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Utility of hepatocytes to model species differences in the metabolism of loxtidine and to predict pharmacokinetic parameters in rat, dog and man

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1. The metabolism of loxtidine (1-methyl-5-[3-[3-[(1-piperidinyl) methyl] phenoxy] propyl] amino-1H-1,2,4-triazole-3-methanol) was studied in freshly isolated rat, dog and human hepatocytes. Metabolism *in vitro* was comparable with previously available *in vivo* data in all three species with the marked species differences observed *in vivo* being reproduced in the hepatocyte model.

2. The major route for the metabolism of loxtidine by rat hepatocytes was N-dealkylation to form the propionic acid and hydroxymethyl triazole metabolites. A minor metabolic route was the oxidation of loxtidine to a carboxylic acid metabolite. The major route of metabolism for loxtidine in dog hepatocytes was glucuronidation with oxidation to the carboxylic acid metabolite being of minor importance. Incubation of loxtidine with human hepatocytes resulted in the drug remaining largely unchanged but with the carboxylic acid metabolite being produced in minor amounts.

3. *In vitro* studies were undertaken with rat, dog and human hepatocytes to determine the Michaelis-Menten parameters V_{max} and K_m for the sum of all the metabolic pathways. These kinetic parameters were used to calculate the intrinsic clearance of loxtidine. Using appropriate scaling factors, the predicted *in vivo* hepatic clearance was then calculated. The predicted intrinsic clearances were 51.4± 12.4, 8.0± 0.8 and 1.0± 0.6 ml/min/kg for rat, dog and human hepatocytes respectively. These data were then used to calculate hepatic clearances of 24.5, 3.1 and 0.2 ml/min/kg for rat, dog and man respectively.

4. *In vivo* hepatic and intrinsic clearances for loxtidine were determined in rat, dog and human volunteers. The hepatic clearances of loxtidine were 26.6, 6.6 and 0.4 ml/min/kg in rat, dog and man respectively and intrinsic clearances were 58.5, 18.6 and 2.0 ml/min/kg in rat, dog and man respectively.

5. The present studies demonstrate that the hepatocyte model may be a valuable *in vitro* tool for predicting both qualitative and quantitative aspects of the metabolism of a drug in animals and man at an early stage of the drug development process.

Introduction

Information on the metabolism of candidate drugs is important in understanding potential species differences in the pharmacological and toxicological properties exhibited by a new chemical entity. The recognition of the limitations in extrapolating animal data to man has increased the pressure for early information on the behaviour of candidate drugs in man. In recent years isolated hepatocytes have been used extensively to study biotransformation reactions and for preliminary studies on the metabolism of a new chemical entity. Such studies have demonstrated that isolated hepatocytes have clear advantages compared with subcellular fractions in the prediction of both routes and rates of metabolism (Begue *et al*. 1983). The use of the hepatocyte system as a predictive model for drug metabolism in different

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species remains an active area of research. Good *in vitro-in vivo* correlations of metabolic patterns have been reported using both isolated animal and human hepatocytes (Le Bigot *et al.* 1987, Oldham *et al.* 1990, Lavrijsen *et al.* 1992, Sandker *et al.* 1994, Pahernik *et al.* 1995) and studies reporting the failure of hepatocytes to predict metabolism have been few (Humpel *et al.* 1989). However, there have been few reports on the comparative metabolism of a single substrate in hepatocytes from the three species central to drug development, namely rat, dog and man (Chenery *et al.* 1987, Ekins *et al.* 1996, Lave *et al.* 1996a, b).

Recently, there has been considerable interest in the development of methods predicting the clearance of drugs *in vivo* from experiments *in vitro* (Houston 1994, Lave *et al.* 1996a, Houston and Carlile 1997, Iwatsubo *et al.* 1997a). Successful predictive methods could lead to significant savings in animals, time and cost during the drug development process. Microsomal (Carlile *et al.* 1997, Iwatsubo *et al.* 1997b, Sanwald-Ducray and Dow 1997) and hepatocyte (Oldham *et al.* 1986, Singh *et al.* 1991, Lave *et al.* 1995, 1996a, b, 1997, Carlile *et al.* 1997) systems have already been used successfully to predict quantitative hepatic elimination *in vivo* for some drugs.

Loxtidine hemisuccinate (1-methyl-5-[3-[3-[(1-piperidinyl) methyl] phenoxy] propyl] amino-1H-1,2,4-triazole-3-methanol) (figure 1) is a potent, long-acting histamine H_2-receptor antagonist (Brittain and Jack 1983). In laboratory animals and human volunteers loxtidine undergoes metabolism by several routes (figure 1) and shows significant species differences (Bell *et al.* 1983). The primary route of metabolism is N-dealkylation in the rat and glucuronidation in the dog. In contrast with animal species, loxtidine is eliminated predominantly by renal clearance in man although a single minor oxidative metabolite has been detected in urine. No evidence for the presence of a glucuronide metabolite was observed.

The observed species differences in metabolism make loxtidine an excellent model for the prediction of xenobiotic metabolism and pharmacokinetics *in vivo*. In the present studies, attempts have been made to model the metabolism of loxtidine and predict hepatic clearance *in vivo* from the data generated in isolated hepatocytes from rat, dog and man.

Materials and methods

Chemicals

^{14}C-Loxtidine hemisuccinate (specific activity 0.06 MBq/mg, radiochemical purity > 98%) (for the position of ^{14}C-radiolabel, see figure 1) was prepared by the Radioisotope Laboratory, Process Research Department, Glaxo Wellcome Research and Development (Ware, UK). Non-radiolabelled loxtidine hemisuccinate (AH23844A, batch no. C215/153/1, purity > 99% as determined by nmr) used for animal pharmacokinetic studies was supplied by Pharmaceutical Research, Glaxo Wellcome Research and Development (Ware, UK).

Authentic non-radiolabelled metabolites of loxtidine: AH24160, GR30450 and AH25227 (figure 1) were synthesized by the Chemical Research Department, Glaxo Wellcome Research and Development Limited, (Ware, UK). β-Glucuronidase (EC 3.2.1.31) and aryl sulphatase (EC 3.1.6.1, glucuronidase free) were purchased from Sigma Chemical Co. (Poole, UK), collagenase (EC 3.4.24.3) was from Boehringer Mannheim (Lewes, UK). Williams' Medium E (WME) without phenol red, L-glutamine and trypan blue were supplied by Flow Laboratories (Rickmansworth, UK). All other chemicals and solvents were of Analar® grade or equivalent.

