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DRUG METABOLIZING CAPACITY *IN VITRO* AND *IN VIVO*—I

CORRELATIONS BETWEEN HEPATIC MICROSOMAL MONOOXYGENASE MARKERS IN β-NAPHTHOFLAVONE-INDUCED RATS

DIANE E. MATTHEW* and J. BRIAN HOUSTON†
Department of Pharmacy, University of Manchester, Manchester M13 9PL, U.K.

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Abstract—Relationships between and within *in vivo* and *in vitro* markers of drug oxidative metabolism have been investigated in rats displaying a wide range of hepatic microsomal monooxygenase activity due to prior treatment with various doses of the inducing agent β-naphthoflavone (BNF). BNF induction produced large dose-related changes in the *in vivo* clearance (CL) of theophylline (TH), antipyrine (AP) and the individual AP metabolite formation clearances, 4-hydroxyantipyrine (4H) and norantipyrine, and the *in vitro* parameters, 7-ethoxycoumarin *O*-deethylase, 7-ethoxyresorufin and P450. No trends were observed with the formation clearance of 3-hydroxymethylantipyrine and 7-methoxycoumarin *O*-demethylase whilst a negative response was observed with aldrin epoxidase. The selectivity of the markers towards BNF induction was coincident with the degree of covariance observed between these parameters. Strong correlations were observed in particular between CL(TH) and CL(4H) and ECOD and EROD indicating the high predictive value of these parameters. These studies demonstrate that under the well controlled conditions which may be imposed in animal environments predictively useful relationships ($r^2 > 0.8$) can be established between *in vitro* and *in vivo* markers of hepatic microsomal monooxygenase activity.

The activity of the hepatic microsomal monooxygenase system (cytochromes P450) is frequently rate limiting in the elimination of drugs and other foreign compounds from the body and/or the formation of active/reactive metabolites. Large differences between animal species, and among different individuals within a given species, exist in cytochromes P450 activities and this has led to the widespread use of probe or marker substrates in both *in vitro* and *in vivo* investigations to quantify these differences.

Numerous cytochrome P450-dependent enzyme assays have been developed to assess microsomal activity. There is a substantial body of knowledge on the relative characteristics of these *in vitro* probes [1]. Particularly useful substrates in resolving specific isozyme changes include 7-ethoxycoumarin *O*-deethylase (ECOD‡), EROD, MCOD and ALE. For *in vivo* studies AP has been widely employed for nearly two decades using either the drug parameters—total clearance or half-life [2], or more recently individual metabolite formation clearances [3], as indices of monooxygenase activity. Quantitation of the three major oxidative metabolites of AP, 4H, N and 3H, has proved to be an advantage over total drug kinetic parameters in several studies in man and rat [4, 5]. Recently TH has been employed as an alternative *in vivo* marker [6].

Attempts to predict the clearances or half-lives of other drugs on the basis of AP kinetics have generally resulted in poor correlations [5, 7]. Similarly correlations between *in vitro* and *in vivo* markers of drug metabolism have not been very successful [8–12]. Multiplicity of the monooxygenase enzyme system together with the well-known overlap in substrate specificities undoubtedly contribute to this lack of success. Therefore, there is a need to systematically examine the relationships between and within *in vitro* and *in vivo* indices. This can be more readily achieved in rats than in man as their environments may be easily controlled and a considerable body of knowledge on the rat cytochrome P450 system exists.

The aim of the present investigations was to produce a wide range of cytochrome P450-dependent enzyme activities in Sprague–Dawley rats using various doses of the inducing agent BNF while maintaining all other extrinsic factors constant. This pool of induced animals was then used to investigate the sensitivity and selectivity of a number of *in vitro* substrates as markers of drug metabolizing enzyme activity. The use of the same animals for *in vivo* and *in vitro* studies removes the inherent insensitivity associated with unmatched comparisons and allows direct intra-rat comparisons to be made. BNF is a

* Recipient of financial support from the Science and Engineering Research Council.

† To whom correspondence should be addressed.

