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Absorption and metabolism of hexamethylmelamine and pentamethylmelamine in rat everted perfused gut segments: correlation with in-vivo data

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The intestinal oxidative metabolism of hexamethylmelamine (HMM) and pentamethylmelamine (PMM) has been studied in microsomes, isolated mucosal cells and intestinal perfused segments. (sub)Cellular systems revealed an almost equal K_m (53–65 μM) and V_{max} (5·6–7·0 nmol min^{-1} g^{-1} intestine) for both compounds. Detailed studies in everted intestinal perfused segments, showed that HMM is metabolized to a far greater extent than PMM (e.g. 11-times, at 80 μM substrate concentration) while PMM transport was 3 times greater than the transport of unchanged HMM. Only when perfused segments were used as an in-vitro tool was a good correlation observed between the in-vivo and in-vitro rate of intestinal metabolism of HMM and PMM. It is concluded that this is due to preservation of structural integrity of the mucosa for both absorptive and metabolic processes.

Hexamethylmelamine (HMM, Fig 1) and pentamethylmelamine (PMM, Fig. 1) both are synthetic s-triazine derivatives. HMM has activity against a wide variety of human solid tumours (Blum et al 1973; Legha et al 1976). But its limited water solubility necessitates oral rather than parenteral administration to man. PMM was developed as a water-soluble analogue for intravenous (i.v.) administration (Clinical Brochure NCI 1978), but appeared to have less antitumour activity and limiting gastrointestinal and central nervous system toxicity compared with HMM (Casper et al 1981; Goldberg et al 1980; Ihde et al 1980).

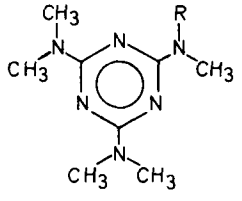

FIG. 1. Structures of hexamethylmelamine (R = CH_3), pentamethylmelamine (R = H) and *N*-hydroxypentamethylmelamine (R = CH_2OH, shown to be an intermediate in hepatic (Gescher et al 1980) and intestinal (Borm et al 1983b) metabolism of HMM to PMM).

We have shown that HMM is subject to extensive gut wall metabolism in-vivo (Klippert et al 1983) and found first-pass effects in rat liver and gut wall of 73 and 71%, respectively.

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PMM, which is HMM less one CH_3-moiety, was not subjected to detectable metabolism (<10%) during its first-pass in the small intestine, while the hepatic extraction ratio was 50% (Klippert 1983; Klippert et al 1985). As the intestinal metabolism of HMM might be related to its (unknown) mechanism of action (Gescher et al 1980; Ames et al 1983) we investigated its in-vitro intestinal metabolism (Borm et al 1983a, b, 1984). In the present study we are concerned with the difference between intestinal first-pass effects of HMM and PMM.

The ability of an organ to remove a substance from a medium perfusing it is dependent on blood flow to the organ as well as enzyme activity. Under first order conditions enzyme activity can be described as a clearance term (Rane et al 1977) Cl_{int}, intrinsic free clearance

$$Cl_{int} = \frac{V_{max}}{K_m} \quad (1)$$

V_{max} and K_m being the Michaelis–Menten parameters of the enzyme system involved. Assuming linear kinetic conditions ($c \ll K_m$) and using the venous equilibration model (Rowland et al 1973; Wilkinson & Shand 1975), the extraction ratio (E) of an organ can be calculated from

$$E = \frac{Cl}{Q} = \frac{f_u.Cl_{int}}{Q + f_u.Cl_{int}} \quad (2)$$

where Cl is the organ clearance, Q is the blood flow through the organ and f_u is the fraction of unbound substance in blood. The value of this model in

predicting hepatic and pulmonary clearance is well established (Wiersma & Roth 1983). To see whether these model systems can also be used to predict the extent of the oxidative first-pass metabolism by the intestine, we have used the rat everted intestinal sac as an in-vitro system to determine extraction ratios for HMM and PMM. The results indicate that the latter system is a useful qualitative and quantitative tool for the assessment of the extraction ratios.

