A novel UV-spectrophotometric method as a reliable alternative for the spectrofluorimetric analysis of lucifer yellow: an application to permeability studies on colon adenocarcinoma-2 cell line

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Background
Human colon adenocarcinoma cell-line (Caco-2) monolayer is a well-established ex vivo model used for estimation of drug-apparent permeability through the human intestinal barrier. Lucifer yellow (LY) is a paracellular permeability marker used generally for examining the integrity of Caco-2 monolayer using spectrofluorimetric detection. No other spectroscopic methods were reported for analysis of LY.
Objective
To introduce a novel validated simple ultraviolet (UV)-spectrophotometric method, for LY determination, as an alternative to the commonly used spectrofluorimetry, as well as to apply the developed UV method to confirm the monolayer integrity of Caco-2 cell line compared with the spectrofluorimetric detection.
Materials and methods
The measurement was carried out at a wavelength of 277 nm. The linearity range was 1.0–12.5 μg/ml with the regression coefficient (R^2)=0.9992. Validation of the method was carried out following the ICH guidelines concerning linearity, precision, accuracy, robustness, and stability and statistically compared with the conventional spectrofluorimetric one. Measurement of cultured Caco-2 cell monolayer integrity was carried out using LY employing spectrofluorimetric and UV-spectrophotometric methods.
Results and conclusion
The calculated *t* test and *F* values indicated that no significant difference existed between both methods regarding precision and accuracy. The developed method has been successfully applied for checking the monolayer integrity of Caco-2 cell line. Comparing permeability percentage results of LY, both methods resulted in LY-permissible values of 2.354 and 2.303% using spectrofluorimetric and UV-spectrophotometric methods, respectively, indicating Caco-2 cell-line monolayer integrity.

Keywords:
Caco-2 monolayer integrity, lucifer yellow, permeability marker, ultraviolet-spectrophotometry

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Introduction

Promotion of promising drug candidates from discovery to development necessitates initial estimation of bioavailability. It is fundamental to assess drug absorption in estimating bioavailability. In modern drug discovery, termination of promising new drug candidates due to unfavorable ADME (absorption, distribution, metabolism, and excretion) profiles, is primarily due to poor permeability/absorption and metabolic instability [1,2]. In order to reach maximum drug absorption, the drug needs to have the highest permeability and concentration at the absorption site. Thus, in vitro evaluation of solubility and permeability can help to predict the extent of drug absorption in vivo, and so, a drug-classification system based on these parameters has been proposed by Amidon *et al.* [3–5].

As a basis for establishing such in vitro–in vivo correlations, US-FDA guidelines introduced the simplification of the biopharmaceutical classification system (BCS) as a key element for the regulation of per-oral drug-dosage forms. Four categories of drug substances are classified in the guidance according to their permeability and solubility properties [6–8]. The BCS serves as a tool to establish dissolution standards for drug products to minimize the in vivo bioequivalence specifications [3,7,9,10]. Moreover, during the formulation stage, awareness of the BCS of the drug under investigation can guide scientists to develop an

appropriate dosage form based on mechanistic, rather than empirical, techniques [7,11,12]. Eventually, in vitro/in vivo correlations can be interpreted with significantly fewer in vivo studies [7,12].

Caco-2 monolayers (human colon adenocarcinoma cell line) are well known to be used as an ex vivo human-absorption surrogate for testing drug compounds administered by oral route [13–16]. In addition Caco-2 cell line is commonly employed as an ex vivo model to assess the apparent permeability of drugs through the human intestinal barrier [17–19]. Caco-2 cells, cultured under standard conditions, form monolayers and differentiate to express carriers, which mRNA-expression level relates well to those found in human enterocytes [18,20]. That is why there are many studies reported in literature, which successfully employed Caco-2 monolayers as a tool for correlation with or the prediction of human absorption of various drug substances [15,21–26].