‡ Abbreviations: ECOD, 7-ethoxycoumarin *O*-deethylase; EROD, 7-ethoxyresorufin *O*-deethylase; MCOD, 7-methoxycoumarin *O*-demethylase; ALE, aldrin epoxidase; 4H, 4-hydroxyantipyrine; 3H, 3-hydroxymethylantipyrine; N, norantipyrine; BNF, β-naphthoflavone; AP, antipyrine; TH, theophylline; CL, clearance, *V*, volume of distributions) SRW, standard rat weight.

well documented specific inducer of the cytochrome P450I (polycyclic aromatic hydrocarbon-inducible) family [13] and a substantial degree of induction of cytochrome P450I (approx. 70-fold [14]) can be readily achieved. This latter feature, together with the relatively narrow substrate specificity of this enzyme [15], made BNF induction a suitable tool for achieving a range of monooxygenase activities for the present studies.

MATERIALS AND METHODS

Preliminary studies. Male Sprague–Dawley rats (N = 24, 230–280 g) were fitted with indwelling cannulae in the right carotid artery and jugular vein [16] under ether anaesthesia. The day after surgery these rats were administered a bolus dose, via the jugular vein, of AP (50 mg/kg) and/or TH (3.25 or 6.5 mg/kg) in normal saline (2 mL/kg). Blood samples were collected from the carotid artery (N = 7–9, 250 μL) over 180 min. Samples were assayed for AP and/or TH, as described.

In vivo *studies.* Male Sprague–Dawley rats (N = 16, 230–280 g) were administered various daily i.p. doses of BNF (0–100 mg/kg) in corn oil (2 mL/kg) for 3 consecutive days. Six doses of BNF (3, 5, 7, 10, 20 and 100 mg/kg) were administered to pairs of rats and four rats received vehicle only. Rats were cannulated in the right carotid artery and jugular vein [16] under ether anaesthesia on the day of the third dose. On the fourth day they were placed in an approved restraining apparatus [17] allowing free movement and access to food and water and facile sampling of blood from the carotid artery. A solution of [*N-methyl*-^{14}C]AP (50 mg/kg, 1 μCi/kg) and TH (6.5 mg/kg) in normal saline (2 mL/kg) was administered as a bolus over 1 min, via the jugular vein.

Blood samples (N = 7–9, 250 μL) were collected over 3 hr and urine over 4 hr. Following completion of sampling, the rats were immediately killed by cervical dislocation, their bladders drained and livers removed. Blood samples were assayed for AP [18] and TH [19] and urine samples for AP and its metabolites [20]. The elimination rate constant, k, for AP disposition was calculated by a log–linear regression of the concentration–time data. The half-life was calculated as $0.693/k$, the total clearance by Dose/AUC (where AUC is the area under the plasma concentration–time curve, itself calculated as Co/k where Co is the concentration at time zero obtained by extrapolation of the log–linear regression line to time zero) and the volume of distribution as Dose/Co. The blood clearances reflect mainly metabolism since both AP and TH are extensively metabolized. Metabolite formation clearances were calculated by dividing the amount of metabolite recovered in the urine 4 hr by the AUC from 0–4 hr.

All clearance and volume of distribution terms are expressed per SRW of 250 g.

In vitro *studies.* Hepatic microsomes were prepared by standard differential centrifugation using a sucrose (0.25 M), Tris–HCl buffer (10 mM, pH 7.4). The final pellet from the 100,000 *g* centrifugation was resuspended in Tris–HCl and stored at −70°.

The following microsomal parameters were determined: total protein [21], cytochrome P450 content [22], ECOD activity [23], MCOD activity [24], EROD activity [25] and ALE activity [26]. It is assumed that if any residual inducer is present in the microsomes then its concentration will be in proportion to dose administered and therefore contribute a constant percentage error.