MATERIALS AND METHODS

Chemicals

HMM (hexamethylmelamine, NCS 13875) was obtained from Offichem (Gieten, The Netherlands). HMM-hydrochloride was prepared as described by Klippert et al (1983) and Van de Vaart–van Zutphen et al (1982). The *N*-demethylated metabolites of HMM were kindly provided by Dr C. J. Rutty (Institute of Cancer Research, Sutton, Surrey, UK). Glucose-6-phosphate dehydrogenase (grade 1), $NADP^+$ and glucose-6-phosphate were purchased from Boehringer Mannheim (Mannheim, FRG). Pethidine hydrochloride was from Brocacef (Maarssen, The Netherlands). All other solvents and reagents were of analytical (p.a.) grade, except for methanol and chloroform (Nanograde, Mallinckrodt, St Louis, USA). Isopropanol, analytical grade (Merck, Darmstadt, FRG) and amylalcohol (Brocacef) were distilled from glass before use.

Animals

Adult male Wistar rats (Cpb:WU) (230–250 g) (TNO Zeist, The Netherlands) were allowed free access to tap water and a commercially available diet (Muracon-1, Trouw, Putten, The Netherlands).

Isolation of cells and preparation of subcellular fractions

Animals were killed by cervical dislocation and the small intestine was immediately flushed free of its contents with ice-cold phosphate buffered saline (mM: NaCl, 90, KH_2PO_4, 9 and $Na_2HPO_4.2H_2O$, 34, to give a solution of pH 7·4). A high-frequency low amplitude vibration method was used to isolate intestinal epithelial cells (see Borm et al 1982, 1983a). Cells were washed twice with phosphate buffered saline and resuspended in Krebs Ringer buffer (mM: NaCl 94, KCl 4·7, $MgSO_4.7H_2O$ 1·2, $CaCl_2.2H_2O$ 2·5, KH_2PO_4 1·2, $NaHCO_3$, 25 and glucose 11·6) saturated with carbogen gas (5% CO_2—95% O_2, v/v), pH 7·4. Microsomal preparations were prepared by homogenization of isolated cells (Borm et al 1983a). isolated cells from 0·125 g intestine ($7·5 \times 10^6$ cells, 3·0 ± 0·2 mg protein) or microsomes obtained from 0·5 g intestine (1·0 ± 0·2 mg protein) were incubated in a final volume of 3·0 ml Krebs Ringer buffer or phosphate-buffer (50 mM, pH 7·4, EDTA: 0·1 mM), respectively. In microsomal incubations the cofactors $NADP^+$ (0·5 mM), glucose-6-phosphate (4·2 mM), $MgCl_2$ (4·2 mM) and glucose-6-phosphate dehydrogenase (0·3 IU ml^{-1}) were included. After a 10 min preincubation the reaction was started by the addition of 0·5 ml HMM hydrochloride or PMM solution in water (final concentration between 1 and 200 μM). The reaction was stopped at 10 min by the addition of 375 μl trichloracetic acid and the mixture was centrifuged at 1000*g*. 100 μl of the supernatant was used for the gas chromatographic assay of demethylation products.

Analysis of HMM, PMM and metabolites

The gas chromatographic assay was that described by Klippert et al (1983) and Hulshoff et al (1980). Supernatant, 50–100 μl, depending on concentration of the sample, was used in the assay. All samples were made up with blank Krebs solution to 100 μl.

Intestinal perfusions

Animals were killed by cervical dislocation, and the small intestine (distal to the duodenum) was excised and immediately flushed free of intestinal contents with ice-cold PBS. Three sequential segments of approximately 10 cm length (0·63 ± 0·09 g, s.d. n = 24) were used for perfusion experiments. Intestinal segments were everted on a metal rod (also used in cell isolation) and mounted in the perfusion apparatus. The intestine was held vertically by attaching a stainless-steel weight (5 g) and submerged in Krebs Ringer buffer, saturated with carbogen gas (5% CO_2—95% O_2, v/v). The serosal side (inside compartment) was perfused with the same medium (flow 5·0 ± 0·4 ml min^{-1}, s.d. n = 24), total volume 30·0 ml. The perfusion apparatus consisted of a glass extraction tube fitted with a Teflon stopper, pierced by four stainless-steel needles (Chowhan & Amaro 1977; Koster & Noordhoek 1983). The entire apparatus was submerged in a water bath at 37 °C. Special inert perfusion tubing (Acid-flex, 2 mm inside diameter, Technicon, Zoetermeer, The Netherlands) was used as HMM absorption occurred in conventional polyethylene tubing. No adsorption occurred in the polystyrene incubation vessels. Results from pilot experiments showed that transport and metabolism data were not dependent upon the origin of the intestinal segment (distal duodenum

