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Limited value of the urinary phenytoin metabolic ratio for the assessment of cytochrome P4502C9 activity *in vivo*

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Relationships between the ratio of *p*-hydroxyphenytoin (p-HPPH), the major metabolite of phenytoin, to unchanged phenytoin excreted in urine (the urinary metabolic ratio or MR) were compared with a number of indices of the metabolic clearances of phenytoin and tolbutamide published previously for seventeen subjects separately administered these known cytochrome P4502C9 (CYP2C9) substrates. Significant correlations ($r_s = 0.50–0.60$, $P < 0.05$) were observed between the phenytoin MR, derived from either 0–24 or 24–48 h urine collections, and inverse areas under the plasma unbound concentration-time curves (measured over various time intervals) of phenytoin and with plasma unbound tolbutamide clearance. Significant correlations ($r_s = 0.59–0.74$) were also observed between the phenytoin MRs and metabolic unbound clearances for *p*-hydroxyphenytoin formation. Despite the significant correlations, variability in tolbutamide and phenytoin metabolic clearance parameters tended to account for $< 50\%$ of the variability in phenytoin MR. Correlations between the renal clearance of phenytoin and the phenytoin MRs suggest that variability in the renal clearance of unchanged drug limits the usefulness of the phenytoin MR for the investigation of factors influencing CYP2C9 activity *in vivo*.

Keywords phenytoin metabolic ratio cytochrome P450 tolbutamide

Introduction

There is increasing awareness of the importance of cytochrome P4502C9 (CYP2C9) in drug metabolism. Substrates for this enzyme include phenytoin [1,2], tolbutamide [2,3], torasemide [4], S-warfarin [5] and a number of non-steroidal anti-inflammatory agents [6–8]. Thus, factors (e.g. drug-drug interactions, genetics) which alter the activity of CYP2C9 potentially affect the therapeutic and toxic responses to these drugs.

Several approaches have been utilised to assess *in vivo* drug metabolising enzyme activity in order to investigate the regulation of individual CYP isoforms. *In vivo* drug metabolising enzyme activity can be measured directly from the unbound partial metabolic clearances of CYP isoform-specific substrates [9–11]. Although this index is theoretically the most correct method for assessing *in vivo* enzyme activity, it is not suitable for population studies given the need for extensive blood and urine sampling. Thus, indirect measures, particularly the ratio of the concentration of metabolite to unchanged drug in urine (the urinary metabolic ratio or MR), have been employed to investigate CYP isoform activities in population studies. It can be demonstrated that the MR approximates the ratio of metabolic partial clearance to renal clearance of unchanged drug when the latter is low relative to hepatic blood flow [10,11]. Assuming renal clearance of unchanged drug remains constant, the MR should reflect metabolic partial clearance and hence the activity of the enzyme(s) responsible for the formation of a particular metabolite.

Here we report the results of a study which investigated the validity of the phenytoin MR as a marker of CYP2C9 activity *in vivo*. The ratio of *p*-hydroxyphenytoin [5-(*p*-hydroxyphenyl)-5-phenylhydantoin; *p*-HPPH], the major metabolite of phenytoin, to unchanged phenytoin excreted in urine was compared with a number of indices of phenytoin and tolbutamide metabolic clearance published previously for seventeen subjects separately administered these known CYP2C9 substrates.

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Methods

Subjects and protocol

Details of the study protocol and the phenytoin and tolbutamide plasma concentration data have been published previously [12]. Briefly, thirteen males (age 19–25 years, weight 55–86 kg) and four females (age 19–21 years, weight 56–66 kg) completed the study. (Although eighteen subjects participated in the original study [12], one male did not comply with the urine collection protocol and data for this subject were excluded from the current analysis.) Subjects were healthy as determined by medical history, physical examination and standard biochemical and haematological parameters. None were smokers or were receiving other drugs at the time of the study, although most admitted to low to moderate alcohol consumption (5–25 g day^{-1}). Subjects provided written, informed consent for their participation in the study, which was approved by the Clinical Investigation Committee of Flinders Medical Centre.

Phenytoin sodium, 300 mg (Dilantin, Parke-Davis, Sydney, Australia) and tolbutamide, 500 mg (Rastinon, Hoechst, Melbourne, Australia) were separately administered to each subject following an overnight fast. Treatment phases were randomised and separated by 3 weeks. Fifteen blood samples (0–32 h post-dose) were collected following tolbutamide administration while sixteen blood samples (0–56 h post-dose) were taken after the phenytoin dose [12]. Additionally, complete urine was collected from each subject from 0–24 h and from 24–48 h following phenytoin administration. During the collection period urine samples were stored at 4° C. At the end of the collection periods, urine volumes were recorded and aliquots were retained and stored at −20° C until analysed.

