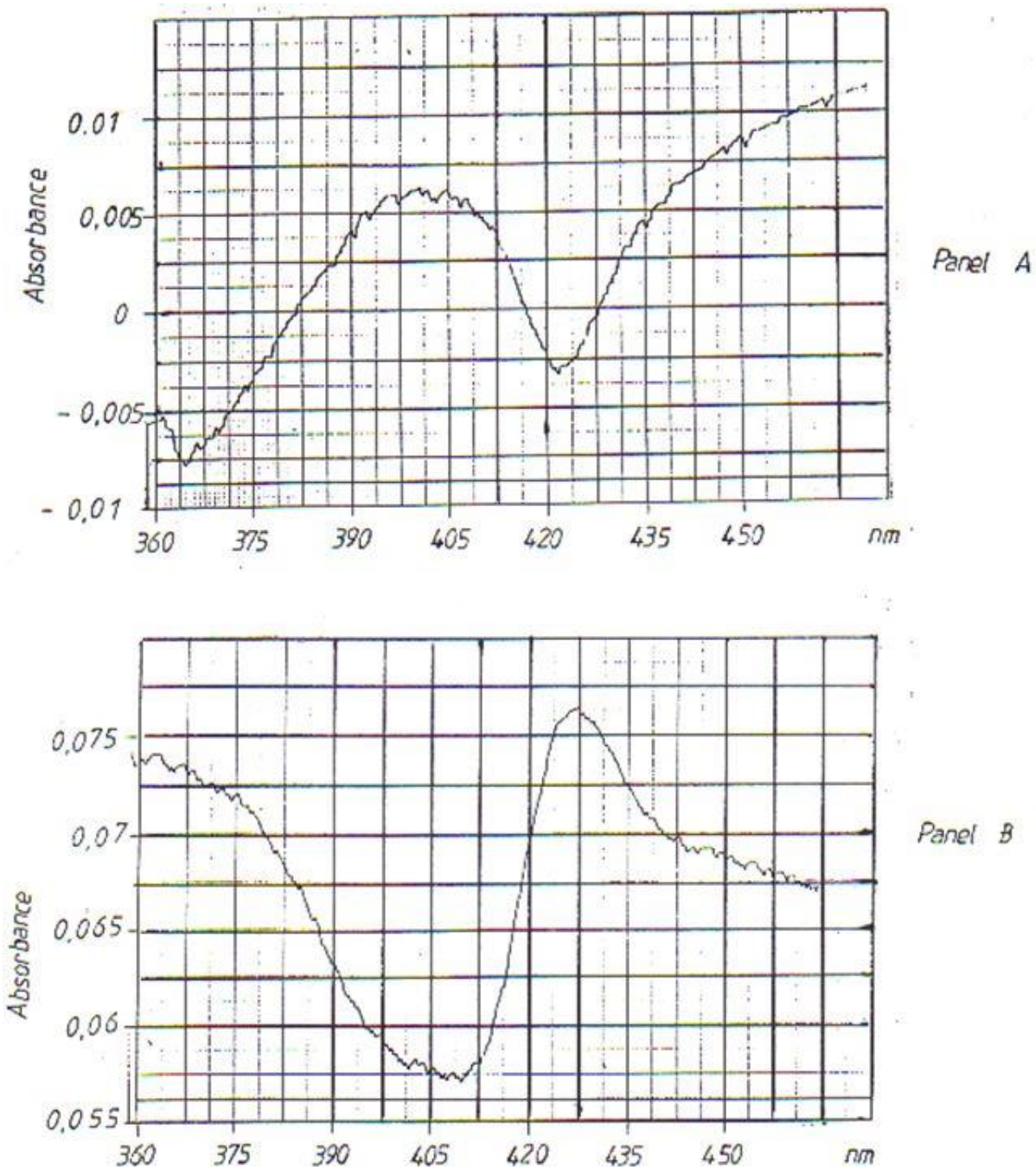


Figure 4. Binding spectrum of compound IV by concentration of 80 mM with pyrazol-induced microsomes (2 mg protein/ml) "Panel A", also, the spectrum of compound III by 80 mM in PB-induced microsomes "Panel B".

In addition, compounds I,II and III did not display any inhibitory effect on the activity of CYP_{450} 3A1 isoenzyme, while compounds IV, V and VI exerted an inhibitory effect on this isoenzyme specific for 0-dealkyation in Dex-induced rat liver microsomes (Figure 2, panel A and B).