When Caco-2 cells are cultured on a transwell plate, they differentiate to give a confluent epithelial cell monolayer made of polarized columnar cells that express microvilli on the apical membrane and tight junctions between adjacent cells [13,27]. It is highly recommended to make, for each batch of seeded cells, a quantitative integrity control of the monolayers via carrying out a transport experiment using a hydrophilic paracellular marker [27,28]. Measurement of the transepithelial electrical resistance (TEER) can be used to check regularly the integrity of the Caco-2 monolayer [29]. Yet, since TEER mainly represents the resistance of the tight junctions and does not sensitively reflect the viability of the cells, the obtained TEER value during a transportation study may be misleading [30]. Alternatively, testing the permeability with the small hydrophilic fluorescent compound lucifer yellow (LY) [27,31], and other paracellular markers, including PEG 4000, dextran, mannitol, and inulin, has proved to be quite convincing [30,32].

While LY is commonly determined fluorometrically [13,31,33–35], the present study introduces an easier and cheaper technique, and at the same time, with similar accuracy. The purpose of the present study is to develop a novel validated simple technique for quantitative determination of LY using ultraviolet (UV) spectrophotometry, which was not studied before. In addition, applying the developed UV method to confirm the monolayer integrity of Caco-2 cell line, which is a prerequisite parameter for testing the permeability of drug substances.

Materials and methods

Materials

LY CH dilithium salt and Dulbecco's modified eagle medium (DMEM) were purchased from Sigma (Munich, Germany). Hank's Balanced Salts Solution (HBSS) was from Bio-West (Nuaille, France). Collagen A was obtained from Santa-Cruz (Heidelberg, Germany). Ethanol was of chromatographic HPLC grade and was bought from Merck (Darmstadt, Germany). DMEM, fetal bovine serum, and penicillin–streptomycin mixture (10 000 U/ml penicillin, 10 000 μg/ml streptomycin) were obtained from Gibco by Life Technologies (Waltham, MA, USA). Inserts (transparent ThinCerts, 6-well, 0.4-μm pore size) were from Greiner Bio-One (Aesch, Switzerland). Caco-2 cells were kindly gifted by professor Teresa Frisan, Karolinska Institute, Sweden, originally obtained from ATCC.

Standard solutions

Stock standard solution (1 mg/ml) of LY in HBSS.

Working standard solution (0.1 mg/ml) of LY in HBSS: prepared from stock standard solution by tenfold dilution with HBSS.

Working solution in HBSS is stored at 4°C for up to 4 weeks without any spectral changes.

Instruments

Fluorescence spectrophotometer: Agilent Technologies, Cary Eclipse G9800A, USA.

Spectrophotometer: Beckman, DU-650, USA.

Procedures

Lucifer yellow spectral characteristics

Fluorescence spectrum: a 5.0 μg/ml solution of LY was scanned (against HBSS as blank) for excitation wavelengths, the available excitations were stored, and then the same solution was scanned for emission at different excitations.

UV–visible spectrum: the absorption spectrum of 5.0 μg/ml LY in HBSS was scanned over the range 200–800 nm against HBSS as a blank.

Linearity

Different aliquots of working standard solution of LY in HBSS (0.1 mg/ml) were accurately transferred into a series of volumetric flasks and then diluted to a volume with HBSS. Standard solutions containing 1.0–12.5 μg/ml of LY in HBSS were obtained.

Spectrofluorimetric method: different LY standard solutions in HBSS were prepared as mentioned. The emission spectra were scanned using an excitation wavelength of 426 nm. A calibration curve was constructed relating the emission-amplitude values of LY at 534 nm to the corresponding concentrations and the regression equation was then computed.

UV-spectrophotometric method: the absorption spectra of the prepared serial solutions were scanned in the range of 200–800. A calibration curve was constructed relating the absorbance values of LY at 277 nm to the corresponding concentrations and the regression equation was then computed.

Statistical analysis

Values of recoveries of concentrations of the proposed UV-spectrophotometric and the conventional spectrofluorimetric methods were statistically compared by calculating Student *t* and *F* values and comparing the calculated with tabulated values.

Caco-2 transport studies

Cell culture

Caco-2 cells were cultured in DMEM media supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin antibiotic at 37°C, 95% humidity, and 5% CO_2.

Transport studies

To coat the inserts (ThinCerts TC inserts, 6-well, 0.4-μm pore size, Geiner Bio-One, Switzerland), collagen-A solution (3.4 mg/ml) was diluted with 70% ethanol (1 : 4), filter-sterilized, and then 500 μl were added to each well. The 6-well plates were left to dry overnight in laminar-flow hood with their lids left ajar.