Chemicals. [*N-methyl*-^{14}C]AP was purchased from the Radiochemical Centre (Amersham, U.K.) with a specific activity of 57 mCi/mmol and radiochemical purity of 99%. Theophylline, methoxycoumarin, antipyrine and β-naphthoflavone were obtained from the Sigma Chemical Co. (Poole, U.K.), ethoxycoumarin and antipyrine metabolites from the Aldrich Chemical Co. (Gillingham, U.K.), ethoxyresorufin from Molecular Probes (Oregon, U.S.A.) and aldrin from Pierce and Warner Chemical Service (Chester, U.K.).

RESULTS AND DISCUSSION

Preliminary studies

Two *in vivo* marker compounds for hepatic microsomal monooxygenase activity, AP and TH, were selected for investigation. In order to optimize the amount of data gleaned from each rat, the feasibility of co-administration was assessed. In addition linearity in the pharmacokinetics of TH at particular dosages was evaluated.

The blood concentration–time curves for both AP and TH administered alone and in combination were virtually superimposable. This was also true for the dose normalized curves obtained from administering different doses of theophylline (3.25 and 6.5 mg/kg). Hence the pharmacokinetic parameters (clearance, volume of distribution and half-life (see Table 1)) for AP and TH obtained in these studies were not significantly different (by t-test). These data indicated that in Sprague–Dawley rats co-administration of AP and TH had no influence on their individual disposition kinetics and the pharmacokinetics of theophylline obeyed first order kinetics at doses of 6.5 mg/kg and below. Both sets of data are consistent with previous investigations by Teunissen *et al.* [19, 27] in Wistar rats.

Effect of BNF administration

In order to achieve a wide range of hepatic microsomal monooxygenase activity various doses of BNF were used (3–80 mg/kg). Table 2 summarizes the effects of BNF induction on the *in vivo* and *in vitro* parameters. Following induction with BNF, large changes were noted for CL(TH) (10-fold), CL(AP) (4-fold), CL(4H) (6-fold) and CL(N) (5-fold). No trends were observed with CL(3H). For the *in vitro* markers large changes were noted for ECOD (14-fold) and EROD (103-fold) and smaller increases in P450 (3-fold) were observed. MCOD showed little response whilst ALE showed a negative response (50% of control). All parameters with the exception of CL(3H) and MCOD showed a graded response to the BNF dose administered. Table 2 shows the response to a comparatively low dose of BNF (5 mg/kg). As most responses were maximal by 20 mg/kg, experiments were centred in the 3–20 mg/kg range. Previous studies on the effect of dose of BNF on aryl hydrocarbon hydroxylase activity [13] reported a

Table 1. Pharmacokinetic parameters describing the disposition of antipyrine and theophylline following separate and coadministration

Treatment	Clearance (mL/min/SRW)	Volume of distribution (L/SRW)	Half-life (min)
Theophylline 3.25 mg/kg (N = 4)	0.90 (0.25)	0.17 (0.03)	138.6 (39.8)
Theophylline 6.5 mg/kg (N = 4)	1.00 (0.27)	0.20 (0.03)	146.4 (27.5)
Theophylline 6.5 mg/kg +	0.99 (0.22)	0.22 (0.04)	161.4 (27.5)
Antipyrine 50 mg/kg (N = 8)	1.88 (0.26)	0.23 (0.04)	84.4 (11.9)
Antipyrine 50 mg/kg (N = 8)	1.60 (0.33)	0.23 (0.02)	102.5 (29.9)

Values are means with SD in parentheses.

Table 2. Effect of BNF induction on various *in vivo* and *in vitro* markers of hepatic microsomal monooxygenase activity

