Determination and prediction of permeability across intestinal epithelial cell monolayer of a diverse range of industrial chemicals/drugs for estimation of oral absorption as a putative marker of hepatotoxicity

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A B S T R A C T
Apparent permeability coefficients (P_{app}) across a human intestinal epithelial Caco-2 cell monolayer were measured for a range of industrial/drug chemicals. A predictive equation for determining in vitro P_{app} values of fifty-six substances was set up using multivariate regression analysis based on in silico-estimated physicochemical properties (molecular weights and water distribution coefficients for apical and basal pH environments) (r = 0.77, p < 0.01). Predicted logP_{app} values of a secondary set of 34 compounds were correlated with the measured values. Under the medicinal logP_{app} values associated with their reported fraction absorbed, a significant inverse non-linear correlation was found between the logarithmic transformed values of observed P_{app} values and reported hepatic no-observed-effect levels of industrial chemicals (r = −0.55, p < 0.01, n = 29). In vitro determination and/or in silico prediction of permeability across intestinal cells could be effective for estimating oral absorption as a putative indicator for hepatotoxicity.

1. Introduction

Current experimental testing methods for estimation of the human risks of industrial chemicals generally require toxicological studies in experimental animals. Such studies include repeated oral doses to rodents for 28 days and employ procedures that adhere to guidance such as Organization for Economic Co-operation and Development test guidelines. Although big toxicity databases have been widely set up, limited numbers of chemicals only possess adequate toxicokinetic data in vivo regarding parameters (such as oral absorption rates) for assessing human potential hazards [1]. The in vitro permeability assay for oral absorption in pharmaceutical research is a kind of established methods and is principally based on using human colon cancer cell line Caco-2 systems [2–6]. Studies that attempted to predict the permeability of drugs and druglike chemicals across Caco-2 cell monolayers have been performed as part of preclinical drug development [7–9]. However, little information has been provided on the oral absorption of industrial chemicals through gastrointestinal absorption and/or the mucosa, which is a necessary phase before such chemicals could exert their potential toxicity. It would be of great benefit for industrial chemicals if it were possible to derive the oral absorption parameters in vivo of general chemicals from established in vitro permeability values.

In the present study, we evaluated the permeability of a broad range of general chemical substances (for which the oral absorption is not commonly investigated) using a pH-dependent Caco-2 monolayer system. A multivariate prediction equation derived from the permeability coefficients of 56 disparate compounds was proposed. The input parameters for this equation were the in silico physicochemical properties of the compounds. This prediction equation was then used to estimate the permeability of a secondary set of 34 compounds. We report herein that the Caco-2 cell permeability coefficients of 28 industrial chemicals and acetaminophen were inversely associated with their hepatic no-observed-effect levels (NOELs).

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2.1. Materials and chemical properties

The chemicals tested for permeability in the Caco-2 cell system (shown in Tables 1 and 2) were of analytical grade and were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan) or from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, nonessential amino acids, penicillin–streptomycin–anphoterin B suspension, and Hank’s balanced salt solution (HBSS) were obtained from Fujifilm Wako Pure Chemical. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2-morpholinohexanesulfonic acid monohydrate (MES) were purchased from Sigma-Aldrich. Cell culture dishes (100 mm) and Transwell plates (12-well, pore size: 0.4 μm, growth area: 1.12 cm²) were obtained from Corning (Corning, NY, USA).

The broad diversity of the tested chemical substances is illustrated...
Table 2

Predicted and Observed logP_app Values of a Secondary Set of 34 Compounds and Their Reported Fraction Absorbed (F_a) and/or Hepatic NOEL Values.