Caco-2 cells were seeded at a density of 260 000 cells/cm^2 into the collagen-A- coated inserts fitted to six-well plates. Prior to all experiments, cells were incubated for 21 days to obtain a tight monolayer. Media were changed every other day. Subsequently, barrier integrity was investigated by the paracellular permeability of LY [31,33].

Measurement of Caco-2 cell-monolayer integrity using lucifer yellow

Paracellular permeability was checked with the tracking of LY dye and the obtained results of less than 3% represented a tight monolayer [36]. For the assay, both compartments of the transwell system were washed with HBSS. Subsequently, 0.1 mg/ml LY in HBSS and pure HBSS was added to the apical and basolateral compartment, respectively. At the end of the exposure period (60 min), the basolateral HBSS medium was collected. The concentration of LY in the collected basolateral HBSS medium was determined by measuring both UV absorbance at wavelength 277 nm and fluorescence (excitation: 426 nm, emission: 534 nm) against HBSS as blank. Measurements were repeated six times. Plain inserts (without cells) were monitored simultaneously following the same procedure [33,34]. The percent permeability was calculated from the obtained values of both assays using the following equation [36]:

$$\%\,\text{Permeability} = \frac{\text{basolateral sample} - \text{blank}}{0.1\,\text{mg/mL LY} - \text{blank}} \times 100.$$

LY permeability of less than 3% was accepted for tight monolayers [36]. Calculated permeability percentage for the UV-spectrophotometric and spectrofluorimetric methods was compared statistically employing paired *t* test.

Results and discussion

LY as a fluorescent marker was formerly and traditionally analyzed spectrofluorimetrically, depending on its native fluorescence [13,33,34]. Although spectrofluorimetry needs a special and rather expensive instrumentation, no other spectroscopic techniques have been reported for LY analysis yet. Therefore, the presented work was an attempt to find a new method for quantitative estimation of LY, and assuring the validity of this method to measure the concentration of LY with a reliable accuracy and precision, compared with the conventional spectrofluorimetric method. Moreover, the aim of this study was to apply the newly introduced spectrophotometric method on permeability testing on Caco-2 cell line.

Linearity

Spectrofluorimetric method: the emission intensities at 540 nm of serial dilutions of LY in HBSS were measured at excitation of 430 nm [13,34]. Figure 1 demonstrates the fluorescence spectrum showing emission at 540 nm. The regression equation relating the emission intensity and the corresponding concentration was found to be A=71.473 C+98.32, R^2=0.9993.

UV-spectrophotometric method: absorbance spectra of standard LY solutions have two maxima, at 277 and 427 nm (Fig. 2). The two wavelengths were checked, and measurements were carried out at both. It was found that analysis of LY was more accurate at 277 nm owing to higher sensitivity and linearity than 427 nm. The calibration range was 1.0–12.5 μg/ml. A linear

relationship [regression coefficient (R^2)=0.9992] was obtained between the absorbance and concentrations. The straight-line equation was established where A=0.0702 C+0.0067.

Method validation

Precision [37]

Validation tests were performed according to ICH recommendations [37]. Table 1 compiled the validation parameters of the proposed UV-spectrophotometric method.

Linearity

Six LY concentrations in the range 1.0–12.5 μg/ml were analyzed in order to evaluate the linearity. Analysis of each concentration was performed in triplicate following the method stated before. Linearity parameters are collected in Table 1.

Accuracy

Previously mentioned methodology was employed in identifying some blind concentrations of LY, so as to check the accuracy. Calculations were performed using the deduced regression equation. Calculated recoveries proposed good accuracy for the described methods (Table 1).

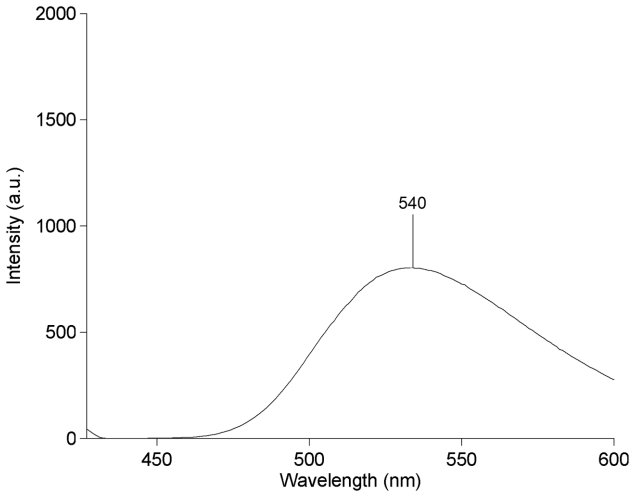
Range

The linearity range was determined by estimation of the actual range according to obedience to Beer's law [38] and the concentration of LY expected in Caco-2 transport studies to obtain linear results (Table 1).