CYPs are a large heme-containing superfamily of monooxygenase enzymes involved in a plethora of O_2 and nicotinamide adenine dinucleotide (NADH)-dependent reaction (Alun et al., 2013), where a CYP is defined as a protein that shows a characteristic absorption of height at a wavelength of 450 nm when the heme-iron is reduced and complexes to carbon monoxide. In addition to the conserved cysteine residue that forms the fifth ligand to the heme of this protein, no other residue is absolutely conserved across all CYPs although the fold is general conserved (Kuppens et al., 2005).

Pyridine effects on rat hepatic cytochromes P_{450}

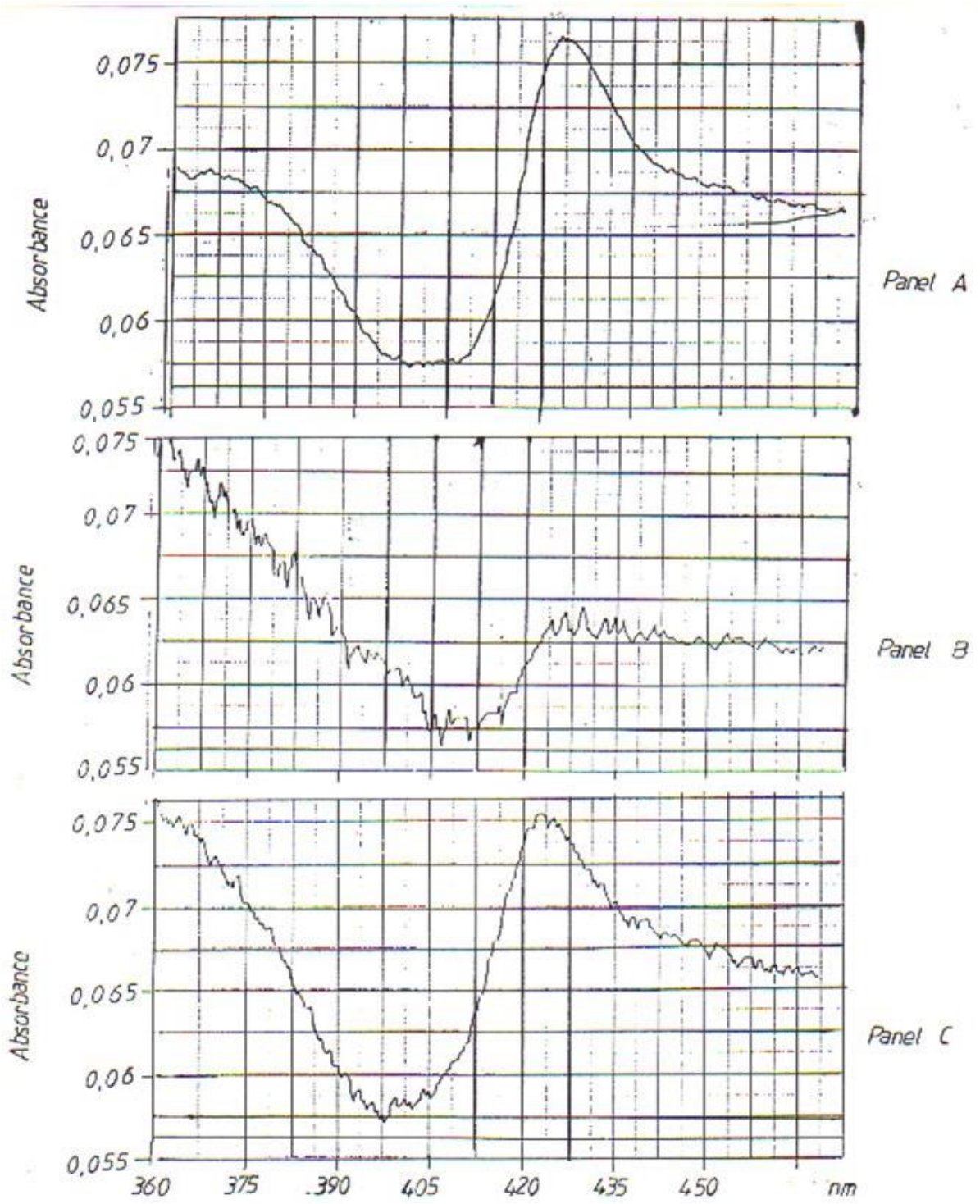


Figure 5. Binding spectrum of compound IV by 80 mM in PB-induced microsomes “Panel A”, and in β-NF-induced microsomes (2 mg protein/ml) “Panel B”. Also the compound IV by 80 mM in Dex-induced rat liver microsomes “Panel C”.

expression were examined at the levels of metabolic activity and were compared with those of CYP 2B1/2 and CYP 2E1 activity as well as CYP 3A protein and mRNA levels increased of rats with pyridine (Kim et al., 2001). CYP 1B1 appears to also have an important role in the activation of environmental procarcinogens.

