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Determination of thalidomide in transport buffer for Caco-2 cell monolayers by high-performance liquid chromatography with ultraviolet detection

Shufeng Zhou[a,*], Yan Li[a], Phillip Kestell[b], James W. Paxton[a]

[a]*Division of Pharmacology and Clinical Pharmacology, Faculty of Medical and Health Sciences, the University of Auckland, 85 Park Road, Grafton Private Bag 92019, Auckland, New Zealand*

[b]*Auckland Cancer Society Research Centre, the University of Auckland, Private Bag 92019, Auckland, New Zealand*

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Abstract

We report simple validated HPLC methods for the determination of thalidomide in the transport buffer for the human colonic cell line (Caco-2) cell monolayers. An aliquot of 50 μl of the mixture was injected onto a Spherex C_{18} column (150×4.6 mm; 5 μm) at a flow-rate of 0.5 ml/min of mobile phase consisting of acetonitrile–10 m*M* ammonium acetate buffer (24:76, v/v, pH 5.5), and thalidomide was detected by ultraviolet detector at a wavelength of 220 nm. Calibration curves for thalidomide were constructed at the concentration range of 0.025–1.0 and 1.0–50 μ*M* in transport buffer. The validated methods were used to determine the transport of thalidomide by Caco-2 monolayers. The transport across the monolayers from the apical (A) to basolateral (B) side was similar to that from B to A side. The apparent permeability coefficient (P_{app}) values of thalidomide at 10–300 μ*M* from the A to B and from B to A side was $2–6\times10^{-5}$ cm/s, with a marked decrease in P_{app} values from A to B side at increased thalidomide concentration. The A to B transport appears to be dependent on temperature and sodium ion. Sodium azide, 2,4-dinitrophenol (both ATP inhibitors), 5-fluorouracil, cytidine and glutamic acid significantly inhibited the transport of thalidomide. These results indicate that the transport of thalidomide by Caco-2 monolayers was rapid, which might involve an energy-dependent mechanism.

Keywords: Caco-2 cell monolayers; Thalidomide

1. Introduction

Thalidomide (α-phthalimidoglutarimide) has been increasingly used as an oral agent for the treatment of a variety of diseases including erythema nodusum leprosum [1–4], Behcet's syndrome [5], and more recently certain malignancies such as multiple myeloma [6,7], renal, breast and prostate carcinoma [8–15]. Clinical trials have confirmed the benefits of thalidomide in relapsed diseases. For example, thalidomide treatment caused objective responses in some patients with malignancies such as relapsed multiple myeloma, and renal carcinoma [6,8, 10,16,17]. The therapeutic value of thalidomide has

*Corresponding author. Present address: Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore. Tel.: +65-68-742-931; fax: +65-67-791-554.

E-mail address: phazsf@nus.edu.sg (S. Zhou).

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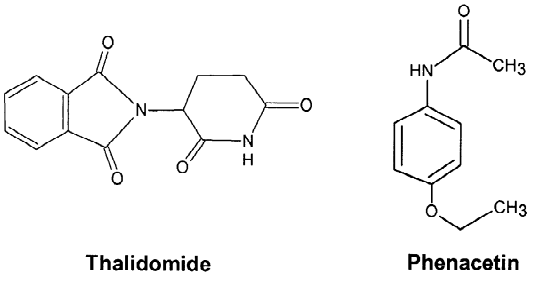
been ascribed to its anti-angiogenic [18,19], immuno-modulating [20–25] and sedative effects [26,27]. Although thalidomide's mechanism of action and pharmacokinetic-pharmacodynamic relationship have not been identified, considerable evaluations in animals and humans have been conducted, and some insights have been generated. Recent studies have revealed that thalidomide inhibited NF-κB induced by various factors such as tumor necrosis factor-α and H_2O_2 [28,29]. Since NF-κB plays an important role in the immune system [30], and confers significant survival potential in a variety of tumors [31–33], the inhibition of NF-κB by thalidomide may be associated with the anti-tumour, immuno-modulating and anti-inflammatory activity [31–34].