to proximal ileum). Therefore no discrimination was made with respect to this factor in the design of the experiments and analysis of the data.

Experimental design and statistical analysis

After 15 min perfusion of the segment with Krebs Ringer, 0·12 ml of a solution containing HMM or PMM in methanol was injected in the mucosal solution (30 ml, outside compartment) achieving final concentrations as required. At 0, 2, 5, 7, 10, 15, 20, 30, 45 and 60 min, 0·5 ml samples were drawn from the mucosal and serosal fluid into polyethylene syringes via spinal needles. Samples were stored in 1·5 ml polystyrene test tubes (Greiner BV, Alphen a/d Rijn, The Netherlands) at −20 °C until analysis by GC. At the end of the experiment the intestinal wet weight was measured. The segments were homogenized using an Ultra-Turrax (Janke & Kunkel KG, Staufen im Breisgau, Switzerland) in 5 ml of Krebs Ringer and assayed for the presence of HMM, PMM and further demethylated products.

With the exception of the determination of K_m- and V_{max}-values in isolated cells and microsomes, and the pilot experiments to establish optimal perfusion concentrations, the main experiment was designed as completely randomized three-factorial, in which the following treatment factors were discriminated. The substrate administered: either HMM or PMM was added to the mucosal side. Dose: the substrates were added in a final concentration of 80, 200 or 1000 μM in methanol (final concentration 0·4%, v/v). Mucus: everted intestinal segments were either mounted immediately after eversion or after eversion followed by vibration (2 mm, 50 Hz) for 2 min in ice-cold phosphate buffered saline. We showed previously (Koster et al 1984) that this procedure was effective in removing all mucus without damaging the morphological structure of the mucosal epithelium. An analysis of variance on all characteristic parameters discussed hereafter revealed that the 2 min previbration ('minus mucus') had no significant effect on any of the parameters, for either substrate or concentration used. As a result all data designed to distinguish the 'mucus' effects were pooled and used in the following evaluation. The data for metabolic clearance were analysed by ANOVA after tranformation to the logarithmic scale, because variances on the arithmetic scale were not distributed homogeneously. Whenever a primary treatment effect was found to be statistically significant, this effect was partitioned in mutually orthogonal comparisons.

K_m- and V_{max}-values were obtained initially by graphical analysis of Eadie Hofstee plots (see also Fig. 4). The values thus obtained were used as first estimates in an iterative program based on non-linear least square regression analysis (Duggleby 1981) to fit the equation $V = V_{max}/(1 + K_m/[S])$. Protein was determined according to Lowry et al (1951).

Kinetic parameters

The (net) transport rate of unchanged parent compound from the mucosal to the serosal side is described by $K_{transport}$ (K_{tra}). The rate by which the intestinal segment metabolizes the substrate is described by $K_{metabolism}$ (K_{met}). K_{met} results from the summation of formation rates of all metabolites on both serosal and mucosal side (eqn 4). (The amount of parent compound 'excreted' as metabolite on the serosal side is *not* included in K_{tra}, but only in K_{met}). Both K_{tra} and K_{met} are expressed in nmol min^{-1} ml^{-1}, and can be calculated from:

$$K_{tra} = \frac{c_{(\text{parent compound})ser}}{\Delta t} \quad (3)$$

$$K_{met} = \Sigma_{i=1}^{n} K_{met,i} \quad (4)$$

$$K_{met,i} = (K_{met,i})_{ser} + (K_{met,i})_{muc} \quad (5)$$

$$= \frac{(c_{met,i})_{ser}}{\Delta t} + \frac{(c_{met,i})_{muc}}{\Delta t'} \quad (6)$$

where, c = concentration at time t, Δt = time interval from lag time till time t, ser = serosal side, muc = mucosal side, $K_{met,i}$ = total rate of formation of the i-th metabolite.