Also, it has been found that, the size and the shape of the aromatic ring system and the placement of the alkyne

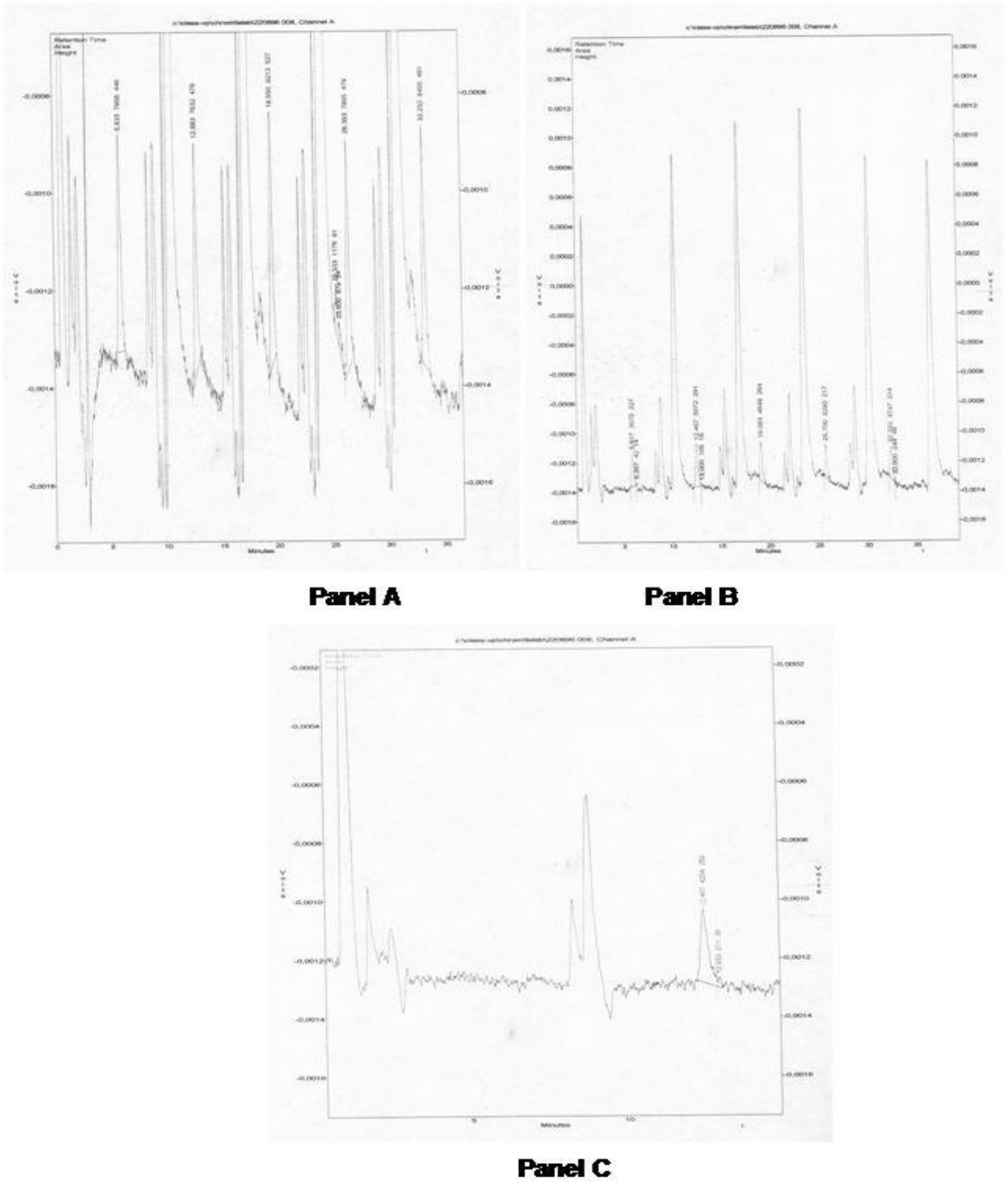


Figure 6. HPLC chromatograms of the extracted samples incubated with control rat liver microsomes. Compounds III and IV by different concentrations [10 and 20 µM] "Panel A", [40 and 80 µM] "Panel B" and "Panel C", the compounds were incubated with control microsomes and NADPH-generating system at 37°C/20 min, $HCLO_4$ 60% was added to terminate the reactions.