Analytical procedures

Plasma unbound and total concentrations of tolbutamide and phenytoin were determined as described previously [12]. Concentrations of phenytoin in urine were analysed using the same procedure as that used for plasma samples, except standard curves were prepared using drug-free urine spiked with phenytoin to give concentrations in the range 0.25–10 mg l^{-1}.

The concentration of p-HPPH in urine was determined by a specific h.p.l.c. method after hydrolysis of the conjugated p-HPPH using β-glucuronidase. Briefly, β-glucuronidase (5000 units, type H1; Sigma Chemical Co, St Louis, MO, USA) was added to 100 µl of urine and the pH was adjusted to 5.4 with acetate buffer (0.1 M). After mixing, the solutions were incubated at 37° C for 1 h. At the completion of hydrolysis, 100 µg of internal standard [5-(4-methylphenyl)-5-phenylhydantoin] and 800 µl of sodium bicarbonate (1 M, pH 7.0) were added to the solution, which was then extracted with ethylacetate (5 ml) by rotary mixing for 20 min. The organic phase was transferred to a conical tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 µl of mobile phase and an aliquot (10 µl) was injected onto the h.p.l.c. The chromatograph was fitted with a Spherisorb reversed phase (C18) column (25 cm × 4.6 mm i.d., ICI Instruments, Melbourne, Australia) which was eluted isocratically with 33% acetonitrile and 67% phosphoric acid (0.05% v/v) at a flow rate of 1.2 ml min^{-1}. Absorbance was monitored at 210 nm. Under these conditions, the retention times for p-HPPH and internal standard were 2.7 and 9.7 min, respectively. Standard curves were prepared using drug free urine spiked with p-HPPH to give concentrations in the range 10–100 mg l^{-1}. Unknown p-HPPH concentrations were determined by comparison of the peak height ratios with those of the standard curve. Using this procedure, the mean (± s.d.) recovery of p-HPPH over the standard curve concentration range was 98.2 ± 1.6%. Within-day ($n = 6$) and between-day ($n = 6$) coefficients of variation were 4.2 and 5.7%, respectively, at an added concentration of 20 mg l^{-1} and 2.9 and 3.4%, respectively, at an added concentration of 80 mg l^{-1}.

Analysis of results

The phenytoin MR for the 0–24 h and 0–48 h urine collection periods was calculated as:

$$MR = \frac{\text{amount of p-HPPH in urine (µmol)}}{\text{amount of phenytoin in urine (µmol)}}$$

Areas under the plasma total concentration-time curves (AUC) for phenytoin and tolbutamide were calculated by the trapezoidal rule with extrapolation to infinite time as described previously [12]. Areas under the unbound plasma concentration-time curves (AUC_u) were determined as the product of individual AUC values and mean unbound fraction for each drug and each subject. Metabolic unbound clearances for p-HPPH formation over the 0–24 and 24–48 h urine collection intervals were calculated as the amount of p-HPPH excreted in urine divided by area under the phenytoin unbound concentration-time curve for the relevant 24 h time period. Renal clearances of phenytoin over these intervals were similarly calculated as the amount of unchanged phenytoin excreted in urine divided by area under the phenytoin (total) concentration-time curve. Correlations between parameters were determined by the Spearman rank method.

Results

The phenytoin MR is theoretically equivalent to the ratio of metabolic clearance for p-HPPH formation (CL_{HPPH}) and renal clearance of unchanged drug (CL_R). Thus, relationships between the phenytoin MRs (determined for the 0–24 and 24–48 h urine collection periods) and indices of CL_{HPPH} and CL_R were investigated. These are summarised in Table 1 and in Figure 1.