The lag time was calculated from the abscissa of the linear least square analysis (see above) or defined as zero if this extrapolation resulted in a negative lag phase. As the lag time of a specific metabolite's appearance does not need to be the same for the mucosal or the serosal side, the symbols Δt and $(\Delta t)'$ are used in eqn (6). Multiplication of the K-values by their corresponding distribution volume (always 30 ml, if the accumulating loss of volume up to 5 ml by subsequent sampling is neglected) yields the total *amount* of drug, transported or metabolized per minute. Subsequent division by the concentration of the parent drug at the mucosal side (c_{muc}) gives the corresponding clearance terms

$$Cl_{tra} = \frac{K_{tra} \cdot 30}{c_{muc}} \quad (7)$$

$$Cl_{met} = \frac{K_{met} \cdot 30}{c_{muc}} \quad (8)$$

in which Cl is the symbol for clearance (in ml min^{-1}).

The initial mucosal concentration (at t = 0) was substituted for c_{muc}, because the amount of converted substrate can be neglected (<10% of the total amount, see also above). Only at low concentrations of HMM the actual clearance is probably underestimated, because at t = 30 almost 17% of the HMM is converted.

RESULTS

General characteristics

A typical example of the appearance of the parent compound and metabolites during the intestinal perfusion experiments is shown in Fig. 2 (HMM, 80 μM initial concentration) and Fig. 3 (PMM, 80 μM initial concentration). All profiles of appearance of metabolites and substrate (serosal side) or the disappearance of the substrate from the mucosal side during 0–30 min could be well fitted ($r \geq 0{\cdot}98$) with linear least square analysis (Snedecor & Cochran 1967), although an exponential curve is expected for first-order kinetics. The reason for the apparent linearity is that at 30 min no more than 7% of the initial amount of PMM (all concentrations) or 7–10% of the HMM (1000–80 μM) initially present, was transported and/or metabolized. Only at low concentrations of HMM (5, 20 μM) almost 17% of the substrate disappeared from the mucosal side, resulting in a better fit by an exponential curve. Linear profiles were obtained regardless of the substrate, concentration or pretreatment. A mass balance was made at low concentrations only (5, 20 and 80 μM), because substrate disappearance could not be quantified at the high background at 200 and 1000 μM, and proved to be correct (100–112% recovery).

Perfusion experiments

Immediately evident from the plots (Figs 2 and 3) are the differences in lag time observed for the parent compounds to 'pass' the intestinal barrier (18·9 min, HMM; 3·1 min, PMM) and rate of total metabolite formation (16·0 and 1·4 nmol min^{-1} g $intestine^{-1}$ for HMM and PMM, respectively). Most of the metabolites formed were in the mucosal compartment. Only small amounts of HMM, PMM or N_2,N_2,N_4,N_6-tetramethylmelamine ($TeMM_2$) could

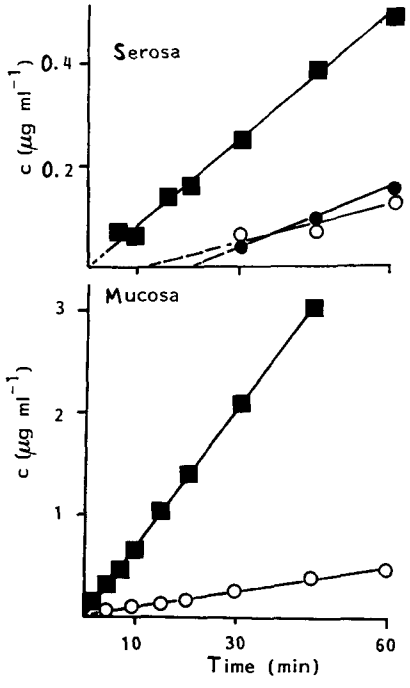

FIG. 2. Appearance of HMM (●), PMM (■) and $TeMM_2$ (○) on the serosal side and of PMM and $TeMM_2$ on the mucosal side of everted isolated perfused segments after administration of 80 μM HMM on the mucosal side. Data points represent individual measurements of any experiment. Note different scale in both panels.