Robustness

Four different solutions of LY (2.0, 5.0, 7.5, and 10.0 μg/ml) were determined in triplicate using the proposed method under different temperatures, namely 25, 30, and 37°C. The method declared to be robust as the found relative SDs was less than 2.0%.

Figure 1



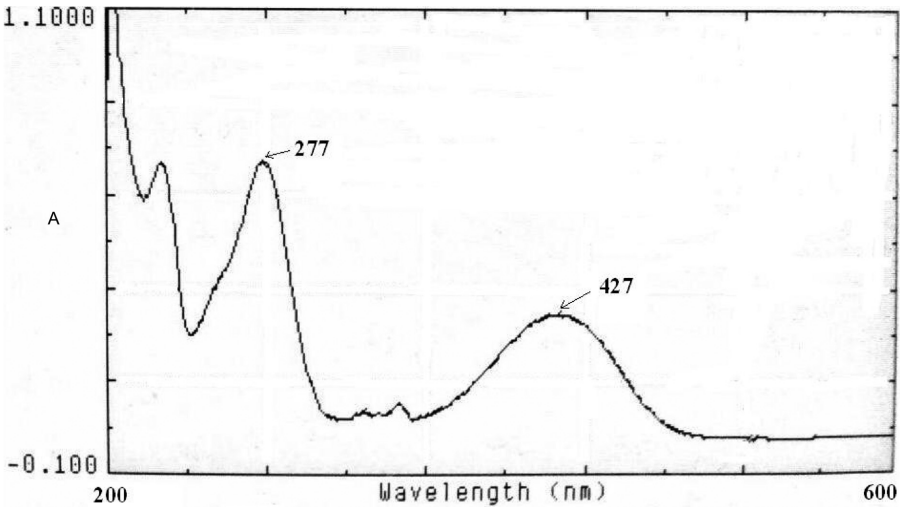
Spectrofluorimetric spectrum of lucifer yellow in HBSS. HBSS, Hank's Balanced Salts Solution.

Table 1 Validation parameters of the proposed ultraviolet-spectrophometric method

Parameters	Value
Linearity (μg/ml)	1.0–12.5
Slope	0.0702
Intercept	0.0067
Correlation coefficient (*r*)	0.9992
Mean±SD	100.121±0.697
Accuracy±SD	100.532±0.253
RSD%[a]	0.427
RSD%[b]	0.709

[a]Relative standard deviation of repeatability. [b]Relative standard deviation of intermediate precision.

Figure 2



UV–visible spectrophotometric spectrum of lucifer yellow in HBSS. HBSS, Hank's Balanced Salts Solution; UV, ultraviolet.

Repeatability and intermediate precision

Three different concentrations of LY (5.0, 7.5, and 10.0 μg/ml) were analyzed in triplicates intradaily and interdaily on three successive days using the proposed method. The relative SD was then calculated (Table 1).

Stability

LY working standard in HBSS has no observable changes in the UV–visible spectrum upon storage for up to 4 weeks at 4°C.

Statistical analysis

The results obtained by the proposed spectrophotometric method and the conventional spectrofluorimetric were statistically compared. The two methods were found similar, that is, having no significant differences owing to the finding that the calculated t test (0.28) and F (1.942) values [39,40] were less than the tabulated ones. Therefore, the proposed method can be used in quantitation of LY instead of the reported one with accepted accuracy, in addition to its simplicity and availability of instrumentation.

Caco-2 cell-monolayer integrity

According to permeability-percentage calculation, it was found that only 2.354 and 2.303% were permeable of LY indicating Caco-2 cell-monolayer integrity after incubation of known concentration of LY in HBSS for 60 min at 37°C on the Caco-2 cultivated on transwell using spectrofluorimetric and UV-spectrophotometric methods, respectively (a permeability of <3% is acceptable [36]). No significant difference (P=0.1374) was found when comparing the values of calculated permeability percentages (means=2.354% and 2.303, for spectrofluorimetric and UV-spectrophotometric methods, respectively), employing paired t test. This successfully confirms the integrity of the Caco-2 cell confluent monolayer.