Preparation of isolated hepatocytes

Adult male Allen and Hanbury in bred random hooded rats (229± 27 g) and adult male or female beagle dogs (10.8± 2.3 kg) were obtained from Biosciences Support Group (Glaxo Wellcome Research and Development Ltd, Ware, UK). Livers were removed from rats anaesthetized with either halothane or enflurane, and from dogs that had been sacrificed by an overdose of pentobarbitone sodium. Human

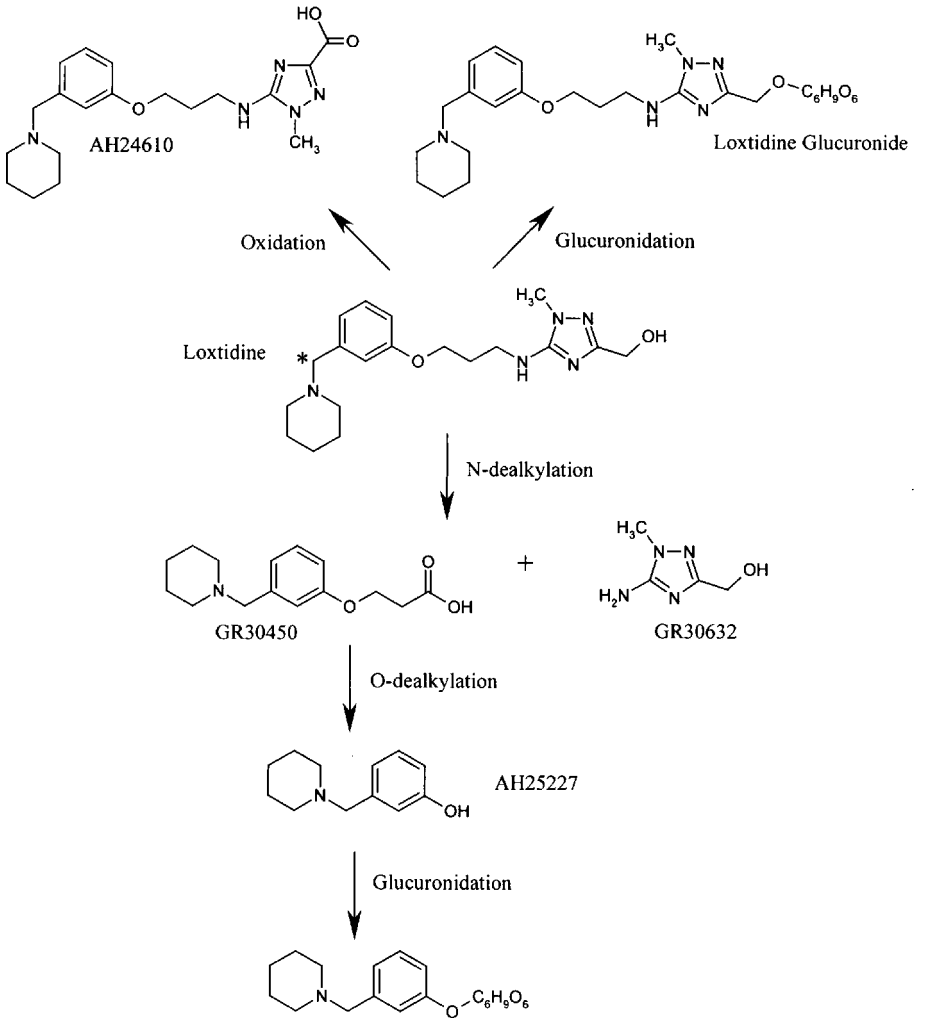

Figure 1. Metabolic pathways of loxtidine (1-methyl-5-[3-[3-[(1-piperidinyl) methyl] phenoxy] propyl] amino-1H-1,2,4-triazole-3-methanol) in rat, dog and man (Bell *et al.* 1983, Jenner *et al.* 1984). *Position of the radiolabel.

liver samples were obtained from patients undergoing hepatectomy or from non-transplanted donor samples with approval from the district Clinical Review Board.

Hepatocytes were isolated as described previously (Bayliss *et al.* 1994) using the biopsy perfusion technique of Oldham *et al.* (1985). For the isolation of dog and human hepatocytes it was necessary to double the units of collagenase activity used in the preparation of rat hepatocytes. Hepatocytes were thrice washed in HEPES buffer containing bovine serum albumin (1% w/v) and re-suspended in WME without phenol red containing L-glutamine (4 mM) at a cell density of 2×10^6 hepatocytes/ml. Viability was determined by trypan blue exclusion and by lactate dehydrogenase (LDH) (EC 1.1.1.27) leakage (Jauregui *et al.* 1981). P450 content of solubilized hepatocytes was determined by the method of Omura and Sato (1964a, b). Hepatocyte suspensions were sedimented by centrifugation (50 *g* for 4 min). The supernatant was removed and the pellet dissolved in Renex 690 solubilizing buffer. The buffer was as described by Warner *et al.* (1978) except that Renex 690 was substituted for Emulgen 911. Protein content was determined by the method of Lowry *et al.* (1951).

Incubation procedures

^{14}C-Loxtidine (in methanolic solution) was placed in polyethylene incubation vials to give final concentrations of 0.5, 1, 5, 10, 25, 50, 100 μM for rat, 0.5, 1, 5, 10, 25, 50, 100, 150 μM for dog and 1, 2.5, 5, 10 μM for human hepatocyte incubations (2 ml incubation volume). A stream of nitrogen gas removed

the solvent. Control (non-viable) hepatocytes were obtained by boiling for 10 min prior to cooling and addition to vials containing ^{14}C-loxtidine (0.5, 1, 10 and 100 μM). Metabolic and kinetic studies commenced with the addition of hepatocyte suspensions (2 ml; 2×10^6 hepatocytes/ml in WME containing glutamine) which had been preincubated at 37° C for up to 30 min. All vials were incubated at 37° C in a shaking water bath (~ 70 oscillations/min) and were purged with water-saturated mixture of oxygen and carbon dioxide (95:5% v/v). Aliquots (200 μl) of the incubations were withdrawn from each vial at 0, 0.25, 0.5, 1.0, 2.0 and 3.0 h after commencement and rapidly frozen in liquid nitrogen and stored at −20° C.

