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ASSESSMENT OF CYTOCHROME P4502E1 INDUCTION IN ALCOHOLIC PATIENTS BY CHLORZOXAZONE PHARMACOKINETICS

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Abstract—Chlorzoxazone is mainly metabolized to 6-hydroxychlorzoxazone (6-OHchlorzoxazone) by the ethanol-inducible cytochrome P450 2E1 (CYP2E1). To evaluate the impact of ethanol consumption on the enzyme induction, the pharmacokinetics of chlorzoxazone and 6-OHchlorzoxazone were studied in alcoholic and control subjects. Fifteen alcoholic male inpatients (all smokers, daily intake 333 ± 191 g of absolute ethanol) and 20 healthy male volunteers (10 smokers and 10 non-smokers, weekly intake <100 g of absolute ethanol) participated in this study. Following a 12 hr fasting period, each subject was orally administered 500 mg of chlorzoxazone. Venous blood and urine samples were collected over a 10 hr period. Areas under the curve of plasma concentration versus time (AUC) of chlorzoxazone and 6-OHchlorzoxazone were calculated. The total plasma clearance of chlorzoxazone was measured as the dose/AUC ratio. The mean total plasma clearance was not different between smoker and non-smoker controls but it was enhanced by 73% in alcoholic patients. These results indicate a negligible and non-significant effect of cigarette smoking in controls but an increased metabolism of chlorzoxazone in alcoholic patients ($P < 0.05$). This increase was corroborated by the 2-fold enhancement of the 6OH-chlorzoxazone/chlorzoxazone AUC ratio, compared to controls. A good correlation was found between this AUC ratio and the 6-OHchlorzoxazone/chlorzoxazone concentration ratio at $t = 2$ hr in patients and in controls ($r = 0.88$ and 0.85, respectively, $P < 0.01$). The concentration ratio increased by 150% in alcoholic patients and decreased by 65% in the seven alcoholics tested after 7 days of alcohol abstinence. It is therefore concluded that the 6-OHchlorzoxazone/chlorzoxazone concentration ratio at $t = 2$ hr could constitute a simple and non-traumatic marker of CYP2E1 induction.

Key words: chlorzoxazone; cytochrome P4502E1; alcoholism; pharmacokinetics

It has been established that the induction of CYP2E1§ enhances the toxicity and carcinogenicity of a number of xenobiotics [1]. This enzyme is ethanol inducible [2, 3] and may partly explain the high incidence of toxic drug effects and cancers in alcoholics [4, 5]. Until recently, the assessment of CYP2E1 in humans has been limited as its determination required a surgical liver biopsy [6, 7]. It is therefore of clinical relevance to develop a non-invasive assay of CYP2E1. A previous study [8] has demonstrated that chlorzoxazone is metabolized *in vitro* by CYP2E1 to 6-OHchlorzoxazone. In light of this, it has been suggested that this drug could be used as a probe for the study of CYP2E1 activity in humans. Chlorzoxazone is a centrally-acting skeletal muscle relaxant, it is well tolerated and seldom produces undesirable side effects. Its hydoxylated metabolite, 6-OHchlorzoxazone, is excreted in urine as a glucuronide conjugate [9].

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§ Abbreviations: 6-OHchlorzoxazone, 6-hydroxychlorzoxazone; CYP2E1, cytochrome P450 2E1; AUC, area under the curve of plasma concentration vs time; CI_T, total clearance: CI_R, renal clearance; C_{max} maximum concentration; t_{max}, time required to reach C_{max}; GGT, gamma-glutamyltransferase; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

In the present study, we examined the pharmacokinetics of chlorzoxazone and its main metabolite in a group of alcoholic patients. These results were then compared with a group of non-alcoholic controls to determine if chlorzoxazone could constitute a useful probe for the *in vivo* evaluation of CYP2E1 induction. In addition, chlorzoxazone metabolism was compared in smoker and non-smoker controls. Tobacco is known to be an inducer of CYP1A and it has recently been observed that chlorzoxazone can act as a substrate for CYP1A [10]. As alcoholics are often smokers, it was necessary to evaluate *in vivo* the specificity of the probe towards CYP2E1.