Table 1 Relationships between urinary phenytoin metabolic ratios and indices of phenytoin and tolbutamide metabolic clearance

	MR $(24–48\ h)$	1 / $AUCu_{PT}$	1 / $AUCu_{PT}$ $(0,\ 24\ h)$	1 / $AUCu_{PT}$ $(24,\ 48\ h)$	CLu_{HPPH} $(0–24\ h)$	CLu_{HPPH} $(24–48\ h)$	CLu_{TOLB}	CL_R $(0–24\ h)$	CL_R $(24–48\ h)$
MR (0–24 h)	0.52	0.65	0.53	0.60	0.70	0.74	0.53	−0.45	−0.33
MR (24–48 h)		0.50	0.51	0.56	0.59	0.64	0.60	−0.31	−0.49

Critical values of r_s ($n=17$): $r_s=0.49–0.645$, $P<0.05$; $r_s>0.65$, $P<0.01$

The phenytoin MRs determined using the 0–24 and 24–48 h urine samples correlated significantly ($r_s=$ 0.49–0.65) with inverse areas under the plasma unbound phenytoin concentration-time curve ($AUCu_{PT}$) calculated for the 0–24 h, 24–48 h and 0-infinity time intervals (taken from ref. 12). Correlation coefficients between the 0–24 and 24–48 h MRs and metabolic unbound clearances for p-HPPH formation (CLu_{HPPH}) corresponding to these intervals were 0.70 and 0.64 ($P<0.01$), respectively. The correlations between the MRs and values of CL_R for the corresponding time intervals ($r_s=$-0.45 and −0.49; $P=0.06$ and 0.045, respectively) were of low or borderline statistical significance. Urine flow and phenytoin CL_R correlated significantly ($P<0.01$) in both the 0–24 ($r_s=0.77$) and 24–48 ($r_s=0.84$) h urine collection periods. Mean recoveries of p-HPPH and unchanged phenytoin over the 0–48 h urine collection period accounted for 62.7% and 0.6% of the phenytoin dose, respectively.

The metabolic clearances of both phenytoin and tolbutamide are dependent on CYP2C9 activity and it might therefore be expected that the phenytoin MRs would correlate significantly with the plasma unbound clearance of tolbutamide (CLu_{TOLB}) measured in the same group of subjects. CLu_{TOLB} correlated significantly with the phenytoin elimination parameters 1/AUCu, 1/AUCu(0, 24 h), 1/AUCu(24, 48 h), CLu_{HPPH}(0–24 h) and CLu_{HPPH}(24–48 h) ($r_s=0.69–0.84$; $P<0.01$) (data not shown). Correlation coefficients between the 0–24 and 24–48 h MRs and CLu_{TOLB} were 0.53 and 0.60 ($P<0.05$), respectively.

Discussion

The hydroxylations of phenytoin and tolbutamide, to form p-HPPH and tolylhydroxy tolbutamide, respectively, are both known to be catalysed by CYP2C9 [1–3]. Metabolic unbound clearance for the formation of either metabolite therefore provides a measure of CYP2C9 activity *in vivo*. Furthermore, since the respective hydroxylation pathways account for 80% or more of the elimination of phenytoin and tolbutamide in humans [13,14], the plasma unbound clearances (and hence inverse AUCu values) should similarly reflect CYP2C9 activity *in vivo*. Although significant correlations were observed between the phenytoin MR (calculated from either the 0–24 or 24–48 h urine collection) and inverse $AUCu_{PT}$ (calculated from 0-∞, 0–24 or 24–48 h), CLu_{HPPH} and CLu_{TOLB}, correlation coefficients ranged from 0.50 to 0.74. Thus, variability in phenytoin and tolbutamide metabolic clearance generally accounts for 50% or less of the variability in the phenytoin MRs measured here. These data suggest that the phenytoin MR is a relatively poor measure of CYP2C9 activity *in vivo*.

As noted earlier, the MR is a composite parameter which is affected by both metabolic clearance and renal clearance of unchanged drug [10,11]. Phenytoin is a reasonably lipophilic compound and, as might be predicted, renal clearance of unchanged phenytoin was shown here to be dependent on urine flow rate. The