Hepatocyte proteins were precipitated using 2 vols acetone and sedimented by centrifugation (12000 *g* for 10 min). The supernatant was removed and reduced to dryness using a sample concentrator (Savant SpeedVac SVC 100, Stratech Scientific, Luton, UK). Concentrated residues were resuspended in methanol (20 μl) and analysed by thin layer chromatography (tlc).

The presence of glucuronide/sulphate conjugates was assessed using specific hydrolysis. β-Glucuronidase/sulphatase (β-glucuronidase free) (15 mg/ml), saccharo-1,4-lactone (10 mg/ml; a specific inhibitor of β-glucuronidase), phenolphthalein glucuronide and phenolphthalein sulphate (5 mg/ml) were dissolved in 0.05 M sodium acetate buffer (pH 5). Incubations with hydrolysing enzymes were carried out for ~ 18 h at 37° C with rat and dog hepatocyte incubations containing ^{14}C-loxtidine. Incubations were terminated with 2 vols acetone and samples processed as previously described. The activity of deconjugating enzymes was validated using the test substrates phenolphthalein glucuronide and sulphate.

Analytical methodology

Quantification of radioactivity. Diluted stock ^{14}C-loxtidine solution (100 μl), aliquots of rat, dog and human hepatocyte incubations (10–100 μl), hepatocyte protein precipitates and acetone washings of precipitates (0.5 ml) were added to plastic scintillation vials containing Pico-Fluor 30 liquid scintillation cocktail (8 ml, Packard Instrument Co.) and their radioactive content determined in a Tracor Analytic Mark III (Model 6882) liquid scintillation system (Tracor Analytic, IL, USA). The external standard channel ratio method was used for the determination of counting efficacy, all counts being corrected accordingly.

Thin layer chromatography. Samples of ^{14}C-loxtidine solution (1–2 μl), non-radiolabelled loxtidine (1 mg base/ml in methanol; 10 μl), authentic loxtidine metabolites AH24610, GR30450 and AH25227 (1 mg/ml in methanol, 10 μl) methanolic extracts of hepatocyte incubations (20 μl) and methanolic extracts of hydrolysed rat and dog hepatocyte incubations (20 μl) were applied to 0.25 mm silica gel G60 F254 prelayered plates (E. Merck, Darmstadt, Germany).

Thin layer plates were developed in glass tanks equilibrated at ambient temperature with the following solvent system: ethyl acetate:propan-2-ol:water:S.G. 0.88 ammonia (50:30:16:4 by vol.). Radioactive areas on the thin layer chromatography plates were located and integrated by use of an Isomess IM 3000 linear analyser (Raytest, Sheffield, UK) linked to an Apple IIe data system.

Autoradiograms of tlc plates were prepared by placing the plates in contact with Hyperfilm β-max X-ray film (Amersham Intl, Amersham, UK) for a minimum of 24 h. The films were developed and fixed according to standard procedures. Reference samples of non-radiolabelled loxtidine and authentic metabolites were visualized on the tlc plates under UV light (254 nm).

Binding of ^{14}C-loxtidine to hepatocyte protein

The non-specific binding of ^{14}C-loxtidine or ^{14}C-labelled metabolites of loxtidine to rat, dog and human hepatocytes was investigated by centrifugation techniques. Rat, dog and human hepatocyte suspensions at time zero and 3 h after incubation with ^{14}C-loxtidine (1, 10 and 100 μM) were centrifuged at 4° C for 5 min (12000*g*) and the supernatant removed. The cell pellet was then washed with WME and solubilized. The radioactive content of the supernatant, pellet wash and the solubilized cell pellet were determined by radioassay to assess recovery of radiolabelled material.

Determination of enzyme kinetic constants. Enzyme kinetic parameters were determined by measuring the rate of disappearance of ^{14}C-loxtidine in rat, dog and human hepatocyte incubations. Estimates of apparent Michaelis-Menten kinetic constants K_m and V_{max} for the metabolism of loxtidine in rat and dog hepatocyte incubations were obtained initially by graphical analysis. The initial kinetic parameters obtained were used as first estimates in an iterative programme based on nonlinear least-squares regression analysis. The actual curve fitting procedure used was the Nelder-Mead Simplex method (O'Neill 1971) to calculate apparent kinetic parameters K_m and V_{max} in the general equation:

$$v_o = \frac{V_{max}[S]}{K_m + [S]}$$

Intrinsic clearance (Cl_{int}) for loxtidine in rat and dog hepatocytes was determined from the ratio of the maximum velocity (V_{max}) and the Michaelis-Menten affinity constant (K_m) as described by the relationship:

$$Cl_{int} = \frac{V_{max}}{K_m}$$

The rate of metabolism of loxtidine by human hepatocytes was significantly slower than by animal hepatocytes and as such required an approach using integrated forms of the Michaelis-Menten equation:

$$\ln\frac{C_o}{C_t} = \frac{V_{max}.t}{K_m} - \frac{(C_o - C_t)}{K_m} \quad (1)$$

$$\frac{t}{C_o - C_t} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}.\frac{\ln(C_o/C_t)}{C_o - C_t} \quad (2)$$

where C_o is the initial concentration and C_t is concentration at time t.

Equation 1 gives a straight line plot when $C_o \ll K_m$, the slope of which gives the intrinsic clearance. Equation 2 yields estimates of K_m, V_{max} and intrinsic clearance (Chenery *et al.* 1987).

Scaling factors similar to those described by Houston (1994) were used to extrapolate *in vitro* Cl_{int} to *in vivo* Cl_{int}. The figures used for rat, dog and human hepatocyte number/g of liver, liver and body weight are detailed in table 1.