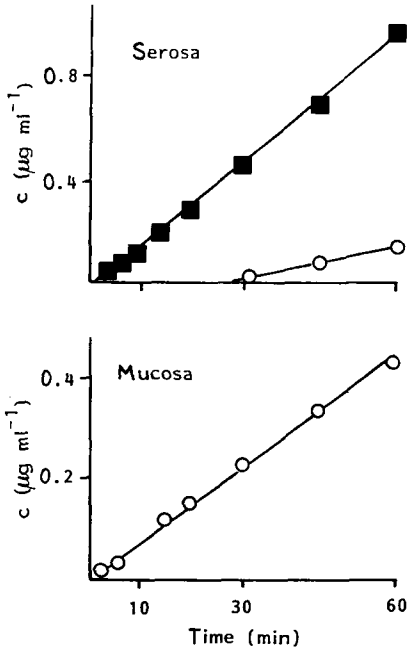

FIG. 3. Appearance of PMM (■) and $TeMM_2$ (○) on the serosal side and of $TeMM_2$ on the mucosal side of everted isolated perfused intestinal segments after administration of 80 μM PMM on the mucosal side. Data points represent individual measurements of any experiment at this concentration. Note different scale in both panels.

be detected in the intestinal segments at the end of experiments. In all experiments the total amount of substance recovered from intestinal tissue was less than 1·7% of the total amount cleared during the experiment, and was therefore neglected. No N_2,N_2,N_4,N_4-tetramethylmelamine ($TeMM_1$) or further demethylation products (trimethylmelamines) were detected either in the incubation media or homogenates.

In Table 1 metabolic (Cl_{met}) and transport (Cl_{tra}) clearance terms are listed, calculated (see legend Table 1) as means of the perfusion experiments involved. HMM and PMM differ significantly in their metabolic clearance ($P < 0{\cdot}001$, ANOVA, df = 11), transport clearance ($P < 0{\cdot}001$, ANOVA, df = 11) and lag time (data not shown) of penetration ($P < 0{\cdot}01$, ANOVA, df = 18). Metabolic clearance of HMM was concentration dependent ($P < 0{\cdot}01$, ANOVA, df = 9 and 2) while being a true constant for PMM (but much smaller) at all concentrations used. Transport clearance (passive diffusion) was a constant for both substrates and PMM transport clearance was 3 times greater than the transport of unchanged HMM.

Determination of K_m and V_{max} values

During the incubation period (10 min) metabolism of HMM and PMM remained restricted to the first demethylated product in cells or microsomes. Both substrates in either in-vitro system yielded linear Eadie Hofstee plots when velocities of *N*-demethylation were plotted against 'metabolic clearance' (v/s). As an example, the Eadie Hofstee plot obtained from the HMM *N*-demethylation by intestinal microsomes is shown in Fig. 4. In Table 2 K_m- and V_{max}-values obtained with microsomes or cells are listed, both parameters showing a striking similarity between HMM and PMM demethylation.

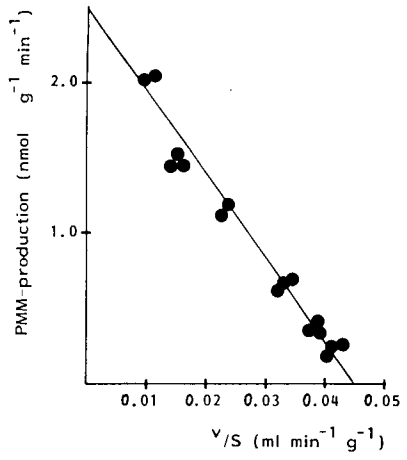

FIG. 4. Eadie Hofstee plot for HMM *N*-demethylase in control rat intestinal microsomes. Microsomes pooled from four animals were used. Triplicate incubations were performed at each concentration (5–200 μM) HMM used. No further demethylation products were detected by GC (see Materials and methods). A straight line (Corr. −0·980, least squares) led to first estimates of 54 μM and 2·45 nmol (min g)$^{-1}$ for K_m and V_{max}, respectively.