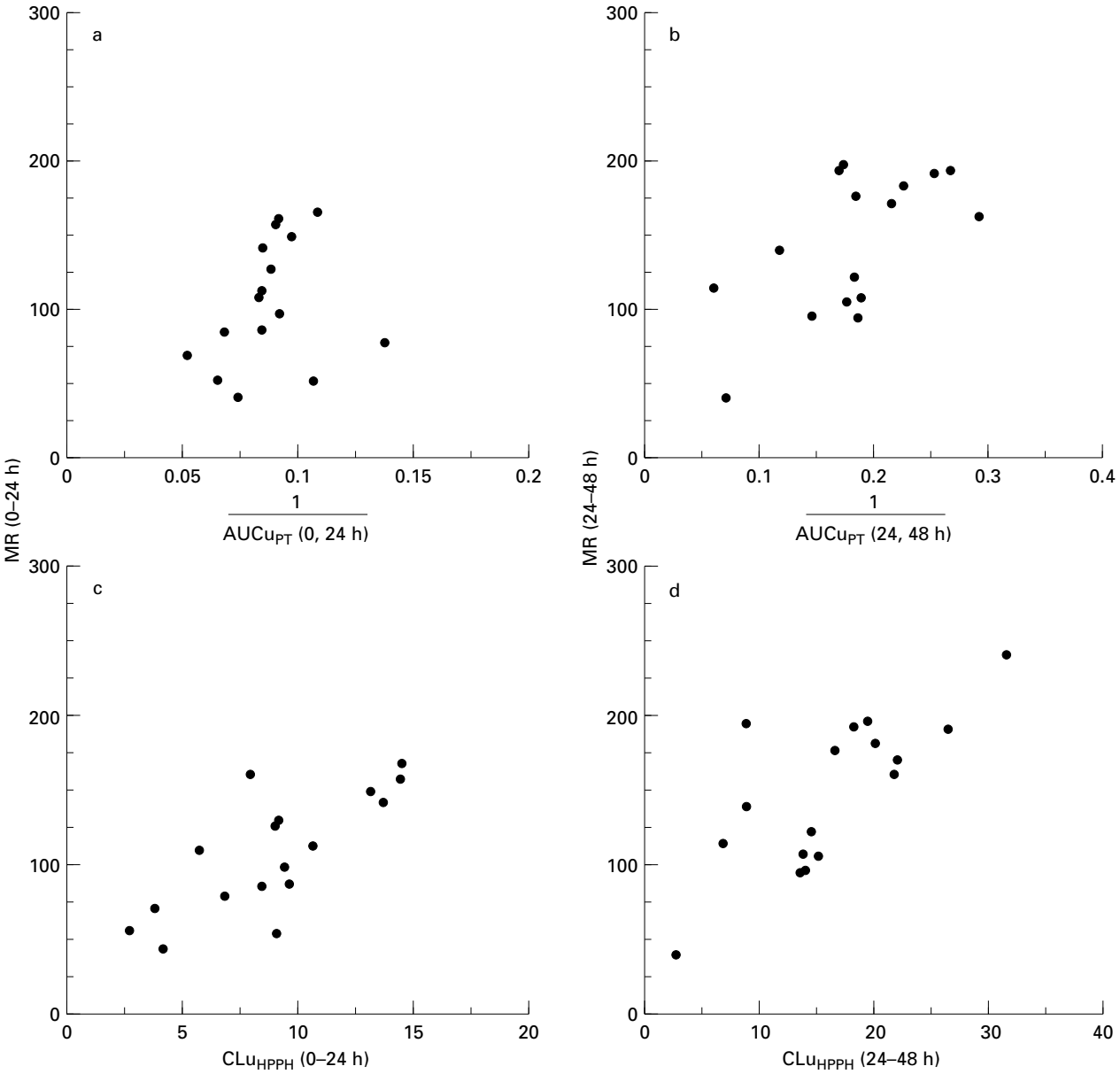

Figure 1 Relationships between the urinary phenytoin metabolic ratios (0–24 and 24–48 h urine collection periods) and inverse areas under the plasma unbound concentration-time 0–24 and 24–48 h curves for phenytoin (a and b) and the metabolic unbound clearances for p-HPPH formation (c and d).

dependence of phenytoin renal clearance on urine flow rate has been reported previously [15]. The urinary excretion of p-HPPH (as the glucuronide conjugate) is less sensitive to changes in urine flow [15,16]. No attempt was made here to standardise urine flow rate, but this factor is rarely controlled in population studies which utilise a MR. In the present work phenytoin renal clearances varied 3.6- and 3.8-fold over the 0–24 h and 24–48 h urine collection periods, respectively (data not shown). Although the range of values of CL_R was smaller than the variability observed for CLu_{HPPH} (Figure 1), an influence of renal clearance on the phenytoin MR was confirmed by significant (or near significant) correlations between these parameters over the two 24 h urine collection periods.

The present study has demonstrated that the phenytoin MR is of limited value for the investigation of factors affecting CYP2C9 activity *in vivo*. Various MRs have proved useful for the assignment of individuals to extensive- or poor-metaboliser phenotypes in pharmacogenetic studies [17–19]. However the phenytoin MR data confirm the results of a previous study from this laboratory [20] which demonstrated the fragility of the MR as a means of detecting changes in metabolic clearance and enzyme activity normally associated with non-genetic influences. The MR is unlikely to serve as a reliable index of metabolic clearance for lipophilic, extensively metabolised drugs with one major pathway of metabolism.

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