| compound | CAS No. | molecular weight | logD_aoretical | logD_basal | predicted* logP_app | observed P_app | observed logP_app | reported human hepatic NOEL mg/kg/day |
|----------|---------|-----------------|----------------|------------|---------------------|---------------|------------------|-------------------------------------|
| acetaminophen | 103-90-2 | 151 | 0.09 | 0.09 | 2.42 | 319 ± 14 | 2.50 | 100 [23] |
| azamethiphos | 35575-96-3 | 325 | 2.58 | 2.58 | 2.14 | 402 ± 18 | 2.60 | 650 |
| bisphenol F | 620-92-8 | 280 | 3.61 | 3.60 | 2.66 | 415 ± 21 | 2.62 | 100 |
| bisphenol S | 80-09-1 | 250 | 1.26 | 1.11 | 2.29 | 503 ± 35 | 2.70 | 200 |
| carbamazepine | 298-46-4 | 236 | 3.64 | 3.64 | 2.54 | 380 ± 14 | 2.58 | 700 |
| 4-chloro-a-cresol | 1570-64-5 | 143 | 2.51 | 2.51 | 2.71 | 754 ± 39 | 2.88 | 250 |
| 2-chloro-phenol | 95-57-8 | 129 | 2.13 | 2.07 | 2.73 | 752 ± 83 | 2.88 | 200 |
| 4-chlorophenol | 106-48-9 | 150 | 2.26 | 2.26 | 2.73 | 431 ± 37 | 2.63 | 500 |
| cinamididine | 51461-61-9 | 252 | -0.79 | -0.58 | 1.92 | 17 ± 2 | 1.22 | 68 [7] |
| coumarin | 91-64-5 | 146 | 0.85 | 0.85 | 2.52 | 806 ± 54 | 2.91 | 100 [13] |
| 4-cumylphenol | 599-64-4 | 212 | 4.99 | 4.99 | 2.77 | 195 ± 34 | 2.29 | 100 |
| dabigatran | 211915-06-9 | 472 | 0.26 | -1.19 | 1.97 | 38 ± 17 | 1.58 | 183 [7] |
| disopyramide | 3737-99-5 | 340 | -0.70 | 0.79 | 1.17 | 14 ± 3 | 1.16 | 83 [7] |
| 7-ethoxy-coumarin | 31005-02-4 | 190 | 1.94 | 1.94 | 2.50 | 750 ± 48 | 2.88 | 300 |
| 3-ethylphenol | 123-07-9 | 122 | 2.75 | 2.75 | 2.80 | 515 ± 50 | 2.71 | 300 |
| 4-ethylphenol | 156-43-4 | 137 | 1.42 | 1.46 | 2.59 | 582 ± 31 | 2.76 | 100 |
| p-fluorophenol | 123-07-9 | 122 | 2.75 | 2.75 | 2.80 | 515 ± 50 | 2.71 | 300 |
| 3-fluorophenol | 123-07-9 | 122 | 2.75 | 2.75 | 2.80 | 515 ± 50 | 2.71 | 300 |
| 4-fluorophenol | 123-07-9 | 122 | 2.75 | 2.75 | 2.80 | 515 ± 50 | 2.71 | 300 |
| 3-hydroxybiphenyl | 580-51-8 | 170 | 3.88 | 3.88 | 2.78 | 284 ± 22 | 2.45 | 300 |
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| 7-hydroxycoumarin | 93-35-6 | 162 | 0.24 | 0.21 | 3.01 | 1030 ± 170 | 3.01 | 300 |
| itopride | 122898-67-3 | 358 | 0.15 | 1.40 | 1.29 | 12 ± 3 | 1.09 | 183 [7] |
| lovastatin | 75330-75-5 | 405 | 4.04 | 4.04 | 2.05 | 21 ± 1 | 1.32 | 31 [7] |
| mfenamic acid | 61-68-7 | 425 | 3.78 | 3.79 | 3.11 | 1804 ± 33 | 3.26 | 300 |
| 2-mercaptopimadazole | 872-35-5 | 100 | -4.91 | -4.91 | 2.02 | 91 ± 3 | 1.96 | 300 |
| methotrexate | 59-05-2 | 454 | -4.60 | -7.46 | 2.03 | 11 ± 2 | 1.04 | 20 [24] |
| 2-methoxy-4-nitroaniline | 97-52-9 | 168 | 1.71 | 1.71 | 2.54 | 552 ± 70 | 2.74 | 100 |
| mirtazapine | 88560-52-8 | 265 | 2.10 | 3.03 | 1.93 | 46 ± 3 | 1.66 | 80 [7] |
| olanzapine | 132559-06-1 | 312 | 3.00 | 3.29 | 2.12 | 35 ± 3 | 1.54 | 300 |
| omeprazole | 73590-58-6 | 345 | 1.60 | 1.63 | 1.96 | 674 ± 69 | 2.83 | 95 [7] |
| p-amino-benzoic acid | 150-13-0 | 137 | 0.40 | -1.08 | 3.06 | 587 ± 40 | 2.77 | 100 |
| p-phentetidin | 156-43-4 | 137 | 1.42 | 1.46 | 2.59 | 582 ± 31 | 2.76 | 160 |
| pravastatin | 81093-37-0 | 425 | -0.11 | -1.57 | 2.08 | 9 ± 1 | 0.95 | 13 [31] |
| 4-tet-butylphenol | 99-71-8 | 150 | 3.70 | 3.70 | 2.82 | 402 ± 19 | 2.60 | 300 |
| verapamil | 52-53-9 | 455 | 0.58 | 2.01 | 0.97 | 23 ± 1 | 1.36 | 100 [7] |