MATERIALS AND METHODS

The protocol of the study was approved on 27 October 1992 by the Institute's ethical committee (Comité de Protection des Personnes se prêtant à la Recherche Biomédicale) of the Morvan Hospital in Brest (France). The participants were informed of the possible side effects of chlorzoxazone and consent was obtained from all the subjects involved in this study. Controls and patients were within 20% of ideal weight according to the Metropolitan Life Insurance Company (Statistical Bulletin 40). In the

Table 1. Biological characteristics of 15 alcoholic patients and 20 controls (10 smokers and 10 non-smokers)

	Patients (N = 15)	Controls (N = 20)
GGT (mU/mL)	228.7 (±231.2)*	14 (±5)
ASAT (mU/mL)	65.5 (±55.4)*	15 (±2)
ALAT (mU/mL)	49.3 (±38.9)*	19 (±6)
Thrombotest (%)	93.6 (±9.7)^{NS}	98 (±2)

Data are means ± SD, *$P < 0.05$ (alcoholics vs controls).

days preceding the study, they had taken no medication which might interfere with CYP2E1 activity (paracetamol, disulfiram, isoniazid). Alcohol consumption was evaluated using a standard questionnaire which detailed daily and weekly ethanol consumption. No alcohol was detectable in the blood of controls or patients once the study was started.

Patients. Fifteen chronically alcoholic male inpatients participated in the study, all of whom had just started a rehabilitation program. Their daily regular ethanol consumption was 333 (±191) g (mean ± SD) of absolute ethanol over a 15.7 (±10.7) year period. They were all smokers (mean cigarettes/day: 21 ± 11). Their mean age was 44.8 (±9) years and they exhibited no clinical or biological evidence of liver cirrhosis. Biological characteristics: GGT, ASAT, ALAT and thrombotest were determined using routine clinical biochemistry.

Healthy volunteers. Twenty healthy male volunteers (10 non-smokers and 10 smokers: mean cigarettes/day = 18 ± 9) participated as controls. Before the beginning of the study, they were screened for contra-indications, using parameters such as blood chemistry analysis, liver function tests, blood cell counts, urine analysis and ECG. One of the selection criteria was that the volunteers should be moderate drinkers (weekly ethanol consumption <100 g). They were not informed of this before selection but were asked to abstain from drinking ethanol for 36 hr before the study. Their mean age was 37 (±6) years.

Biological characteristics of patients and controls are given in Table 1.

Kinetics of chlorzoxazone and 6-OHchlorzoxazone. After a 12-hr fast, the patients and the volunteers were orally administered a 500 mg pellet of chlorzoxazone at 8.00 a.m. To measure plasma chlorzoxazone and 6-OHchlorzoxazone concentrations, venous blood samples were drawn before drug intake and 0.5, 1, 1.5, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 hr thereafter. Urine was collected in fractions of 0–2, 2–4, 4–6, 6–8, and 8–24 hr following drug intake. In addition, seven of the alcoholic patients were administered a 500 mg pellet of chlorzoxazone after 7 days of total alcohol abstinence and a single blood sample was drawn 2 hr after drug administration.

Chemicals. Chlorzoxazone tablets were obtained from Goldline Laboratories (Miami, FL, U.S.A.). This drug is a centrally acting skeletal muscle relaxant which has been used for over 26 years, it is well tolerated and seldom produces undesirable side effects. In rare instances it may cause drowsiness, dizziness and gastrointestinal disturbances such as nausea.

Analytical chlorzoxazone was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and 6-OHchlorzoxazone was synthetized by R. Peter (University of Erlangen, Germany). *Helix Pomatia* juice, containing 1,000,000 Roy units of sulfatase and 100,000 Fishman units of β-glucuronidase per mL, was obtained from IBF Biotechnics (Paris, France). Other chemicals of analytical grade were obtained from Merck (Darmstadt, Germany).