Parameter	Control value	Induction with BNF 5 mg/kg	Fold increase over entire BNF dose range	N
CL(TH)*	1.11 (0.17)	3.57 (0.28)	9.6	16
V(TH)†	0.24 (0.03)	0.22 (0.03)	—	16
$T_{1/2}$(TH)‡	154.1 (33.7)	43.9 (8.6)	0.1	16
CL(AP)*	1.87 (0.32)	4.18 (0.71)	3.5	16
V(AP)†	0.22 (0.04)	0.27 (0.04)	—	16
$T_{1/2}$(AP)‡	82.6 (14.7)	44.4 (4.3)	0.3	16
CL(4H)*	0.23 (0.11)	0.61 (0.06)	6.3	15
CL(N)*	0.15 (0.06)	0.52 (0.06)	5.4	15
CL(3H)*	0.19 (0.08)	0.27 (0.03)	1.2	15
ECOD§	0.320 (0.049)	1.001 (0.211)	14	16
EROD§	0.056 (0.015)	1.732 (0.496)	103	16
P450‖	0.374 (0.023)	0.662 (0.083)	2.5	16
MCOD§	0.192 (0.018)	0.360 (0.030)	1.3	16
ALE§	1.770 (0.252)	2.536 (0.344)	0.4	16

Values are means with SD in parentheses.
* mL/min/SRW.
† L/SRW.
‡ min.
§ nmol/min/mg protein.
‖ nmol/mg protein.

more shallow dose–response relationship requiring 80 mg/kg for maximal response. It is acknowledged that the data discussed are the net consequence of induction of both P450IA members (P450IA1 and P450IA2) whose substrate affinities may differ.

All concentration–time curves for AP and TH showed a monoexponential decline. The marked decrease in half-life and increases in clearance confirmed the sensitivity of both compounds to BNF induction. No alteration in apparent volume of distribution was observed for either AP or TH (Table 2) following BNF induction. The lack of change in CL(3H) confirms earlier observations on the refractory behaviour of this particular antipyrine pathway (in contrast to 4H and N) to induction by BNF [28]. Formation clearances for individual TH metabolites were not determined since other investigators have shown a high degree of covariance between the demethylation and 8-oxidation of TH in rat [27]. Furthermore, contrary to studies in man where selective metabolite modification had been demonstrated [6, 29], treatment with the classic inducers PB and 3-methylcholanthrene produced no selective effects in rats [30].

Relationships between and within in vitro *and* in vivo *markers*

Table 3 summarizes the relationships between and within the markers of hepatic microsomal monooxygenase activity in the pool of 16 animals.

Table 3. Relationship between and within *in vivo* and *in vitro* markers of hepatic microsomal monooxygenase activity

CL(AP)	CL(4H)	CL(N)	CL(3H)	ALE	MCOD	P450	ECOD	EROD	
0.884	0.944	0.890	0.129	−0.613	0.350	0.866	0.951	0.910	CL(TH)
	0.941	0.987	0.395	−0.310	0.622	0.781	0.808	0.764	CL(AP)
		0.956	0.372	−0.551	0.399	0.783	0.930	0.890	CL(4H)
			0.409	−0.348	0.621	0.826	0.827	0.845	CL(N)
				0.269	0.418	0.019	0.149	0.040	CL(3H)
					0.322	−0.426	−0.648	−0.539	ALE
						0.546	0.287	0.441	MCOD
							0.796	0.897	P450
								0.915	ECOD

Correlation statistics are shown. Critical values for statistical significance ($P < 0.001$) are 0.725 ($N = 16$) and 0.704 ($N = 15$). For correlations involving CL(4H), CL(3H) and CL(N) $N = 15$, for all others $N = 16$.