Hepatic clearance (Cl_H) was calculated from *in vitro* data using the 'well stirred' liver model as described by the following relationship (Morgan and Smallwood 1990):

$$Cl_H = \frac{Q(f_u - Cl_{int})}{(Q + f_u Cl_{int})}.$$

Dose preparation and in vivo *animal experimentation*. Non-radiolabelled loxtidine hemisuccinate, 15.5 or 232.8 mg was dissolved in 5 or 20 ml isotonic saline for intravenous administration to either rat or dog. Concentrations of dosing solutions were, therefore, 2.65 mg and 9.97 mg/ml expressed as loxtidine base. The dose volume of the administered drug was 2 and 0.5 ml/kg bodyweight for rat and dog respectively. Hooded rat (272± 33 g) and beagle dog (11.9± 2.1 kg) were denied access to either Laboratory Animal Diet or SDS Diet A (Special Diet Services, Witham, UK) but were allowed water *ad libitum* for ~ 8 h prior to the administration of loxtidine. During this time predose urine and blood samples were obtained from both rat and dog. Animals received a single intravenous target dose of 5 mg loxtidine/kg by administration into either the lateral tail vein of the rat or the cephalic vein in the foreleg of the dog. The range of doses administered to rat (n = 6) and dog (n = 2) were 5.25 –5.37 and 4.9 –5.1 mg/kg bodyweight respectively.

All animals were housed individually in stainless steel metabolism cages designed for the separate collection of urine and faeces and were maintained in thermostatically controlled environments with a 12-h light/dark cycle. Water was made available *ad libitum* throughout the experimental period, as was animal diet from 2 h post-dose. Urine was collected from rats (n = 2) and dogs (n = 2) for 0–24 h post-dose in containers cooled by solid carbon dioxide.

Samples of blood (100 μl) were taken from the tail vein of rats (n = 4) at 0, 2, 10, 20, 30, 45 min, 1, 2, 3, 4, 6, 8, 12 and 24 h post-dose and placed into heparinized microtainers and centrifuged at 3000 *g* for 6 min to separate plasma from red cells.

Samples of blood (5 ml) were taken from the jugular vein of the dog at 0, 5, 15, 30, 45 min, 1, 2, 3, 4, 6, 8, 12 and 24 h post-dose. The blood was placed in heparinized containers (Becton-Dickinson and Co., Rutherford, NJ, USA) and centrifuged at 1700 *g* for 15 min at 4° C and the plasma removed. All samples were stored at –20° C until analysed.

Human volunteer studies. Human pharmacokinetic data were obtained from four healthy male volunteers aged 25–37 years (76.2± 7.95 kg) who had received varying doses of loxtidine. Each subject received an intravenous bolus dose (0.025–0.5 mg/kg) of loxtidine. One volunteer received two separate intravenous doses of loxtidine (0.25 and 0.5 mg/kg) separated by 48 h. Blood samples were taken at 1, 2, 3, 4, 5, 6 and 24 h post-dose and the plasma was separated by centrifugation and then analysed by radioimmunoassay. Urine was collected for 24 h.

Radioimmunoassay. A radioimmunoassay for loxtidine with a detection limit of 4 ng/ml (Harrison *et al.*, 1983) was used. Some of the metabolites of loxtidine cross-react in the assay system; therefore, the parent drug was selectively extracted from plasma or urine prior to assay. Samples were adjusted to pH 10 with 0.2 M sodium borate buffer and extracted with water saturated dichloromethane. The phases were

separated and the dichloromethane reduced to dryness. The residue was re-suspended in 0.5 M phosphate buffer (pH 7.4). Rat, dog or human plasma samples, rat and dog urine (100 μl), loxtidine standards and quality control samples were analysed as described by Harrison *et al*. (1983).

Protein binding studies. Whole blood was obtained from rat, dog and human volunteers by venepuncture. Blood was immediately transferred to heparinized tubes and the plasma separated by centrifugation (1700 *g* for 15 min at 4° C). All experimentation was performed using freshly prepared plasma. Dialysis was performed at 37° C for ~ 6 h in two-chamber dialysis cells using Spectrapor 2 membrane tubing (mw cut-off 12000–14000; Spectrum Medical Industries, Los Angeles, CA, USA). Dialysis cells were rotated throughout the 6 h. Duplicate determinations were carried out at each drug concentration. ^{14}C-Loxtidine was placed in buffer for one determination and in plasma for the second and then dialysed against control plasma or buffer respectively. After dialysis the radioactive content of the plasma and buffer was determined by radioassay and the percentage protein binding determined using the following equation

$$\frac{\text{Bound} + \text{Free}(x) - \text{Free}(y)}{\text{Bound} + \text{Free}(x)} \times 100\%,$$

where (x) is the radioactivity in the plasma compartment and (y) the radioactivity present in the buffer compartment.

Pharmacokinetic analysis. Pharmacokinetic parameters of loxtidine in rat (n = 3), dog (n = 2) and human volunteers (n = 5) were determined on each of the individual loxtidine plasma profiles after intravenous administration. The maximum plasma concentration (C_{max}) after intravenous administration was determined by data inspection and extrapolation back to time zero. The area under the plasma concentration time curves ($AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$, where t is the last data point), plasma half-life ($t_{\frac{1}{2}}$), elimination rate constant (k_{el}) and volume of distribution (V) were calculated using SIPHAR (SIMED, Creteil, France). The plasma AUC was calculated using the linear trapezoidal rule. Pharmacokinetic parameters such as total plasma clearance, renal clearance, hepatic clearance and intrinsic clearance were determined in each species from the dose administered, the $AUC_{0\text{-}\infty}$, urinary concentrations of drug and *in vitro* plasma protein binding using standard relationships (Labaune 1989).

Results

Viability of hepatocyte preparations

^{14}C-Loxtidine did not appear to have any significant effect on cell viability. The viability of rat, dog and human hepatocytes as determined by trypan blue exclusion was initially 86.8± 3.4% (n = 12), 90.2± 4.4% (n = 12) and 87.9± 2.7% (n = 6) respectively. After 3 h incubation at 37° C in the presence and absence of ^{14}C-loxtidine (100 μM) the viability decreased to 71.8± 7.3 and 71.9± 3.6% in rat hepatocytes (n = 12); 70.9± 3.2% and 72.5± 3.7% in dog hepatocytes (n = 6), and 73.7± 4.9 and 71.7± 2.9% in human hepatocytes (n = 3). Cytochrome P450 concentrations in rat, dog and human hepatocytes were 0.33± 0.04, 0.13± 0.03 and 0.06± 0.02 nmol/10^6 cells respectively. Protein concentrations were 1.39± 0.16, 1.05± 0.13 and 1.61± 0.36 mg/10^6 cells for rat, dog and human hepatocytes respectively.