Chlorzoxazone and 6-OHchlorzoxazone determination. To liberate 6-OHchlorzoxazone from its conjugates, 0.5 mL of plasma or 0.5 mL of 1/100 diluted urine were hydolysed overnight at 37° with 20 μL of *Helix pomatia* juice. Proteins were then precipitated with 4 mL of 0.6 N perchloric acid. Chlorzoxazone and 6-OHchlorzoxazone were extracted twice with 4 mL ethyl acetate. The organic phases were evaporated to dryness under a stream of nitrogen. The residues were dissolved in 250 μL of mobile phase and 20 μL were injected on a C18 column (Nucleosil, 5 μM, 250 × 4.6 mm). The compounds were separated by HPLC with a mobile phase consisting of acetonitrile–0.5% acetic acid (30:70, v/v), at a flow rate of 1 mL/min and were detected at 287 nm. The retention times for chlorzoxazone and 6-OHchlorzoxazone were 6.5 and 17 min, respectively. Peak area measurements were used for quantification and compared to standard solutions (0.5 to 20 μg/mL) of chlorzoxazone and 6-OHchlorzoxazone. The limit of detection in biological samples was found to be 0.5 μg/mL. Within and between run precision was below 5 and 10%, respectively for both compounds at three concentration levels (0.5, 10 and 20 μg/L). The procedure was accurate to within 0.3–6%. Endogenous and exogenous interferences were negligible.

Pharmacokinetic analysis. The maximum concentrations (C_{max}) of chlorzoxazone and 6-OHchlorzoxazone and the time required to reach them (t_{max}) were recorded for each subject. The areas under the curve of plasma concentration versus time (AUC) were evaluated using the trapezoidal rule, and the total clearance of chlorzoxazone (Cl_T) was calculated as the dose/AUC ratio [11]. The 6-OHchlorzoxazone/chlorzoxazone ratio was calculated from the AUCs. The ratio of 6-OHchlorzoxazone and chlorzoxazone present in the plasma was also calculated at $t = 1.5$ hr and $t = 2$ hr. The total amount of drug excreted in urine was estimated as being the sum of the amounts excreted in each sample. The renal clearance (Cl_R) of 6-OHchlorzoxazone was calculated for each fraction as being the excretion rate to plasma concentration ratio at mid-point time [11].

Statistical analysis. The values for C_{max}, Cl_T, AUC, total excreted levels in urine and 6-OHchlorzoxazone/chlorzoxazone ratios were compared between the alcoholic and control groups by means of a non-parametric Mann–Whitney U test. The correlation between the 6-OHchlorzoxazone/chlorzoxazone AUC ratios and the plasma con-

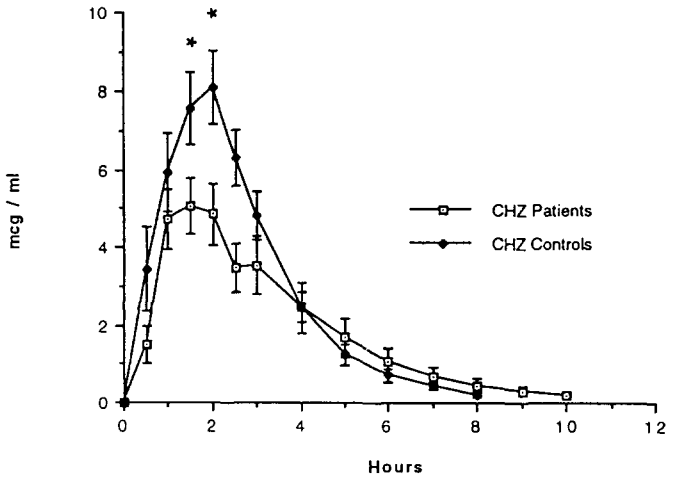

Fig. 1. Plasma chlorzoxazone concentrations vs time in 15 alcoholic patients and 20 controls after ingestion of 500 mg of chlorzoxazone (mean ± SEM).*P < 0.05 alcoholics vs controls.

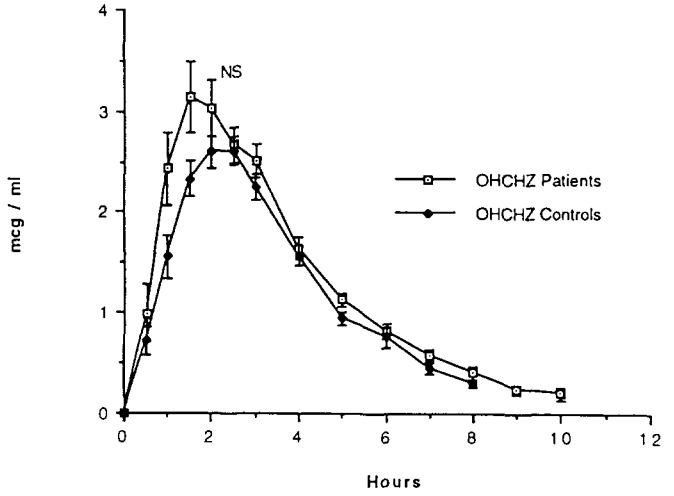

Fig. 2. Plasma 6-OHchlorzoxazone concentrations vs time in 15 alcoholic patients and 20 controls after ingestion of 500 mg of chlorzoxazone (mean ± SEM).

centration ratios at $t = 1.5$ hr and $t = 2$ hr was assessed by calculation of the correlation coefficient.