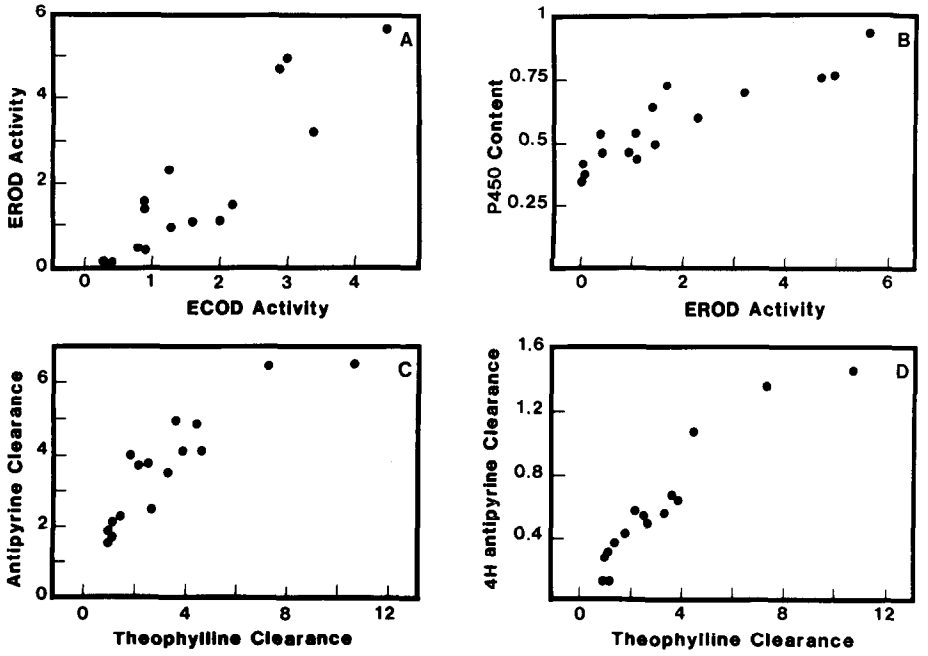

Fig. 1. Relationships between various markers of hepatic microsomal monooxygenase activity. (A) EROD and ECOD activities; (B) EROD activity and P450 content; (C) CL(AP) and CL(TH); (D) CL(4H) and CL(TH).

Although correlation coefficients greater than 0.725 are highly statistically significant ($P < 0.001$) for this sample size, it has been recently stressed [3] that for predictive purposes very high correlation coefficients are required since it is the square of the correlation coefficient which is a measure of the explained variance in one variable by the other variable.

In vitro *markers*

The relationships within the *in vitro* markers may be explained by the selective nature of BNF induction; little change in MCOD and ALE activity in contrast to large changes in ECOD and EROD-associated cytochrome P450. The latter two activities were highly correlated with each other and total P450 (Table 3). The particularly strong relationship between ECOD and EROD (Fig. 1) is indicative of the high affinity of both compounds for the major cytochrome inducible by BNF, P450I. The high correlation between EROD and total P450 (Fig. 1) is of particular interest since it reflects the changes in the qualitative nature of the P450 complement following BNF induction. Although total P450 increases by only three-fold with maximal BNF dosing, the increase in P450I isozyme is approximately 70-fold [14]. Since MCOD activity was unaffected by BNF induction, it was not surprising that correlations between this and the other *in vitro* parameters were poor. Unlike the other parameters, ALE activity decreased with increasing BNF dose administered. This is consistent with suppression of the particular isozymes responsible for aldrin epoxidation. Thus negative but low correlations were observed between ALE and the other parameters.