When the metabolic clearance data obtained in the intestinal perfusion system is plotted (Table 1) against the rate of appearance of total metabolites, a linear ('Eadie Hofstee') plot is also obtained for HMM (Fig. 5) but not for PMM because its metabolic clearance is a constant, independent of concentration. So using intestinal segments as an in-vitro model to study PMM metabolism no saturation kinetics (Michaelis-Menten) were observed at

Table 1. Metabolic and transport clearances calculated from analysis of intestinal perfusion experiments.

Substrate	Mucosal concentration μM	Cl_{met}[a]	Cl_{abs}[a]	Cl_{tot}[a]	E_g[b] (Cl_{met}/Cl_{tot})
PMM	All concentrations[c] used (20–1000 μM)	17·5 ± 2·1 (12)	47·5 ± 3·0 (11)	63·8 ± 3·2 (11)	0·27 ± 0·15 (11)
HMM	5	234 (1)	17·2 (1)	300 (1)	0·78
	20	250 (1)	16·5 (1)	382 (1)	0·88
	80	200 ± 17·5 (4)	21·2 ± 6·3 (4)	221·3 ± 13·2 (4)	0·90 ± 0·14
	200	117·7 ± 8·6 (4)[d]	21·2 ± 4·1 (4)	132·9 ± 6·9 (4)[d]	0·84 ± 0·07
	1000	34·2 ± 3·0 (4)[d]	11·2 ± 2·5 (4)[d]	45·4 ± 5·3 (4)[d]	0·75 ± 0·09

[a] Clearance terms are expressed in μl min^{-1} (g intestine)$^{-1}$ and presented as the mean ± s.e.m. of the number of perfusion experiments involved (in parentheses). Metabolic (Cl_{met}) and transport (Cl_{tra})-clearance are defined in the section *kinetic parameters*. Total clearance (Cl_{tot}) was assayed by the disappearance of the substrate from the mucosal compartment at 5, 20 and 80μM only. At 200 and 1000 μM Cl_{tot} is calculated as the sum of Cl_{met} and Cl_{tra}.

[b] E_g denotes the intestinal extraction ratio as described in eqn (9).

[c] An analysis of variances (ANOVA) revealed no effect of previbration and/or concentration on Cl_{met}, Cl_{tra}, Cl_{tot} and lag-times. For this reason all data were pooled and the mean ± s.e.m. of the ANOVA is listed.

[d] Significantly different from the same parameter at different concentrations (at least $P < 0{\cdot}01$, ANOVA).

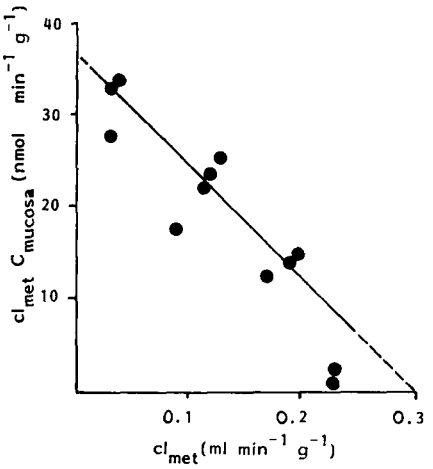

FIG. 5. 'Eadie Hofstee'-plot for the rate of metabolism of HMM ($Cl_{met}.S = V_{met}$) in intestinal perfused segments. Separate data from each perfusion experiment (5–1000 μM) are shown. A straight line (Corr. −0·836, least squares) led to the first estimates: K_m (app) = 113·3 μM and V_{max} (app): 27·2 nmol (min g)$^{-1}$.

concentrations up to 1 mM at the mucosal side; but, it could be that the concentrations used were lower than K_m (therefore $K_{m_{app}} \gg 1$ mM, Table 2).