RESULTS

Fifteen alcoholic inpatients (all smokers) and 20 healthy controls (10 smokers and 10 non-smokers) were given a tablet of 500 mg of chlorzoxazone in order to compare the pharmacokinetics of chlorzoxazone and its main metabolite 6-OHchlorzoxazone. None of the subjects reported significant side effects from chlorzoxazone intake. The levels of GGT, ASAT and ALAT were significantly higher in alcoholic patients (Table 1).

The mean plasma chlorzoxazone concentrations reached a peak approximately 2 and 1.5 hr after oral administration of the drug in patients (t_{max} = 1.7 ± 0.5) and controls (t_{max} = 1.5 ± 0.5), respectively. These concentrations were lower in patients than in controls ($P < 0.05$) at $t = 1.5$ and $t = 2$ hr (Fig. 1). In contrast, the mean plasma concentration of 6-OHchlorzoxazone peaked earlier in patients (t_{max} = 1.7 ± 0.5) than in controls (t_{max} = 2.1 ± 0.5) and were slightly higher although not significantly different in patients (Fig. 2).

Pharmacokinetic parameters are summarized in Table 2. C_{max} and AUC values for chlorzoxazone and 6-OHchlorzoxazone did not differ between smoker and non-smoker controls. In contrast, chlorzoxazone C_{max} and AUC values were almost 2-fold lower in patients than in smoker or non-smoker controls ($P < 0.05$). Consequently, the total

Table 2. Pharmacokinetic parameters of chlorzoxazone (CHZ) and 6-hydroxychlorzoxazone (6-OHCHZ) in 15 alcoholic patients and 20 controls (10 smokers and 10 non-smokers) after oral ingestion of 500 mg of chlorzoxazone

	Patients (N = 15)	Controls (N = 20)	Smoker controls (N = 10)	Non-smoker controls (N = 10)
CHZ C_{max} μg/mL	6.29 (±3.01)*	9.95 (±4.31)	10.00 (±5.04)	9.90 (±3.72)
CHZ AUC μg/mL.hr	16.32 (±10.08)*	24.75 (±12.21)	24.97 (±16.07)	24.53 (±7.54)
CHZ CI_T L/hr	41.06 (±20.18)*	23.74 (±9.23)	25.33 (±11.36)	22.15 (±6.72)
6-OHCHZ C_{max} μg/mL	3.39 (±1.21)^{NS}	2.88 (±0.65)	2.89 (±0.72)	2.87 (±0.63)
6-OHCHZ AUC μg/mL.hr	12.96 (±3.30)^{NS}	11.43 (±2.32)	10.65 (±1.70)	12.21 (±2.67)
6-OHCHZ CI_R L/hr	31.7 (±9.6)^{NS}	32.6 (±8.0)	34.6 (±4.4)	29.8 (±7.3)
6-OH-CHZ/CHZ AUC RATIO	1.06 (±0.59)*	0.53 (±0.21)	0.52 (±0.19)	0.54 (±0.24)
6-OHCHZ/CHZ t = 2 hr RATIO	0.95 (±0.66)*	0.38(±0.16)	0.38 (±0.18)	0.37 (±0.15)
Total 6-OHCHZ in urine mg	413.8 (±88.6)^{NS}	380.11 (±182.4)	406.7 (±132.18)	353.36 (±88.47)

Data are means ± SD, *P < 0.05 (15 alcoholics vs 20 controls). NS, not significant.