Thalidomide is a derivative of glutamic acid and contains two amide rings and a single chiral center (Fig. 1). The isomers of thalidomide interconvert rapidly at physiological pH in vivo [35]. Some stereoselective differences in the pharmacokinetics and pharmacodynamics of thalidomide have been reported [36,37]. The *S*-isomer has been associated with the teratogenicity of thalidomide [38,39]. The pharmacokinetics of thalidomide has been considerably investigated in animals, whereas its pharmacokinetic study in humans has been hindered by the absence of a proper intravenous formulation, due to its instability and poor solubility in water. Thalidomide is unstable in aqueous medium at physiological pH and biological matrices such as plasma and blood, undergoing rapid and spontaneous hydrolysis [35,39–42]. Although in vitro study indicates that thalidomide is biotransformed by cytochrome P450 (CYP2C) [43–46], the CYP-catalyzed metabolism plays a minor role in the elimination of thalidomide in vivo. Some thalidomide metabolites such as 5′-OH-thalidomide have been reported to inhibit angiogenesis [19,38,47]. As a biological response modifier, thalidomide is often given orally for protracted treatment regimens. Oral absorption of thalidomide (typically 100 mg) is slow, with time to maximum plasma concentration at 2–4 h and an estimated bioavailability of 80–100% [48–52]. With larger doses (≥200 mg), its absorption rate decreases considerably, and thus a variable and lower bioavailability is expected [41,48,52–54]. This has been partly ascribed to the poor solubility of thalidomide in intestinal fluids. However, other mechanism (e.g. reduced intestinal transport due to saturable absorption kinetics) cannot be ruled out.



Thalidomide Phenacetin

Fig. 1. The chemical structure of thalidomide and phenacetin.

Chromatographic methods have been developed and used to quantify thalidomide and its metabolites in biological matrices during pharmacokinetic and metabolic studies [50,53,55–58]. These methods can often fulfil the requirement of quantification of thalidomide and/or its metabolites in plasma and liver subcellular fractions. However, they have the drawbacks of complicated and time-consuming sample treatments and analysis procedures, which increase the risk of degradation of thalidomide in the samples. Indeed, sample instability due to inappropriate handling may contribute to the large variability in the pharmacokinetic parameters of thalidomide in humans [59]. Although a few enantiomer-selective HPLC methods have been developed and used to separate enantiomers of thalidomide [35,60–63], chiral separation was not considered in many studies. In an attempt to characterize the transport of thalidomide by the human colon cancer cell line (Caco-2 cells), which have been widely used to investigate drug permeability and transport [64], we developed simple HPLC methods for the determination of thalidomide in Hanks balanced salt solution (HBSS) used as transport medium. We report here on the developed and validated HPLC methods for thalidomide determination.

2. Experimental

2.1. Chemicals

Thalidomide (purity>99%, determined by HPLC) was provided by Celgene (Warren, NJ, USA).

Phenacetin (used as internal standard, I.S.), sodium azide, 2,4-dinitrophenol, diclofenac, ibuprufen, celecoxib, dimethyl sulphoxide (DMSO), cytidine, adenine, thymidine, guanine, DL-glutamic acid, diprodomole, papaverine, 5-fluorouracil, 2-[*N*-morpholino]ethanesulfonic acid, cyclophosphamide and ifophosphamide were obtained from Sigma–Aldrich (Auckland, New Zealand). 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) and *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA) were synthesized by the Auckland Cancer Society Research Centre of the University of Auckland. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin-ethylenediaminetetraacetic acid, nonessential amino acids, penicillin and gentomycin were all from Life Technologies (Auckland, New Zealand). HBSS (1 l) with 25 m*M* *N*-[2-hydroxyethyl]piperazine-*N*′-[4-butanesulfonic acid] (HEPES) and 25 m*M* glucose was prepared by dissolving 0.14 g $CaCl_2$, 0.40 g KCl, 0.06 g KH_2PO_4, 0.047 g Mg_2Cl, 0.10 g $MgSO_4 \cdot 7H_2O$, 8.00 g NaCl, 0.35 g $NaHCO_3$, 0.048 g Na_2HPO_4, 4.5 g D-Glucose, and 5.95 g HEPES in 1 liter Milli-Q water, and pH was adjusted to 7.4 using 1 *M* NaOH or 1 *M* HCl. HBSS was sterilized by filtering through a 0.1 μ*M* filter. [^{14}C]Mannitol (specific activity of 351 mCi/mmol, radiochemical purity of 98.6%) was obtained from Amersham Pharmacia (Auckland, New Zealand). All other chemicals and reagents were of analytical or HPLC grade as appropriate.