* Predicted using the following equation: 

\[ \text{logP}_{\text{app}} = 2.9 - 0.0032 \times \text{(molecular weight)} + 0.49 \times (\text{logD}_{\text{aoretical}}) - 0.38 \times (\text{logD}_{\text{basal}}) \]

Observed \( \text{P}_{\text{app}} \) value represents the mean of triplicate determinations with standard deviation in this study.

Fig. 1. Coordinate values in a two-dimensional plane illustrating variety in the chemical space for the primary set of 56 compounds (open circles) and the secondary set of 34 (solid circles) compounds evaluated using Caco-2 permeability assays.

in a two-dimensional plane depicting the wide chemical space (Fig. 1), as described previously [10]. Briefly, the structures described by 196

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substance in a final concentration of < 0.1 % dimethyl sulfoxide (originally dissolved in dimethyl sulfoxide and diluted with Hank's balanced salt solution) was applied to the apical side of Caco-2 cells cultured on Transwell plates. Caffeine and Lucifer yellow were used as positive and negative permeability controls, respectively. The amounts of the test substances in permeation samples from the basal sides were measured by high-performance liquid chromatography or liquid chromatography–mass spectrometry [10]. The experiment for each chemical substance was performed in triplicate determinations.

2.3. Statistical analysis

Univariate and multivariate linear regression analyses were performed using Prism software (GraphPad Software, San Diego, CA, USA). The relationships among log\(P_{\text{app}}\), values of chemicals experimentally determined in vitro, their physicochemical properties estimated in silico, and reported in vitro toxicological properties [the no-observed-effect level (NOEL)] for hepatotoxicity taken from the Hazard Evaluation Support System Integrated Platform in Japan and literature were investigated [11,12].

3. Results

The \(P_{\text{app}}\) values of more than 50 disparate types of chemicals (Fig. 1) were measured and are shown in Table 1. The observed \(P_{\text{app}}\) values of 56 chemicals varied in the range 5–1490 nm/s. The physicochemical properties (MW, log\(P\), log\(D_{\text{apical}}\), and log\(D_{\text{basal}}\)) of the 56 chemicals were estimated using in silico methods and are shown in Table 1. To investigate the feasibility of establishing a predictive equation, we carried out various analyses to identify the relationships between log\(P_{\text{app}}\) values and the compounds’ physicochemical parameters. Univariate linear regression analyses revealed that, under the present conditions, the observed log\(P_{\text{app}}\) values (Table 1) were correlated with the corresponding MW (\(r = 0.48, p < 0.01, n = 56\), Fig. 2A), log\(P\) values (\(r = 0.31, p < 0.05, n = 56\), Fig. 2B), log\(D_{\text{apical}}\) values (\(r = 0.53, p < 0.01, n = 56\), Fig. 2C), and log\(D_{\text{basal}}\) values (\(r = 0.41, p < 0.01, n = 56\), Fig. 2D). Because log\(P\) values univariately showed a low correlation coefficient, further analyses were performed with the rest of three chemical parameters, MW, log\(D_{\text{apical}}\) and log\(D_{\text{basal}}\) values. Bivariate analyses established that log\(P_{\text{app}}\) values were correlated with the MW and log\(D_{\text{apical}}\) values (\(r = 0.67, p < 0.01, n = 56\), Fig. 2E), MW and log\(D_{\text{basal}}\) values (\(r = 0.66, p < 0.01, n = 56\), Fig. 2F), and log\(D_{\text{apical}}\) and log\(D_{\text{basal}}\) values (\(r = 0.60, p < 0.01, n = 56\), Fig. 2G) in combination. Moreover, log\(P_{\text{app}}\) values were multivariately correlated with the MW, log\(D_{\text{apical}}\) and log\(D_{\text{basal}}\) values in combination (\(r = 0.77, p < 0.01, n = 56\), Fig. 2H), which led to the following equation: Predicted log\(P_{\text{app}}\) value = 2.9 – 0.0032 × (MW) + 0.49 × (log\(D_{\text{apical}}\)) – 0.38 × (log\(D_{\text{basal}}\)). These results suggest that multiple physicochemical properties are the determinants of the permeability coefficient of a variety of chemicals in the pH-dependent Caco-2 monolayer assays.