Recovery of radioactivity from hepatocyte incubates

The extraction efficiency of radiolabelled material from rat (n = 3), dog (n = 3) and human (n = 2) hepatocyte incubations was 92± 2, 98± 2 and 98% for each species respectively. Less than 1% of the total radioactivity in the incubations from any species remained in the precipitate. The remainder of the radiolabelled material (up to 6%) was associated with the precipitate washings indicating a full recovery of radiolabelled material. These data indicate that very little if any non-specific binding of loxtidine or metabolites occurs.

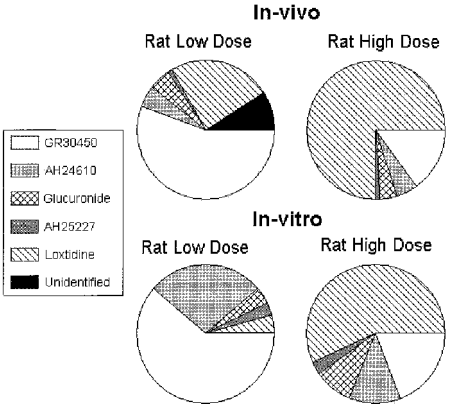

Figure 2. Correlation of loxtidine metabolites formed in rat hepatocytes (1 μM and 100 μM) incubated for 3 h and rat *in vivo* following intravenous administration (5 and 50 mg/kg) of loxtidine (rat *in vivo* data from Bell *et al*. 1983).

Stability of ^{14}C-loxtidine in isolated hepatocyte suspensions

No break down of ^{14}C-loxtidine occurred in control (non-viable) or 3 h incubations with either rat, dog (0.5 and 100 μM) or human (1 and 10 μM) hepatocytes as determined by tlc (data not shown).

Rat hepatocyte incubations

The major radioactive metabolite observed at all concentrations was the propionic acid metabolite (GR30450) which comprised 61.3± 3.6 and 19.1± 8.6% of the radioactivity in the 3 h incubation when the initial concentrations of ^{14}C-loxtidine were 1 and 100 μM respectively (n = 6) (figure 2). The carboxylic acid metabolite (AH24610) of loxtidine was detected in all rat incubations at all concentrations studied. The carboxylic acid represented 27.4± 2.9 and 12.1± 3.0% of the radioactivity present after 3 h incubation with 1 and 100 μM ^{14}C-loxtidine respectively (figure 2). Metabolites that chromatographed with a similar R_f to loxtidine glucuronide represented < 9.7% of the radioactivity in rat hepatocyte incubations. Trace amounts (up to 3% of radioactivity present) of the phenolic metabolite (AH25227), were also observed. The metabolism of ^{14}C-loxtidine (1 and 100 μM) was completely inhibited in the presence of SKF525A (100 μM) suggesting that the metabolism of loxtidine in rat hepatocytes is mediated via the cytochrome(s) P450.

Dog hepatocyte incubations

In dog hepatocyte incubations, the major radioactive metabolite confirmed by enzyme hydrolysis was the glucuronide conjugate of loxtidine. The aglycone formed after hydrolysis of the dog hepatocyte co-chromatographed with ^{14}C-loxtidine. Incubation of the dog hepatocyte suspension containing the metabolite in the presence of saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase, resulted in the major metabolite remaining unhydrolysed. This metabolite represented 36.1± 11.1 and 28.1± 9.1% of the radioactivity in the 3-h incubations when the

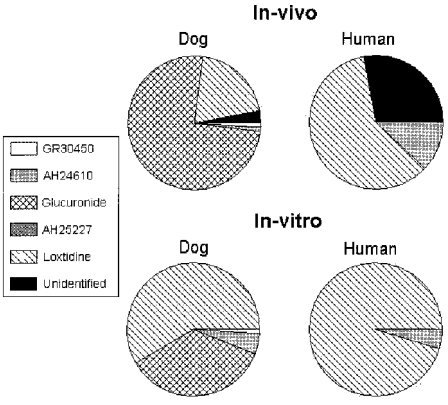

Figure 3. Correlation of loxtidine metabolites formed in dog and human hepatocytes (1 μM) incubated for 3 h and dog and human volunteers following intravenous administration of loxtidine (*in vivo* data from Bell *et al.* 1983, Jenner *et al.* 1984).

initial concentrations of ^{14}C-loxtidine were 1 and 100 μM respectively (figure 3). The other radioactive products were oxidative metabolites comprising up to 6% of the total. The major oxidative metabolite was the carboxylic acid metabolite, AH24610. The ratio of metabolites did not change over the concentration range used.

Human hepatocyte incubations

Only one metabolite was detected in human hepatocyte incubations that co-chromatographed with the authentic standard of the carboxylic acid (AH24610) metabolite. This metabolite was observed at all concentrations of ^{14}C-loxtidine studied representing 5.1± 2.0, 4.7± 1.8 and 4.1± 1.8% of the radioactivity on the tlc plate. No evidence for the formation of the other metabolites of ^{14}C-loxtidine seen in animal species was detected in any of the human hepatocyte incubations (figure 3).

Enyme kinetic analysis of loxtidine metabolism

Initial velocity data determined experimentally were modelled using mono-phasic-fitting procedures. Computer generated Lineweaver-Burke transformations of the experimentally determined initial velocities for rat and dog hepatocytes were used to visualize the data. The mean apparent K_m and V_{max} for the metabolism of ^{14}C-loxtidine by rat hepatocytes were 9.9± 4.0 μM and 0.114± 0.032 nmol/min/10^6 cells respectively. The apparent kinetic constants for the metabolism of ^{14}C-loxtidine in dog hepatocytes were 441± 257 μM and 0.512± 0.341 nmol/min/10^6 cells respectively. Using the appropriate scaling factors, which included hepatocytes/g tissue, liver and body weight, final means for the Cl_{int} of loxtidine by rat and dog hepatocytes calculated from *in vitro* data were 51.4± 12.4 and 8.0± 0.8 ml/min/kg respectively (table 1).