2.2. Cell culture

Caco-2 cells at passage 19 obtained from the American Type Culture Collection (Rockville, MD, USA) were maintained by serial passage in plastic culture flasks (Life Technologies, Auckland, New Zealand). The cells were cultured in DMEM with 10% fetal bovine serum, 1% nonessential amino acids, and 100 U/ml penicillin and gentomycin. The cells were grown in an atmosphere of 5% CO_2/95% air at 37 °C and given fresh medium every 3 or 4 days. For transport studies, the cells were seeded in 0.4-μm pore-size 12 mm I.D. Transwell polycarbonate inserts (Corning Costar Corp., Cambridge, MA, USA) in 12-well plates at a density of 1.5×10^5 cells/insert. The culture medium (0.5 ml in the insert and 1.5 ml in the well) was replaced every 48 h for the first 6 days and every 24 h thereafter. The transepithelial electric resistance (TEER) of the monolayers was examined routinely before and after the experiment using the Millicell®-ERS system (Millipore, Bedford, MA, USA). Cells at passage 20–28 were used for transport experiments 20–31 days after seeding. In addition to routine TEER measurements, the paracellular transport marker [^{14}C]mannitol was used to confirm the integrity of Caco-2 monolayers. The monolayers were used for the transport study when the transepithelial electrical resistance exceeded 300 Ω.cm^2 and the leakage rate of [^{14}C]mannitol was <1% per h.

2.3. Transport experiments

The transport of thalidomide by Caco-2 monolayers was investigated using the methods described previously [65,66] with some minor modifications. Briefly, the cell monolayers were washed twice with HBSS containing 25 m*M* HEPES (pH 7.4) prior to the transport experiments. After each wash, the plates were incubated at 37 °C for 30 min, and the TEER was measured. HBSS solution on both sides of the cell monolayers was then removed by aspiration. For the measurement of the apical (A) to basolateral (B) transport, 0.5 ml of HBSS containing thalidomide (5–300 μ*M*) was added on the A side, and 1.5 ml of HBSS without the drug was added on the B side. The inserts were moved to wells containing fresh incubation medium every 15 min for 4 h. This is a procedural substitute for collecting serial samples from an accepting chamber, which will reduce the risk of thalidomide degradation, as the collected samples were acidified and chilled immediately. For the measurement of B to A transport, 0.5 ml of HBSS containing thalidomide (5–300 μ*M*) was added on the B side, and 0.5 ml of HBSS without the drug was added to the A side. The inserts were then incubated at 37 °C, and the incubation medium (HBSS) in the A side was replaced by fresh medium at 15-min intervals. Thalidomide solutions were freshly prepared by dissolving in DMSO, and phenacetin solutions prepared using methanol and stored at −20 °C. All experiments were conducted in triplicate. The amounts of thalidomide in a 0.4-ml aliquot of sample collected from each time point were determined by HPLC with ultraviolet detection.

The effect of apical pH (5.5–7.4) on the transport

of 100 μ*M* thalidomide from the apical to the basolateral side was examined under constant pH of the basolateral side (7.4). The pH of the apical side was altered by substituting appropriate amounts of HEPES in the incubation medium by equimolar (25 m*M*) 2-[*N*-morpholino]ethanesulfonic acid. In experiments to investigate the effect of Na^+ on the transport of thalidomide (100 μ*M*) from A to B across the Caco-2 cell monolayers, the sodium chloride in the HBSS was replaced by equimolar amounts (140 m*M*) of potassium chloride. The permeability of thalidomide (100 μ*M*) from A to B was measured after incubation for 30 min at 4 or 37 °C. In experiments to investigate the effect of various compounds on the apical to basolateral transport of 10 and 100 μ*M* thalidomide, compounds such as diclofenac, DMXAA, DACA, nucleotides, verapamil, and cimetidine (all at 100 μ*M*) were added to the incubation medium on the apical side of the cell monolayers. All inhibitors were made fresh immediately prior to each experiment and dissolved in DMSO and added to the apical side. Vehicle was used for the control inserts.

2.4. Determination of thalidomide by HPLC

2.4.1. HPLC instrumentation

A Hewlett-Packard (HP) 1100 HPLC system was used to quantify thalidomide in HBSS solution. The system consisted of an injector (model G1313A), a binary pump (model G1312A), and a diode array detector (model G1365B) at an operation wavelength of 220 nm (Hewlett-Packard, Avondale, CA, USA). All data were collected and processed by the HP1100 ChemStation software. A Luna C_{18} guard column was positioned ahead of the 5-μm Luna analytical column (150×4.6 mm; Phenomenex, Torrence, CA, USA). The mobile phase (flow-rate of 0.5 ml/min) consisting of acetonitrile–10 m*M* ammonium acetate buffer (24:76, v/v, pH 5.5) was degassed immediately before use.