functional group were important for in activation The cytochrome P_{450} (or CYP) isoenzymes are a group of heme-containig enzymes embedded primarily in the lipid bilayer of the endoplasmic reticulum (ER) of hepatocytes, it takes part in the metabolism of many drugs, steroids and carcinogens (Zakia, 2008). It has been estimated

that 90% of human drug oxidation can be attributed to six main enzymes (CYP 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4/5).

In contrast, compound VII showed a inhibitory effect by the low concentrations of the substrate erythromycin (10 and 15 mM), while the high concentrations (20 to 25 mM) has no inhibitory effect. These results indicated that, compound IV has most potency on the activity of 3A1 isoenzymes, as well as, compounds V and VI has slightly potency inhibitory effect. These finding revealed that the phenyl ethyl amine group of the compound IV and the phenanthrene ring of compounds V and VI involved the potential inhibitory effect against CYP_{450} 3Al isoenzymes.

It was suggested that, the amino group "-NH_2" of compound IV was responsible for the inhibitory activity of these isoenzmes. Human cytochrome P_{450} IA1, 1A2, and 1B1 are known to have overlapping substrate specificities, many polycyclic hydrocarbons containing acetylanic side chains were examined as inhibitors of the three enzymes using 7ethoxy-resoufin 0-deethylation (EROD) as the enzyme assay in all cases. Several compounds have inhibitory effect at low nanomolar concentrations "Phenanthrene and 2-Ethnyl pyrene" nearly completely inhibited P_{450} 1B1 at concentration at which no P_{450} 1A1 inhibition was noted. All the above compounds also inhibited P_{450} 1A2. Several polycyclic hydrocarbons devoid of acetylenic groups were also inhibitory with respect to all three P_{450}s. Several polycyclic hydrocarbons and their oxidation products are very inhibitory with respect to human CYP_{450}s 1A1, 1A2, and 1B1 (Zhang et al., 2006; Mori et al., 2006).

It was investigated that the Cytochrome P_{450} 1B1 is involved in the metabolism of Procarcinogens and Xenobiotics. Human CYP 1B1 protein has been detected in a variety of tumors but is not detected in adjacent normal tissues or in liver (Rochat et al., 2001). This suggests that CYP 1B1 could biotransform anticancer agents specifically in the target cells. The interaction between CYP 1B1 commonly used anticancer drugs was screened using an ethoxyresorufin deethylase assay. These results indicated that the activity of isoenzymes CYP_{450} 2D1 have a dose-dependent effect. Also, the inhibitory properties of compound IV against CYP_{450} 2D1 may be due to the amino group in the structure (Korytko et al., 2000). They found that polycyclic aromatic hydrocarbons (PARS) are a ubiquitous class of environmental contaminants. They showed that CYP 6D1 is the major isoenzyme involved in phenanthrene metabolism, the majority of phenanthrene metabolism was inhibited by CYP 6D1. This study increases the number of known substrates of CYP 6D1, and identifies polyaromatic hydrocarbon as a potential substrates of CYP 6D1, it was investigated that P_{450} enzymes primarily catalyze the chemically challenging reactions of C-H bond oxidation but other activities are known, including dealklation, desaturation, C-H bond formation, dehydration, decarboxylation and skeletal rearrangements. The phenanthrene ring of compounds V and VI involved the potential inhibitory effect against CYP_{450} 3A1 isoenzymes.

The further experiment in which rat microsomes were prepared and analyzed for the protein content, the CYP level and erythromycin N-demethylase activity of the control group and pretreated groups indicated that the extract can stimulate the activity of Cytochrome P_{450} isoforms, and changes in the pharmacokinetics of diazepam resulting from extract are related to an increase in metabolic activity of CYP 3A (Yumoto et al., 2001).