The slow rate of metabolism of ^{14}C-loxtidine by human hepatocytes and the limited data points did not allow for a full evaluation of apparent Michaelis-Menten parameters. Therefore, an integrated form of the Michaelis-Menten equation was used to calculate Cl_{int} as described in the methods. The Cl_{int} of loxtidine by human hepatocytes was determined as 1.0± 0.6 ml/min/kg (table 1) when using the appropriate scaling factors.

Table 1. Estimates of Michaelis–Menten parameters and the evaluation of intrinsic and hepatic clearance in rat, dog and human hepatocytes.

Species	Km (μM)	V_{max} (nmol/min/10^6 cells)	Number of hepatocytes/g liver[1]	Cl_{int}[2] (ml/min/g liver)	Liver wt (g)	Body wt (kg)	Cl_{int}[3] (ml/min/kg)	Cl_H[4] (ml/min/kg)
Rat (n = 6)	9.9± 4.0	0.114± 0.032	120×10^6	1.45± 0.28	9.72± 1.75	0.275± 0.04	51.4± 12.4	24.5
Dog (n = 6)	441± 257	0.512± 0.341	240×10^6	0.276± 0.028	345.8± 87.4	11.9± 3.0	8.0± 0.8	3.1
Human (n = 6)	56.3± 33.6	0.015± 0.006	120×10^6	0.041± 0.024	1694[5]	70.0	1.0± 0.58	0.2

1. Figures are determined from Mizuma *et al.*, (1982), Pang *et al.*, (1985) and Arias (1988), or by assessment of nmol CYP450 content of hepatocyte preparations and microsomal protein concentrations.
2. Cl_{int} calculated from the ratio of apparent V_{max} and K_m.
3. Total Cl_{int} determined after scaling.
4. Cl_H *in vitro* calculated using the 'well stirred' model.
5. Calculated from Boxenbaum (1980).

Table 2. Pharmacokinetic parameters of loxtidine in rat, dog and man following intravenous administration.

Species	Wt (kg)	Dose (mg/kg)	C_{max} (ng/ml)	AUC_{0-t}[1–3] (ng/ml.h)	$AUC_{0-\infty}$ (ng/ml.h)	$t_{\frac{1}{2}}$ (h)	V[4] (L/kg)	A_eU_{0-24}[5] (ug)	% Protein binding
Rat (n = 3)	0.255	5.36	3998	3482	3573	0.8	1.8	366	26.3± 2.6
	0.242	5.34	2125	1989	2074	0.8	3.2	328	
	0.259	5.31	1940	2231	2372	1.0	3.3		
	0.225± 0.012	5.34± 0.03	2688± 801	2567± 801	2673± 793	0.9± 0.1	2.7± 0.8		
Dog (n = 2)	14.4	4.87	4229	9238	9749	3.1	2.2	11342	57.8± 2.5
	13.6	5.06	3649	7037	10140	2.4	1.7	17025	
Man (n = 5)	74.5	0.025	56	379	408	6.7	0.6	952	77.8± 1.7
	84.9	0.05	230	1134	1225	7.0	0.4	2480	
	74.5	0.05	128	946	975	5.0	0.4	1943	
	69.3	0.25	1215	3683	3782	5.3	0.5	8127	
	69.3	0.50	1782	6747	6919	4.9	0.5	18740	
	76.2± 7.9					5.8± 0.1	0.5± 0.1		

1. Rat plamsa AUC calculated from 0 to 4 h post-dose.
2. Dog plasma AUC calculated from 0 to 12 h post-dose.
3. Human plasma AUC calculated from 0 to 24 h post-dose.
4. V = volume of distribution.
5. A_e = amount of drug excreted in 0–24 h post-dose urine (U).

Table 3. Calculation of clearances of loxtidine after intravenous administration to rat, dog and man.

Species	Cl_P[1] (ml/min/kg)	Cl_R[2] (ml/min/kg)	Cl_H[3] (ml/min/kg)	Fraction unbound (f_u)	Liver blood flow (Q)[4] (ml/min)	Cl_{Int}[5] (ml/min/kg)
Rat	35.1± 9.2	8.5	26.6	0.74	69	58.5
Dog	8.3	1.7	6.6	0.42	44	18.6
Man	1.0± 0.2	0.5± 0.1	0.4± 0.1	0.22	25	2.0± 0.6

1. $Cl_P = \frac{\text{Dose}}{\text{AUC}}$ after intravenous administration.
2. $\frac{\text{Amount of drug excreted unchanged in urine to } t}{\text{AUC}_{0-t}}$.
3. $Cl_H = Cl_P - Cl_R$.
4. Data from Boxhenbaum (1980).
5. $Cl_{int} = \frac{Q\,Cl_H}{f_u(Q - Cl_H)}$.

Pharmacokinetic analysis

Pharmacokinetic analysis of loxtidine plasma concentrations was performed on each individual data set obtained from rat, dog and man. The mean rat, dog and human pharmacokinetic parameters are presented in table 2. The AUC for the loxtidine plasma concentration-time curve in the rat accounted for ~ 91–93% with only 7–9% extrapolated. For one dog, the extrapolation was only 9%; however, the extrapolated AUC for the second dog was 34%. This was due to an extrapolation from the 4 h point to infinity rather than from 8 h to infinity. The human volunteer study was designed to assess the efficacy of loxtidine and as such the initial time point was 1 h after intravenous administration. Although the first available time point was 1 h after administration the data are sufficient to describe the distribution phase of the intravenous plasma profile, and thus allow an accurate extrapolation and estimation of C_{max}. The contribution of the extrapolated AUC between 0 and 1 h in human volunteers was 11–20% of the total and was considered significant. All AUC-derived pharmacokinetic parameters in man were calculated using total AUC extrapolated to time zero by the computer program.

The mean half-life of loxtidine was 0.9± 0.1, 2.7 (2.4–3.1) and 5.8± 1.0 h in rat, dog and man respectively (table 2). Loxtidine had a moderate volume of distribution (2–3 l/kg) in rat and dog and a low volume of distribution (0.4–0.6 l/kg) being approximately equivalent to total body water in man (table 2).