Calculation of extraction-ratios

Hepatic and intestinal extraction ratios were calculated from K_m- and V_{max}-values using the well-stirred model (eqn 1 and 2), substituting liver blood flow (80 ml min^{-1} kg^{-1}) and mucosal blood flow (2·5 ml min^{-1} kg^{-1}) for Q respectively (eqn 2) and putting f_u to unity. Calculated hepatic extraction ratios (Table 2) are in good agreement with the in-vivo first-pass effects of HMM (73%; Klippert et al 1983) and PMM (50%; Klippert et al 1984). Intestinal extraction ratios for HMM and PMM were predicted to be 0·6 using isolated cells (or microsomes) as an in-vitro tool.

Unlike the hepatic situation, the intestinal predictions are not in good agreement with in-vivo observed intestinal first-pass metabolism. A satisfactory prediction was made for the intestinal extraction ratio of HMM (E_g = 0·71; Klippert et al 1983), but PMM was shown not to be subject to any detectable first-pass metabolism in the rat small intestine (Klippert et al 1984).

When the apparent 'K_m' and 'V_{max}'-values obtained with the intestinal perfusion system were used (see also Fig. 5) again a reasonable intestinal extraction ratio for HMM was obtained, while there was no value for PMM. However, when the metabolic clearance (Cl_{met}) is substituted in eqn (2) for intrinsic clearance (Cl_{int}), assuming 32 g intestine kg^{-1} rat and a mucosal blood flow of 2·5 ml min^{-1} kg^{-1}, the intestinal extraction ratio for PMM is 0·138 at all concentrations studied. From the latter method, HMM would be 30 to 75% extracted during its first-pass through the intestinal wall, depending on substrate concentration. These extraction ratios are in reasonable agreement with the in-vivo data.

The success of the method described led us to the calculation of an 'alternative' extraction ratio derived from the intestinal perfused segments by means of the equation

$$E_g = \frac{Cl_{met}}{Cl_{met} + Cl_{tra}} = \frac{Cl_{met}}{Cl_{total}} \qquad (9)$$

which is basically the same as eqn (2), in which not only intrinsic clearance (V_{max}/K_m) is substituted by

Table 2. Values for the Michaelis-Menten parameters of intestinal and hepatic HMM or PMM *N*-demethylase derived from different in-vitro systems and used for prediction of first pass metabolism.

			HMM			PMM		
Organ	In-vitro system	(n)	$K_{m,app}$[a]	V_{max}[b]	E[c]	$K_{m,app}$	V_{max}	E
Liver	Microsomes[a]	(1)	26·5	63·6	0·60	83	95·0	0·40
Small intestine	Microsomes[d]	(1)	55	5·6	0·57	ND[f]	ND[f]	
	Isolated cells	(2)	65	7·0	0·58	53	6·7	0·62
	Intestinal segments	(12)	140 ± 20[e]	39·0 ± 2·9[e]	0·78	≫1 mM		

[a,b] $K_{m,app}$- and V_{max}-values are expressed in μM and nmol min^{-1} (g organ)$^{-1}$, respectively.

[c] The intestinal extraction ratio (E_g) was calculated using eqn (1) and (2), substituting a mucosal blood flow (Q) of 2·5 ml min^{-1} kg^{-1} and assuming 32 g small intestine per kg rat. The hepatic extraction ratio (E_H) was calculated using the same model, using a portal vein blood flow of 80 ml min^{-1} kg^{-1} and assuming 48 g liver per kg rat.

[d] V_{max} was corrected for microsomal recovery (45%, see Borm et al 1984). N.B. Hepatic microsomes were prepared from isolated hepatocytes conform to intestinal microsomal preparation (Borm et al 1984).

[e] Both mean and s.e.m. were calculated from the Duggleby plot.

[f] ND means Not Determined.

(n) is the number of batches or segments for the determination of K_m and V_{max}.

metabolic clearance (Cl_{met}) but also Q is replaced by the transport clearance, Cl_{tra}. Using the data obtained with the intestinal perfused segments in eqn (9) values are obtained as listed in Table 1. A large difference in the intestinal extraction ratio (E_g) is predicted between PMM (0·27 ± 0·05) and HMM (0·83 ± 0·06) consistent with the in-vivo data. Moreover, the extraction ratios were shown to be independent of mucosal substrate concentration.