Conclusion

A novel UV-spectrophotometric method was developed and validated for the analysis of LY as an alternative for the conventional spectrofluorimetry. The newly introduced method was efficiently applied, for the first time with reliable accuracy and precision, to check the permeability across the Caco-2 cell line cultivated according to the reference guidelines [33]. The integrity of monolayer was confirmed by the standard impermeable marker LY. The simplicity of the UV-spectrophotometric determination was fascinating and the accuracy was satisfactory for the application of LY as a permeability marker for cell culture using Caco-2.

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Conflicts of interest

There are no conflicts of interest.

References

1 Bohets H, Annaert P, Mannens G, Anciaux K, Verboven P, Meuldermans W, *et al.* Strategies for absorption screening in drug discovery and development. Curr Top Med Chem 2001; 1:367–383.

2 Lin J, Sahakian DC, De Morais S, Xu JJ, Polzer RJ, Winter SM. The role of absorption, distribution, metabolism, excretion and toxicity in drug discovery. Curr Top Med Chem 2003; 3:1125–1154.

3 Amidon GL, Lennernäs H, Shah VP, Crison JR. A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. Pharm Res 1995; 12:413–420.

4 Pade V, Stavchansky S. Link between drug absorption solubility and permeability measurements in Caco-2 cells. J Pharm Sci 1998; 87:1604–1607.

5 Golfar Y, Shayanfar A. Prediction of biopharmaceutical drug disposition classification system (BDDCS) by structural parameters. J Pharm Pharm Sci 2019; 22:247–269.

6 FDA. Guidance for industry: waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), MD, USA. 2017.

7 Löbenberg R, Amidon GL. Modern bioavailability, bioequivalence and biopharmaceutics classification system. New scientific approaches to international regulatory standards. Eur J Pharm Biopharm 2000; 50:3–12.

8 Ghadi R, Dand N. BCS class IV drugs: highly notorious candidates for formulation development. J Control Release 2017; 248:71–95.

9 Dressman JB, Amidon GL, Reppas C, Shah VP. Dissolution testing as a prognostic tool for oral drug absorption: immediate release dosage forms. Pharm Res 1998; 15:11–22.

10 Bou-Chacra N, Melo KJC, Morales IAC, Stippler ES, Kesisoglou F, Yazdanian M, *et al.* Evolution of choice of solubility and dissolution media after two decades of biopharmaceutical classification system. AAPS J 2017; 19:989–1001.

11 Meyer MC, Straughn AB, Jarvi EJ, Wood GC, Pelsor FR, Shah VP. The bioinequivalence of carbamazepine tablets with a history of clinical failures. Pharm Res 1992; 9:1612–1616.

12 Benet LZ. The role of BCS (biopharmaceutics classification system) and BDDCS (biopharmaceutics drug disposition classification system) in drug development. J Pharm Sci 2013; 102:34–42.

13 Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 1989; 96:736–749.

14 Hilgers AR, Conradi RA, Burton PS. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm Res 1990; 7:902–910.

15 Saha P, Kou JH. Effect of bovine serum albumin on drug permeability estimation across Caco-2 monolayers. Eur J Pharm Biopharm 2002; 54:319–324.

16 Lechanteur A, Almeida A, Sarmento B. Elucidation of the impact of cell culture conditions of Caco-2 cell monolayer on barrier integrity and intestinal permeability. Eur J Pharm Biopharm 2017; 119:137–141.

17 Lennernäs H. Human intestinal permeability. J Pharm Sci 1998; 87:403–410.

18 Gram LK, Rist GM, Lennernäs H, Steffansen B. Impact of carriers in oral absorption: permeation across Caco-2 cells for the organic anions estrone-3-sulfate and glipizide. Eur J Pharm Sci 2009; 37:378–386.

19 Sánchez BA, Calpena CA, Mallandrich M, Clares B. Validation of an ex vivo permeation method for the intestinal permeability of different BCS drugs and its correlation with caco-2 in vitro experiments. Pharmaceutics 2019; 11:638.