To verify the multivariate prediction equation, log\(P_{\text{app}}\) values for a secondary set of 34 compounds (Table 2) were predicted using the above equation in silico before \(P_{\text{app}}\) values were measured in in vitro experiments. The Caco-2 cell permeability coefficients of these additional 34 compounds were determined and are shown in Table 2. Estimated log\(P_{\text{app}}\) values were well correlated with the experimentally observed log\(P_{\text{app}}\) values (\(r = 0.78, p < 0.01, n = 34\), Fig. 3). Under the present conditions, the predicted \(P_{\text{app}}\) values of 23 and 27 of the 34 additional compounds were within twofold and threefold errors, respectively, of the experimentally observed values. Under these conditions, predicted log\(P_{\text{app}}\) values of some medicines, namely olanzapine, lovastatin, methotrexate, pravastatin, and cimetidine were overestimated in comparison with the observed values, presumably because of partly contributions of active efflux pump in the experimental environment.

To investigate the relevance of in vitro pH-dependent Caco-2 monolayer systems to in vivo absorption rates, the relationship was examined between the measured log\(P_{\text{app}}\) values for pharmaceutical drugs and their reported absorption (fraction absorbed, \(F_a\)) in humans (Fig. 4). A significant sigmoidal correlation was observed between the

![Fig. 2. Relationships between log\(P_{\text{app}}\) values experimentally observed in the Caco-2 cell system and those calculated using univariate (A–D), bivariate (E–G) and multivariate (H) linear regression analyses of the primary set of 56 compounds, as a function of physicochemical properties (MW, log\(P\), log\(D_{\text{apical}}\), and log\(D_{\text{basal}}\)). Each observed log\(P_{\text{app}}\) value represents the mean of triplicate determinations with standard deviation as shown in Table 1. Solid and dashed/dotted lines indicate linear regression and twofold/threefold ranges, respectively.](image-url)
experimental log\(P_{\text{app}}\) and reported \(F_u\) values \((r = 0.61, p < 0.01, n = 28)\); a similar nonlinear shape has been previously reported for this relationship \([5,13]\). Furthermore, under the present conditions, a significant inversely non-linear relationship was found between the logarithmic transformed values of observed \(P_{\text{app}}\) and reported hepatic NOELs of industrial chemicals and acetaminophen \((r = -0.55, p < 0.01, n = 29; \text{Fig. 5B})\), but not with the calculated log\(P\) \((r = -0.27, p = 0.2, n = 29; \text{Fig. 5A})\).

4. Discussion

Conditions that mimic the in vitro pH gradient found between the gastrointestinal lumen and plasma have been shown to well reflect human oral absorption of drugs in the gut \([6,14]\). Furthermore, simple pH-dependent Caco-2 monolayer systems have proven advantageous in predicting in vivo drug absorption as a part of pharmaceutical research \([2,5,13,15-19]\). It has been reported recently that Caco-2 permeability coefficients for 768 diverse drugs and druglike compounds could account for passive diffusion across the mucosal epithelium \([9]\) using a minimal set of physicochemical descriptors (octanol–water logD, pKa, hydrogen bonding potential, and molecular size), a model has been successfully set up to predict Caco-2 permeability coefficients \([9]\). However, the pharmacokinetics and/or toxicokinetics of industrial chemicals are not usually investigated as part of their extensive acute toxicity studies \([1]\). Therefore, the relationship between \(P_{\text{app}}\) and the hepatic NOEL values of chemical substances were examined in the present study.