DISCUSSION

When a drug is placed in the intestinal lumen, several steps may be rate-limiting for its passage across the wall (i.e. bioavailability): (i) the uptake through the mucosal barrier and intracompartmental diffusion as free drug; (ii) the metabolic conversion of the drug by enzymes (K_m, V_{max}); and (iii) the release of free drug and/or (possibly inhibiting) metabolites on the serosal and/or mucosal side. Therefore extrapolation of in-vitro data to predict intestinal first-pass effects is an uncertain process at best.

In contrast with the satisfactory results obtained for hepatic first-pass effects, the observed in-vivo difference in intestinal first-pass of HMM and PMM could not be anticipated when eqn (2) was used to predict their in-vivo intestinal extraction ratios. The predicted value for HMM (E_g = 0·58, cells or microsomes) seems in reasonable agreement with in-vivo data, but the value for PMM was predicted to be similar. Clearly, relating enzyme kinetic parameters with mucosal blood flow (Klippert et al 1982) is not sufficient to estimate in-vivo first-pass effects in the small intestine. Organ clearance as described by eqn (2) seems to be valid only in the situation (HMM as substrate) where transport clearance is much smaller than metabolic clearance. For HMM, metabolic clearance is always greater than transport clearance (2- to 14-fold) and total clearance by intestinal perfused segments (1·5–9·3 ml min^{-1} kg^{-1} body weight) is greater than mucosal blood flow (2·5 ml min^{-1} kg^{-1} body weight). PMM transport clearance, however, is about 3 times its metabolic clearance when assayed in the perfusion system. As a result, when a confined incubation system (isolated cells) in which transport, rate of diffusion and possible polarity in drug metabolism are definitely overlooked is used, there is an *over*estimation of the metabolism of PMM. The advantage of the intestinal extraction ratio calculated by eqn (9) is that transport clearance is determined in the same experiment and not a parameter (like mucosal blood flow) assayed in a different (in-vitro) system or taken from literature. Also, it is immediately recognized that mucosal blood flow is a factor determining transport clearance, but that it is not the only one.

When isolated perfused segments are used, the intracellular substrate concentration (and therefore metabolic clearance, Cl_{met}) is determined by the rate at which the substrate is absorbed, K_{in} (mucus passage, absorption at microvilli), by the rate of its passage out, K_{out} (passage through the basolateral membrane, several muscle layers), and the intrinsic free clearance, $Cl_{int}(V_{max}/K_m)$. Obviously, the rapid passage of PMM is the reason that no saturation of PMM metabolism is observed with concentrations up to 1000 μM. Saturation at this point would certainly be expected on basis of the observed cellular K_m (53 μM).

We used everted intestinal segments to establish if mucus had an effect on the absorption (and 'therefore' on metabolism) of HMM and/or PMM. That no effect was observed could be due to the fact that rat intestinal segments produce mucus rapidly, thereby nullifying the effect of the 2 min previbration on mucus during the 15 min equilibration period. Eversion does not appear to affect functional (Chowhan & Amaro 1977) or morphological integrity (Koster et al 1984); but, undoubtedly, the everted intestinal perfused segments differ from the in-situ (for review, see Windmueller & Spaeth 1981) and in-vivo situation. The absence of intestinal motility and of an intact mucosal flow may profoundly affect uptake and/or release of drug and metabolites.

All this implies that we are underestimating transport clearance in particular. Assuming the latter is twice as high in-vivo, while metabolic clearance remains the same, substitution in eqn (9) would lead to intestinal extraction ratios of 0·71 and 0·16 for HMM and PMM, respectively. In-vitro–in-vivo correlation clearly improves.

We have shown that everted perfused intestinal segments can be used as a simple in-vitro system predicting intestinal metabolism. Absorption and metabolism are 'competing' processes, eventually determining the intestinal first-pass. This competition was shown previously in the rat in-situ (vascularly) using perfused rat intestinal segments (Bock et al 1979) for benzo(a)pyrene (slow absorption, high metabolism) and naphthalene (rapid absorption, low metabolism). Clearly, determination of intrinsic clearance in a confined in-vitro system can result in false positive predictions.

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