20 Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell A-L, Karlsson J. Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. Drug Metab Dispos 2007; 35:1333–1340.

21 Stockdale TP, Challinor VL, Lehmann RP, De Voss JJ, Blanchfield JT. Caco-2 monolayer permeability and stability of Chamaelirium luteum (false unicorn) open-chain steroidal saponins. ACS Omega 2019; 4:7658–7666.

22 Pérez-Sánchez A, Cuyàs E, Ruiz-Torres V, Agulló-Chazarra L, Verdura S, González-Álvarez I, *et al.* Intestinal permeability study of clinically relevant formulations of silibinin in Caco-2 cell monolayers. Int J Mol Sci 2019; 20:1606.

23 Yee S. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharm Res 1997; 14:763–766.

24 Sun F, Adrian M, Beztsinna N, van den Dikkenberg JB, Maas-Bakker RF, van Hasselt PM, *et al.* Influence of PEGylation of vitamin-K-loaded mixed micelles on the uptake by and transport through Caco-2 cells. Mol Pharm 2018; 15:3786–3795.

25 Artursson P, Palm K, Luthman K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev 1996; 22:67–84.

26 Price E, Kalvass JC, DeGoey D, Hosmane B, Doktor S, Desino K. Global analysis of models for predicting human absorption: QSAR, in vitro, and preclinical models. J Med Chem 2021; 64:9389–9403.

27 Nikulin S, Knyazev E, Poloznikov A, Shilin S, Gazizov I, Zakharova G, *et al.* Expression of SLC30A10 and SLC23A3 transporter mRNAs in Caco-2 cells correlates with an increase in the area of the apical membrane. Mol Biol 2018; 52:577–582.

28 Lehr C-M. Cell culture models of biological barriers: in vitro test systems for drug absorption and delivery. Florida, USA: CRC Press; 2002.

29 Vila L, García-Rodríguez A, Cortés C, Marcos R, Hernández A. Assessing the effects of silver nanoparticles on monolayers of differentiated Caco-2 cells, as a model of intestinal barrier. Food Chem Toxicol 2018; 116:1–10.

30 Mukherjee T, Squillantea E, Gillespieb M, Shao J. Transepithelial electrical resistance is not a reliable measurement of the Caco-2 monolayer integrity in Transwell. Drug Deliv 2004; 11:11–18.

31 Beisl J, Varga E, Braun D, Warth B, Ehling-Schulz M, Del Favero G, *et al.* Assessing mixture effects of cereulide and deoxynivalenol on intestinal barrier integrity and uptake in differentiated human caco-2 cells. Toxins 2021; 13:189.

32 Sun H, Chow EC, Liu S, Du Y, Pang KS. The Caco-2 cell monolayer: usefulness and limitations. Expert Opin Drug Metab Toxicol 2008; 4:395–411.

33 Hubatsch I, Ragnarsson EG, Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nat Protoc 2007; 2:2111.

34 Konsoula R, Barile FA. Correlation of in vitro cytotoxicity with paracellular permeability in Caco-2 cells. Toxicol In Vitro 2005; 19:675–684.

35 Ujhelyi Z, Fenyvesi F, Váradi J, Fehér P, Kiss T, Veszelka S, *et al.* Evaluation of cytotoxicity of surfactants used in self-micro emulsifying drug delivery systems and their effects on paracellular transport in Caco-2 cell monolayer. Eur J Pharm Sci 2012; 47:564–573.

36 Pratt J, Venkatraman N, Brinker A, Xiao Y, Blasberg J, Thompson DC, *et al.* Use of zinc finger nuclease technology to knock out efflux transporters in C2BBe1 cells. Curr Protocols Toxicol 2012; 52:23. 2.

37 ICH. Validation of analytical procedures: methodology: US FDA, Federal Register; 1997. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/q2b-validation-analytical-procedures-methodology. [Accessed 10 August 2021].

38 Skoog DA, West DM. Instrumental analysis, principles of instrumental analysis. 6th ed. Philadelphia, USA: Saunders College Publishing; 2007.

39 Ostle B. Statistics in research. Ames, Iowa, USA: The Iowa State University Press; 1960.

40 Ho J, Tumkaya T, Aryal S, Choi H, Claridge-Chang A. Moving beyond P values: data analysis with estimation graphics. Nat Methods 2019; 16:565–566.