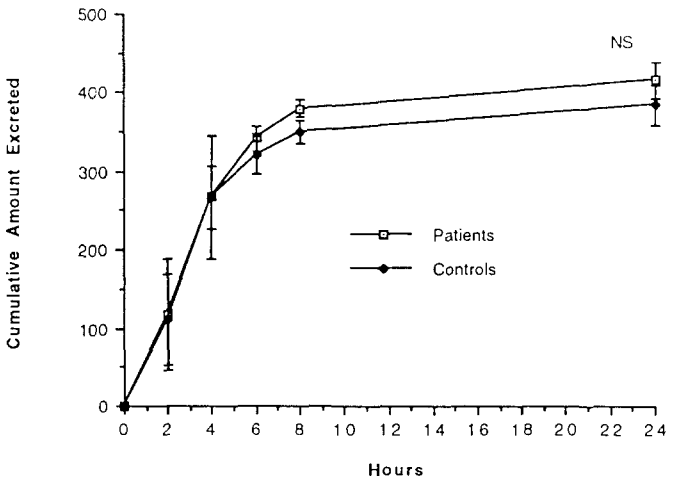

Fig. 3. Cumulative amount of 6-OHchlorzoxazone excreted in the urine of 15 alcoholic patients and 20 controls after ingestion of 500 mg of chlorzoxazone (mean ± SEM).

clearance of chlorzoxazone was significantly higher in the patients than in either control groups ($P < 0.05$). The C_{max} values for 6-OHchlorzoxazone did not significantly increase in alcoholics compared to controls.

The total amount of 6-OHchlorzoxazone excreted in the urine did not differ between patients (mean ± SD: 413.8 ± 88.6 mg) and smoker (406.9 ± 132.2) or non-smoker controls (363.4 ± 88.5 mg). The amounts excreted in each urine fraction were also comparable between the groups (Fig. 3). Similarly, the renal clearances were not different in patients or in controls. No chlorzoxazone was found to be excreted in the urine.

In order to eliminate the influence of differing absorption levels or inequalities in the volume of distribution of the drug between subjects, the 6-OHchlorzoxazone/chlorzoxazone AUC ratio was calculated. It was found to be significantly greater in the patients than in the controls (1.06 ± 0.59 vs 0.53 ± 0.24 $P < 0.05$). The 6-OHchlorzoxazone/chlorzoxazone ratio was also calculated from the plasma concentrations at t = 1.5 hr (0.91 ± 0.66 vs 0.37 ± 0.16 $P < 0.05$) and at t = 2 hr (0.95 ± 0.66 vs 0.38 ± 0.16 $P < 0.05$). These concentration ratios were significantly correlated to the AUC ratios: at t = 1.5 hr, the correlation coefficient was 0.84 in patients and 0.80 in controls ($P < 0.01$) and at t = 2 hr, 0.88 and 0.85, respectively ($P < 0.01$). The concentration ratio at t = 2 hr was used to test seven alcoholics on the day following their entrance to the hospital and after 7 days of alcohol abstinence. It decreased from 1.39 (±0.67) to 0.5 (±0.24), $P < 0.001$ (Fig. 4). Patients A and B most likely stopped their alcohol consumption a few days before hospitalization.

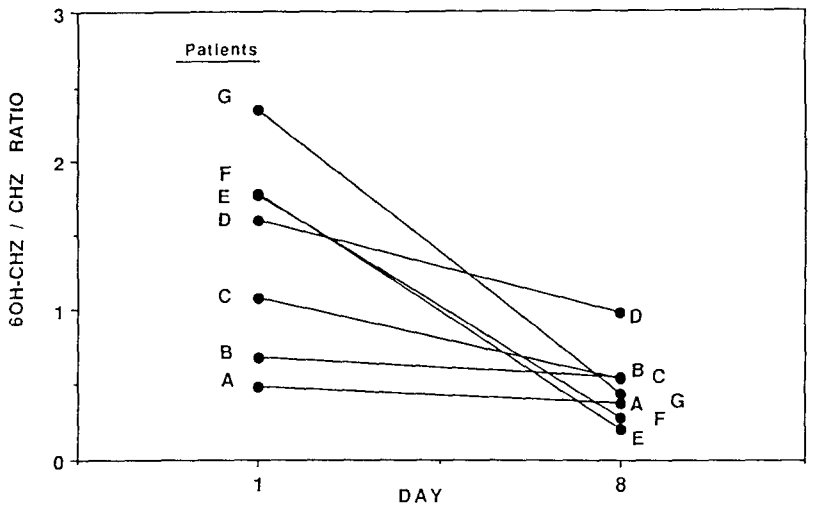

Fig. 4. Plasma concentration ratio 6-OHchlorzoxazone/chlorzoxazone at $t = 2$ hr in seven alcoholic patients on the day following their entrance to the hospital and 7 days after alcohol abstinence.