In our previous report, suitable concentrations of albumin for in vitro assays of drug oxidations by human liver microsomal cytochrome P450 2C enzymes could be multivariately estimated using the drugs’ physicochemical properties in combination \([20]\). In the current study, multivariate regression analysis with three physicochemical properties in combination (reflecting the experimental apical and basal pH conditions in the current monolayer cell assays) showed that the in silico predicted and in vitro measured \(P_{\text{app}}\) values of a total of 90 chemicals were well correlated (Fig. 2). These results suggest that our proposed multivariate regression equation using the physicochemical properties of compounds in combination could predict the permeability coefficients across the Caco-2 cell sheets of a wide variety of chemicals. Analysis of the combined 90 tested chemical substances allowed us to update the multivariate equation as follows: Predicted log\(P_{\text{app}}\) value = 3.0 – 0.0038 × (MW) + 0.41 × (log\(D_{\text{apical}}\)) – 0.30 × (log\(D_{\text{basal}}\)). The reason why some predicted log\(P_{\text{app}}\) of drugs were out of threefold areas are not known under the present conditions, presumably because of some contributions of active efflux/influx pump in the actual experimental Caco-2 environment. Predictions for any uptake/eﬄux transport potential of substances in the current models using simple physiological parameters may have some limitation at present and would be another big project expected in this research area. In another viewpoint, a multivalent equation fortified with more chemical descriptors might be solved for good prediction in future.

The \(P_{\text{app}}\) values obtained from experiments in this study could reﬂect the in vivo intestinal absorption of known medicines (Fig. 4), although some absolute values were different in in vitro systems (Tables 1 and 2). Our current inverse correlation between logarithmic transformed values of reported NOEL and measured \(P_{\text{app}}\) values of general chemicals was able to apply for a drug, acetaminophen (Fig. 5). The in vivo oral absorption, rather than partition coefficients, is considered to be one of the many determinant factors predicting the pharmacokinetics and/or potential hepatotoxicity (Fig. 5) of intentionally or unintentionally orally ingested chemical substances. In the present study, if one (5-amino-2-chlorotoluene-4-sulfonic acid) and two points, respectively, from two compounds implying moderate absorption would be omitted in correlation assays shown in Fig. 5B, both the non-linear correlation coefﬁcients were still signiﬁcant \((r = -0.49, p < 0.01, n = 28; \text{and } r = -0.39, p < 0.05, n = 27)\). Under the present relationship...
analyses, although NOEL values of chemicals are generally determined in discreet numbers dependent on animal dosing levels, continuous variable in vitro apparent permeability data \( P_{app} \) of industrial chemicals would be one of the divergent determinant factors predicting in vivo potential hepatotoxicity, in comparison with chemical lipophilicity (log P). It could be of use to have more NOEL values of chemicals from any toxicity/ regulatory databases with similar evaluation criteria to help correlations between the \( P_{app} \) and NOEL values to the toxicity conclusions. Anyway, it should be noted that chemical exposure levels via intestinal absorption after oral doses should be one of the primary key steps and following species-specific metabolic activations in livers and their mechanistic modifications would be the secondary critical points to understand potential hepatic risk from multiple exposures in chemical toxicology. Gastrointestinal epithelial Caco-2 cells have been also reportedly used in the other toxicological research such as cytotoxic effects of pesticides in combination [21] or gene expression profiles by nanosilver [22].

Consequently, being able to predict the permeability of a diverse range of industrial chemicals across the intestinal epithelial cell monolayer using their physicochemical properties in combination could be of use for estimating systemic exposure via oral absorption as one of putative toxicokinetic markers of hepatotoxicity. With a view to predicting hepatic toxicity after oral absorption of chemicals as a part of risk assessment, simple physiologically based pharmacokinetic models (consisting of gut, liver, and central compartments) were recently used to estimate the plasma/hepatic concentrations of chemicals after virtual intestinal absorption after oral doses should be one of the primary key steps and following species-specific metabolic activations in livers and their mechanistic modifications would be the secondary critical points to understand potential hepatic risk from multiple exposures in chemical toxicology. Gastrointestinal epithelial Caco-2 cells have been also reportedly used in the other toxicological research such as cytotoxic effects of pesticides in combination [21] or gene expression profiles by nanosilver [22].

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