Total plasma clearance (Cl_P) of loxtidine in rat, dog and man was 35.1± 9.2, 8.3 and 1.0± 0.2 ml/min/kg respectively. Renal clearance (Cl_R) of loxtidine determined from the amount of loxtidine excreted in urine during time t in rat, dog and man was 8.5, 1.7 and 0.5± 0.1 ml/min/kg respectively (table 3). Thus, from the relationship $Cl_P = Cl_R + Cl_H$ the hepatic clearance (Cl_H) of loxtidine was determined as 26.6, 6.6 and 0.4± 0.1 ml/min/kg in rat, dog and man respectively (table 3). In the above relationship, hepatic clearance was assumed to be the sum of metabolic and biliary clearance. The biliary clearance of loxtidine was shown to be negligible in rat and dog (Manchee and Bell, unpublished data); therefore in the present studies hepatic clearance was taken to equal metabolic clearance. The apparent extraction ratio (ER) of loxtidine (ER = Cl_H/Q, where Q is hepatic blood flow) *in vivo* was therefore 0.38,

Table 4. Comparison of model-dependent parameters for the hepatic elimination of loxtidine derived *in vitro* and *in vivo* in rat, dog and man.

	Species		
Parameter	Rat	Dog	Man
Predicted *in vitro*			
Cl_{int} (ml/min/kg)	51.4	8.0	1.0
$Cl_H^{(1)}$ (ml/min/kg)	24.5	3.1	0.2
$Cl_H^{(2)}$ (ml/min/kg)	29.2	3.2	0.2
$ER^{(1,3)}$	0.36	0.07	0.01
In vivo			
Cl_H (ml/min/kg)	26.6	6.6	0.4
$Cl_H^{(1)}$ (ml/min/kg)	58.5	18.6	2.0
$Cl_H^{(2)}$ (ml/min/kg)	45.4	17.0	2.0
$ER^{(3)}$	0.38	0.15	0.02

1. Determined using the 'well stirred' model of hepatic elimination.
2. Determined using the 'parallel tube' model of hepatic elimination.
3. Extraction ratios were calculated using liver blood flows of 69, 44 and 25 ml/min/kg for rat, dog and man respectively (Boxenbaum 1980).

0.15 and 0.02 in rat, dog and man respectively, suggesting that the extraction ratio of loxtidine was moderate in rat but low in dog and man (table 4).

Binding of ^{14}C-loxtidine to rat, dog and human plasma proteins

The non-specific binding of ^{14}C-loxtidine to the dialysis equipment including the dialysis membrane was 3–5% of the initial radioactivity. Binding to rat plasma proteins was similar at all drug concentrations studied (24–30%). However, the binding of ^{14}C-loxtidine to both dog and human plasma proteins was concentration-dependent, becoming saturated at the highest concentrations of ^{14}C-loxtidine studied (10 000 ng/ml). Thus, the binding of ^{14}C-loxtidine to dog plasma proteins was 54–59% at the lower drug concentrations but was reduced to 43% at the highest drug concentration. The binding of ^{14}C-loxtidine to human plasma proteins was 76–80% at the lower drug concentrations and 63% at the highest drug concentration. Only the protein bindings at the lowest concentrations of drug were used in any subsequent calculation. The fraction of loxtidine unbound (f_u) was, therefore, 0.74, 0.42 and 0.22 in rat dog and human respectively (table 3).

Calculation of in vivo *intrinsic clearance*

Loxtidine is a low to intermediate extraction ratio compound in rat, dog and man, therefore the 'well-stirred' liver model (Morgan and Smallwood 1990) was applied to calculate Cl_{int} from *in vivo* measurements of hepatic clearance (Cl_H) using the following relationship:

$$Cl_{int} = \frac{Q, Cl_H}{f_u(Q - Cl_H)},$$

where f_u is the unbound fraction of drug in plasma and Q is the liver blood flow. Hepatic blood flow was calculated from the relationship $Q = 0.0554 \times B^{0.804}$, where B = body weight (Boxenbaum 1980). Using the above model the *in vivo* intrinsic clearances of loxtidine in rat, dog and human were 58.5, 18.6 and 2.0± 0.6 ml/min/kg respectively (table 3).

Discussion

The results of the present study show that in rat, dog and man the metabolites of loxtidine produced by isolated hepatocytes closely parallel those produced *in vivo*. Thus, the major route for the metabolism of loxtidine in rat hepatocytes was N-dealkylation to form the propionic acid metabolite. This metabolic pathway has been shown to be saturable *in vivo* (Bell *et al*. 1983) and saturation was also observed in rat hepatocyte incubates in this study where the relative extent of N-dealkylation was greatest at the lowest concentration of loxtidine studied. Other workers have demonstrated saturation of metabolic pathways in rat hepatocytes. For example, isolated rat hepatocytes have been used to reproduce the *in vivo* dose-dependant metabolism of diphenylhydantoin (Inaba *et al*. 1975) and propranolol (McCormick *et al*. 1988). In contrast with the rat, the major route of metabolism of loxtidine in the dog *in vivo* is conjugation with glucuronic acid. This was also observed in dog hepatocytes, the metabolite being confirmed by enzyme hydrolysis. The metabolism of loxtidine in the dog is independent of dose up to and including 50 mg loxtidine/kg (Bell *et al*. 1983); this finding was confirmed *in vitro* by the results of this study where the extent of metabolism remained constant through out the concentration range examined. Administration of loxtidine to man has revealed that the drug is eliminated predominately by renal clearance with minimal metabolic clearance. A single metabolite has been identified, the carboxylic acid (unpublished data). During the present studies, loxtidine was incubated with hepatocytes isolated from six different human samples. In each case, loxtidine remained largely unchanged with only one metabolite produced. The metabolite formed by human hepatocytes co-chromatographed with an authentic standard of the carboxylic acid metabolite, thus reflecting the *in vivo* situation.