DISCUSSION

Previous studies [9, 12] have shown that chlorzoxazone is mainly metabolized to 6-OHchlorzoxazone which is subsequently excreted in the urine as a glucuronide conjugate. *In vitro* experiments have determined that CYP2E1 is the enzyme responsible for the 6-hydroxylation of chlorzoxazone [8]. Recent studies performed in humans with a CYP2E1 inhibitor such as disulfiram [13] and an inducer such as isoniazid [14] have confirmed the involvement of CYP2E1 in the metabolism of chlorzoxazone. It has recently been shown, however, that chlorzoxazone can also be metabolized by CYP1A *in vitro*. This cytochrome is induced in humans by cigarette smoking. The results of this study indicate that the contribution of this isozyme should be minor *in vivo*, due to its lower affinity for the chlorzoxazone substrate [10]. Indeed, a negligible participation of CYP1A in the chlorzoxazone metabolism can be assumed as no difference in chlorzoxazone metabolism was found between smoker and non-smoker controls.

Alcoholic patients, whose CYP2E1 is induced by chronic ethanol consumption, should metabolize chlorzoxazone to 6-OHchlorzoxazone faster than non-alcoholics. The results of this study demonstrate that the total clearance of chlorzoxazone was indeed significantly greater in alcoholic patients than in controls. This difference is the result of an increased metabolism of chlorzoxazone as demonstrated by the lower C_{max} and AUC of chlorzoxazone in alcoholics associated with an earlier t_{max} and an equivalent AUC for 6-OHchlorzoxazone. Furthermore, the increased clearance of chlorzoxazone was not the consequence of an impaired absorption since the total amount of metabolite excreted in urine did not differ between the groups. In addition, the renal elimination of the metabolite was comparable between alcoholic and control patients.

Based on the pharmacokinetics of chlorzoxazone metabolism, we attempted to define an ideal blood sampling time period for which chlorzoxazone and 6-OHchlorzoxazone concentrations would reflect the CYP2E1 induction status. The drug to metabolite concentration ratio 2 hr after drug ingestion is well correlated to the AUC ratio. It is 2.5-fold higher in patients than in controls and decreases in patients after alcohol abstinence. This confirms the major participation of drinking habits in the increase of this ratio as smoking habits did not change during this period. This ratio cannot be calculated from urine as no chlorzoxazone is excreted in the urine. We therefore suggest that the plasma ratio might be used as a useful, simple and non-traumatic indicator of the induction of CYP2E1 as its enhancement in alcoholic patients appears most likely to be a result of CYP2E1 induction.

CYP2E1 is known to be induced in humans by ethanol consumption [2, 3] and in animals by many other compounds or physiological states [15]. Diet also plays an important role in regulating induction levels of CYP isozymes [16]. Since CYP2E1 is directly involved in the metabolism of acetaminophen [17], nitrosamines [18], carbon tetrachloride [19], or other industrial solvents such as benzene [20], or pyridine [21], the individuals with higher levels of CYP2E1 may have a higher risk of toxicity from these agents. On the other hand, several polymorphic restriction sites have been shown on the CYP2E1 gene [22–24]. Whether these mutations lead to different activities of CYP2E1 is not yet known. The utilization of chlorzoxazone metabolism as a non-invasive probe of human CYP2E1 is of great interest since it may help to determine the importance of the human CYP2E1 in the bioactivation and detoxification of chemicals oxidized by the CYP isozyme.

Conclusion

The results presented here suggest that chlorzoxazone is an adequate probe to measure the

CYP2E1 induction *in vivo* and that the evaluation of the metabolite/parent drug ratio 2 hr after dosage constitutes a good indicator of this induction. It may be used as a simple and non-invasive marker for phenotyping this cytochrome. The most frequent inducer of CYP2E1 is ethanol, therefore the induction of CYP2E1 should be taken into account in alcoholic patients for two reasons: firstly to avoid the prescription of drugs which would be metabolized to reactive compounds, and secondly to allow adjustment of drug dosage. Evaluating the variations in the level of CYP2E1 is of major importance if we are to understand the role of ethanol on the toxicity and carcinogenicity of many compounds.

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