In vivo *markers*

A marked level of covariance was demonstrated

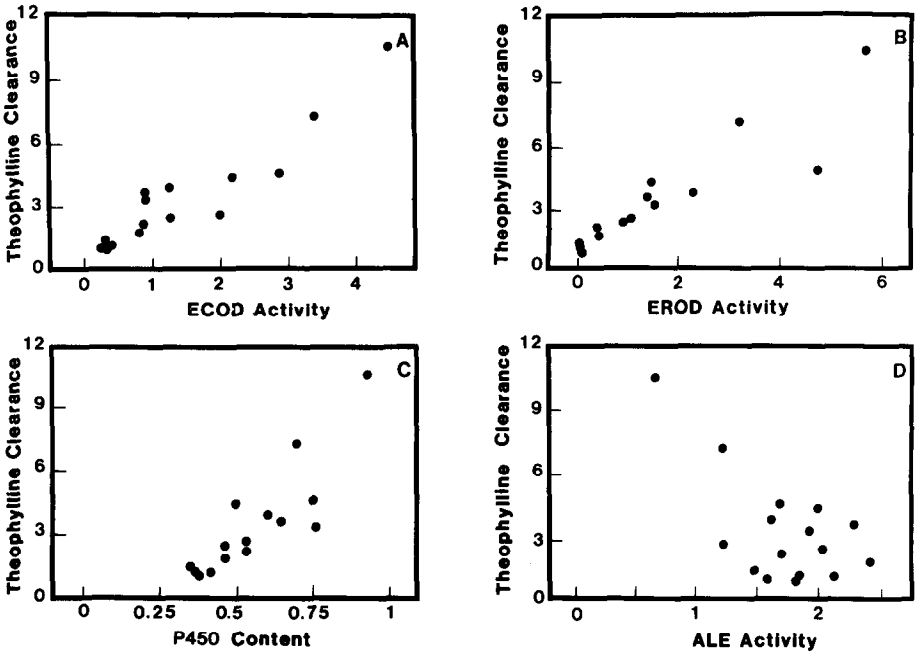

Fig. 2. Relationship between CL(TH) and four markers of *in vitro* activity. (A) CL(TH) and ECOD activity; (B) CL(TH) and EROD activity; (C) CL(TH) and P450 content; (D) CL(TH) and ALE activity.

between all of the *in vivo* parameters with the exception of CL(3H). Breimer *et al.* [5] postulated that different enzymes might be involved in the formation of each of the metabolites of AP and that measurement of the rate of formation of these metabolites might provide better indices of drug metabolizing status than clearance of the parent compound. The differences observed with the present data tend to confirm this theory. CL(TH) correlates to a greater extent with CL(4H) than with CL(N) or CL(AP) (see Table 3 and Fig. 1). There was a tendency for all AP clearances (AP, 4H and N) to plateau at high CL(TH). This behaviour indicates that CL(TH) is a more sensitive marker for the BNF-type of induction [31, 32] demonstrating, on average, twice the change in its CL than is evident in the AP clearances (see Table 2). The lack of correlation between CL(3H) and other CL parameters is consistent with the selective induction patterns of BNF on AP metabolite kinetics discussed previously.

In vitro–in vivo *markers*

Both ECOD and EROD provide a good prediction of *in vivo* clearance. In particular CL(TH) and CL(4H) correlate very highly with both ECOD and EROD. Figure 2 illustrates the data for CL(TH) plotted against four different measures of microsomal activity. P450 as an *in vitro* marker whilst less highly correlated than ECOD and EROD provides a reasonable prediction of *in vivo* activity. In contrast ALE and MCOD (data not plotted) were of little predictive value as expected from the preceding data. As shown in Fig. 3 the determination of CL(4H) provides a distinct improvement on CL(AP) or CL(N). The latter two clearances were of similar value in predicting microsomal activity. Thus by partitioning CL(AP) into CL(4H) an approximate 20% increase in the predictive value of ECOD was achieved. A similar improvement was seen with EROD but not the other microsomal markers.

Conclusions

By using various doses of BNF a wide range of cytochromes P450 activity has been achieved in rat. Using this pool of rats good correlations ($r > 0.9$) or predictive value ($r^2 > 0.8$) have been demonstrated between particular *in vitro* and *in vivo* markers of hepatic microsomal monooxygenase activity. The *in vivo* parameters CL(TH) and CL(4H) and the microsomal ECOD and EROD activities proved to be sensitive probes for BNF induction and showed a high degree of covariance. It must be stressed that for the above markers sensitivity to and selectivity for BNF induction of P450I are not linked. AP is equally responsive to induction of other P450s in addition to P450I, for example phenobarbitone induction of P450IIB [28]. CL(TH) [30, 33] and ECOD activity [23, 33] are also markedly influenced by phenobarbitone induction albeit to a lesser extent than BNF induction.