The present studies have, therefore, shown that rat, dog and human hepatocytes are a good quantitative model for the *in vivo* metabolism of loxtidine. The marked species differences observed *in vivo* were reproduced in the hepatocyte model. Thus, the results from the present studies add to the growing number of published data demonstrating a good correlation between the drug metabolite profiles detected in hepatocyte preparations, and those observed *in vivo* (Green *et al*. 1986, Chenery *et al*. 1987, Le Bigot *et al*. 1987, Chenery 1988, Guillouzo *et al*. 1988, McCormick *et al*. 1988, Seddon *et al*. 1989, Fabre *et al*. 1990, Jajoo *et al*. 1990, Lacarelle *et al*. 1991, Lavrijsen *et al*. 1992, Sandker *et al*. 1994, Pahernik *et al*. 1995). To our knowledge loxtidine is at present unique in that other examples of *in vitro-in vivo* correlations do not exhibit such marked species differences in metabolic pathways. Furthermore, many other workers have failed to extend their studies to one of the major species used in the pharmaceutical industry, namely the dog. Although no one animal model represents an exact model for the metabolism of loxtidine in man, both rat and dog form the loxtidine metabolite observed in man. In summary, the hepatocyte model offers a versatile system providing the opportunity to elucidate metabolic pathways of new chemical entities in animals and man at an early stage of drug development and may provide an opportunity for establishing the most appropriate toxicology species.

The potential of the hepatocyte system for the prediction of drug metabolism pathways has been established for a variety of chemical entities. However, interest in the use of hepatocytes to predict quantitative pharmacokinetic parameters of drug biotransformation has grown significantly in recent years using rat (Oldham *et al*. 1986, Chenery *et al*. 1987, Singh *et al*. 1991, Houston 1994, Ashforth *et al*. 1995,

Hayes *et al*. 1995, Zomorodi *et al*. 1995, Carlile *et al*. 1997), or, less frequently, human hepatocytes (Lave *et al*. 1995, 1996a, b, 1997). Only recently has there been a greater interest in comparative studies. This has lead to a significant increase in the number of published studies where the *in vivo* intrinsic clearance of a new chemical entity in rat, dog and man have been predicted from *in vitro* data (Chenery *et al*. 1987, Lave *et al*. 1995, 1996a, b). In the present study intrinsic and the subsequent hepatic clearance was determined for loxtidine using rat, dog and human hepatocyte data. These *in vitro* data were confirmed by studies *in vivo* in rat, dog and human volunteers.

Under first-order conditions, intrinsic clearance can be calculated from the ratio V_{max}/K_m. The extent of drug binding to cellular material including proteins can influence the apparent kinetic parameters by altering the effective substrate concentration at the drug metabolizing enzymes. Otherwise, the calculation of intrinsic clearance data is independent of the various models of hepatic elimination (Morgan and Smallwood 1990). Since, the binding of loxtidine to rat, dog and human hepatocyte proteins was negligible and therefore did not significantly affect the apparent Michaelis-Menten parameters derived *in vitro*, the intrinsic clearance was expressed directly as apparent V_{max}/apparent K_m.

To compare Cl_{int} determined *in vitro* with *in vivo* data, the ratio V_{max}/K_m was scaled to give units of ml/min/kg. Scaling requires consideration of factors such as numbers of hepatocytes per g liver, animal liver weight and body weight. It is the uncertainty of these scaling factors, particularly the number of cells per g liver that make the prediction of intrinsic and hepatic clearance from *in vitro* data precarious. Houston (1994) has reviewed the use of scaling factors. Several workers have discussed the appropriate methods to determine the number of hepatocytes per g rat liver and Houston (1994) and Carlile and co-workers (1997) have reviewed the approaches taken. The current studies used figures of 120×10^6, 240×10^6 and 120×10^6 hepatocytes per g rat (Mizuma *et al*. 1982, Pang *et al*. 1985), dog and human liver (Arias 1988). These data were confirmed by relating the microsomal protein content of 1 g liver, the nmol P450 per mg microsomal protein and the nmol P450 per 10^6 hepatocytes for each species.

Scaling of Cl_{int} for loxtidine gave 51.4, 8.0 and 1.0 ml/min/kg in rat, dog and human hepatocytes. Using the 'well-stirred' liver model (Morgan and Smallwood 1990) these Cl_{int} values provide Cl_H of 24.5, 3.1 and 0.2 ml/min/kg. These values compare favourably with the Cl_H derived *in vivo*, 26.6, 6.6 and 0.4 ml/min/kg in rat, dog and human (table 4) demonstrating the ability of the model system to predict *in vivo*.

The predicted apparent hepatic ER (ER = Cl_H/Q) of loxtidine derived from *in vitro* data was 0.36, 0.07 and 0.01 in rat, dog and man using blood flows of 69, 44, 25 ml/min/kg respectively (Boxenbaum 1980). These data compare well with the ER of 0.38, 0.15 and 0.02 determined from *in vivo* studies in rat, dog and man. ER was low in dog and human hepatocytes, vindicating the use of the 'well-stirred' model. However, the predicted apparent hepatic extraction ratio in rat (0.36) was close to a value that is considered to be intermediate. Pharmacokinetic data were also evaluated using the 'parallel tube' liver model (table 4) (Morgan and Smallwood 1990). The use of the 'parallel tube' model resulted in little change in the Cl_H determined for dog and human. A small difference was observed for the Cl_H predicted for the rat when using the 'parallel tube' model (29.2 ml/min/kg) compared with the 'well-stirred' model (24.5 ml/min/kg), suggesting that in the

case of high extraction compounds the choice of the model to determine Cl_H may be important. However, the hepatic extraction ratio predicted for loxtidine from data *in vitro* using either model was not substantially different.

In the absence of pharmacokinetic data in animals and man *in vivo*, the present studies with isolated hepatocytes would have accurately predicted the species differences in the hepatic clearance of loxtidine. These data would have indicated that loxtidine has increased metabolic stability in man compared with animal species because of the lower hepatic clearance value in man. Taken together, these data suggest that relatively little loxtidine would be eliminated by hepatic metabolism in man implying that the half-life of loxtidine may be increased when compared with animal species, which is in fact the case. The half-life increases from < 1 h in the rat to 6 h in man.

This type of kinetic information produced from predictive *in vitro* systems early in the development process could help to eliminate the possible pharmacokinetic shortfalls some new chemical entities encounter when they are first administered to man during the later stages of drug development.

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