The results of ethoxyresorufin-0-dealkylation reaction "EROD" in β-NF-induced rat liver microsomes for CYP_{450} 1A1/1A2 "Figure 3B" exerted that compounds III and IV most potent on the activity of these iso enzymes at all concentrations of "ER" ethoxy resorufein (Hemaiswarya and Doble, 2006; Zafar and Sharif, 2003), they found that, the human cytochrome P_{450} 2B6_metabolizes, among numerous other substrates as phenanthrene ring. This compound extremely high specificity for 2B6 and did not bind to other human and rodent P_{450}s or inhibit the metabolism of phenanthrene catalyzed by human 1A2, 2A6, 2C8, 2C9, 2D6, 2E1, 3A4 and 3A5. The degree of inhibition by the 2B6 specific defines the concentration of 2B6 to phenanthrene metabolism in human liver. Compounds I and II have an inhibitory effect only at the high concentration from ER (8 to 16 µM). It has been found that the hydrolysis of ester group of the compounds I and II to free acid metabolites formed would possess no CYP_{450} inhibitory properties. It was found that the free acid compound was indeed characterized by a lack of any inhibitory activity against CYP_{450} 1A 2 from β-NF pretreated rat liver microsomes (Alexidis et al., 1996; Bradford, 1976).

In addition, compounds II, III and IV have an inhibitory effect on the activity of CYP_{450} 2B isoenzymes in PB-induced rat liver microsomes by using pentoxyresorufin 0- dealkylation "PROD" (Figure 4B). The most potent inhibitor was the compound IV and the less potent inhibitor was the compound II, while the intermediate inhibitor was the compound III against the activity of CYP_{450} 2B isoenzyme. The other compounds did not display any effect on 2B-isoenzyme. It was investigated that, aryl acetylene compounds play a role as inhibitors of cytochrome P_{450}-dependent alkoxyresorufin dealkylation activities in liver microsomes prepared from rats exposed to β-NF or PB. Many of the acetylenes investigated produce pseudo-first order time-dependent and NADPH-dependent losses of the dealkylation activities characteristic of mechanism-based irreversible in activation (suicide inhibition). Ethynes are more effective suicide inhibitors of CYP_{450} 2B-dependent dealkylation than the corresponding propynes. Also, it was found that, the loses of P_{450}-dependent activity produced by aryl acetylenes were not accompanied by corresponding decrease in the measured P_{450} absorption spectra (Bradford, 1976; Chearwae et al., 2004; Kuppens et al., 2005).

Our results revealed that, the compounds III, IV, V, VI and VII have an inhibitory effect by concentration of 6 µM on the activity of CYP_{450} 2E1 of ρ-nitrophenol hydroxylation reaction in pyrazol-pretreated rat liver microsomes. Compound VI was found to be a very potent inhibitor of CYP_{450} 2E1, the less potent inhibitor was the compound VII as shown in Figure 4D. In contrast, the compounds I and II did not revealed any effect on this isoenzyme. Based on the above presented results, we predicted that the phenanthren structure of compounds V and VI may be affected the activity of CYP_{450} 2El in rat liver microsomes. Also, we can conclude that the acetylene groups of the compounds III and IV have a responsibility for the inhibitory activity towards CYP_{450} 2E1 isoenzyme. It was found that, CYP 2E1 isoenzyme catalyzing the metabolism of phenanthrene, the quantitatively determined contribution of human 2E1 to the metabolism of the above substrates ranged from 25 to 75%. Thus, human CYP 2E1 isoenzyme was responsible for the phenanthrene metabolism in human.

CONCLUSION

In conclusion, seven newly synthesized ethnyl derivatives were examined for their inhibitory properties against different CYP_{450} isoforms using differentially induced rat liver microsomes. It was found that all compounds under investigation at high concentrations inhibited the activities of CYP 1A and 2B isoenzymes, except compound I which showed no effect for CYP 2B. Also, it was investigated that compounds III and IV at high concentration (80 µM) revealed an interaction with the catalytic active site of CYP_{450}. Type I binding was observed for compound IV, while compound III exerted type II or reverse type I binding. As well as, at a concentration of 80 µM, compound IV completely inhibited Dex-O-demethylation, while compound III at this concentration had no effect. Also, it was demonstrated that the effect of the compounds at a single concentration of 6 µM against different concentration of erythromycin appeared to be variable for CYP 3A1 activity on dealkylation in Dex-induced rat liver microsomes. Our results revealed that compounds IV; V and VI had no inhibitory effect, while compounds I, II, III and VII showed a specific inhibitory effect. The ethnyl-derivatives compounds proved to be potentially variable activities by different responses on CYP_{450} isoforms in rat liver microsomes.

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