Between 1977 and 1981 several investigators documented poor correlations between *in vitro* and *in vivo* markers of human cytochrome P450 activity [8–12]. In retrospect this lack of success is not surprising and may be explained, at least in part, by the poor selection of markers and the lack of appreciation of the multiplicity of cytochromes P450. In all cases the *in vivo* marker was AP total clearance and, as discussed earlier, individual metabolite formation clearances have subsequently been documented to

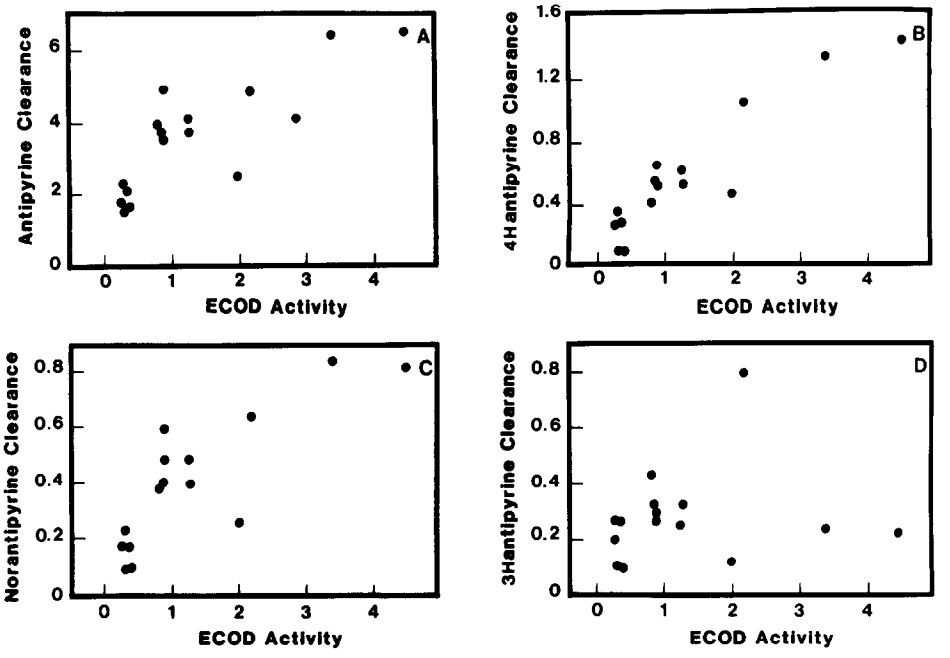

Fig. 3. Relationship between AP and its individual metabolite formation CLs and ECOD activity. (A) CL(AP) and ECOD activity; (B) CL(4H) and ECOD activity; (C) CL(N) and ECOD activity; (D) CL(3H) and ECOD activity.

be of greater value. Microsomal activity was assessed in these studies using either total cytochrome P450 content or marker assays which in the main have now been superseded by more sensitive and selective assays of particular isozyme(s) activity. Many of these correlation studies employed human biopsy material from suspected liver diseased patients and hence the pathology, as well as the representative nature of the sample taken, is open to question. Similar investigations carried out using animal tissues [31, 32, 34] were without these concerns but the range of activity observed in the basal state was too narrow to allow useful correlations to be assessed.

More recent work with human microsomes has centered on comparing activities towards different marker substrates. Strong correlations have been demonstrated for substrates subject to regio-selective hydroxylation including biphenyl [35], warfarin [36] and 2-acetylaminofluorine [37] but not between other markers. Notable exceptions to this trend are the correlations between EROD and N-demethylation of various methylxanthines [29] and between the 4-hydroxylation of debrisoquine and the 2-hydroxylation and 10-hydroxylation of desmethylimipramine and nortriptyline, respectively [38].

In animal experiments conditions can be carefully controlled and hence the imponderables resulting from multifactorial modification of monooxygenase activity in the human work are not a major problem. This situation and the adopted experimental design contributed to the success of the present correlation studies. The same rats were used for both the *in vitro* and *in vivo* measurements and markers were co-administered to minimize inter- and intra-individual variability. The use of BNF in addition to producing a wide range of enzyme activity also alters the complement of cytochromes P450 by selective induction of P450I. These investigations are the first to demonstrate strong and predictively useful correlations between *in vitro* and *in vivo* markers of hepatic microsomal monooxygenase activity.

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