2.4.2. Sample preparation

Transport studies were conducted by incubating thalidomide with HBSS at either apical or basolateral side of Caco-2 monolayers. An aliquot (400 μl) was collected and mixed with 2 vol. (800 μl) of ice-cold acetonitrile/methanol mixture containing 2% acetic acid (v/v) and 10 μ*M* phenacetin. This protected thalidomide from spontaneous degradation. After vortexing for 20 s, the mixtures were centrifuged at 3000 *g*×10 min. The supernatant were collected and dried under nitrogen flow using a Speedvac concentrator (Savant Instruments Inc., Farmingdale, NY, USA). The residue was reconstituted with 100 μl mobile phase and 50 μl injected onto the HPLC.

2.4.3. Calibration curves

Calibration curves were constructed over the concentration range of 0.025–1.0 μ*M* and 1.0–50 μ*M* separately. Sample preparation of standards was the same as for unknown samples. The ratio of peak area of thalidomide to that of internal standard, and linear least-squares regression analysis weighted according to the reciprocal of peak area ratio squares was conducted to determine the slope, intercept and coefficient of determination by Prism 3.0 program (Graphpad Software, CA, USA).

2.4.4. Sensitivity and selectivity

The limit of quantification (LOQ) was defined as the minimum concentration which could be determined with acceptable accuracy (i.e. recovery between 80 and 120%) and precision (coefficient of variation (CV)<20%) [67]. The limit of detection was the amount which could be detected with a signal-to-noise ratio of 3. The selectivity of the method was examined by determining if interfering chromatographic peaks were present in blank HBSS or in the presence of various drugs, including DMXAA, DACA, diclofenac, ibuprufen, celecoxib, cytidine, adenine, diprodomole, papaverine, thymidine, guanine, DL-glutamic acid, 5-fluorouracil, cyclophosphamide, and ifophosphamide.

2.4.5. Accuracy and precision

Quality control (QC) samples containing thalidomide were prepared from weighing independent of those used for preparing calibration curves. Final concentrations of low, medium and high QC samples were 0.025, 0.1 and 0.5 (for calibration curve over 0.025–1.0 μ*M*), and 2.5, 25 and 250 μ*M* (for calibration curve over 1.0–50 μ*M*), respectively. These samples were prepared on the day of analysis in the same way as calibration standards. The performance of the HPLC method was assessed by

analysis of 24 QC sample (four each of low, medium, and high concentrations) on a single assay day to determine intra-day accuracy and precision, and 24 QC samples (four each of low, medium, and high concentrations) on each of four consecutive assay days to determine inter-day accuracy and precision.

2.4.6. Stability

Thalidomide at 50 μ*M* was incubated in HBSS at different pH values (5.4, 6.0, 6.5, 7.0 and 7.4) at 37 °C over 24 h. At indicated time points, an aliquot of 50 μl of the stock solution was collected and processed as standard samples.

2.5. Data analysis

Data were the mean±SD. The apparent permeability coefficient (P_{app}) is expressed in cm/s, and calculated as

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{60} \times \frac{1}{A} \times \frac{1}{C_0}$$

where $\Delta Q/\Delta t$ is the permeability rate (nmol/min); A, the surface area of the membrane (cm^2); and C_0, the initial concentration in the donor chamber (nmol/ml). Samples from 30 min point were used for P_{app} calculations. The initial statistical analysis to evaluate the differences in the mean kinetic parameters among the different groups was carried out by a one-way analysis of variance (ANOVA) with a post-hoc test. Student's unpaired *t*-test was conducted for the between-group comparisons with a significance level of $P<0.05$.

3. Results

3.1. Validation of HPLC methods

We report here on validated HPLC methods for the determination of thalidomide in HBSS. Representative chromatograms from HBSS with added thalidomide and I.S. are shown in Fig. 2. Under the chromatographic conditions used for the analysis of thalidomide, the retention time for thalidomide and internal standard was 5.5 and 6.8 min, respectively. The total chromatography run time was 8.5 min.

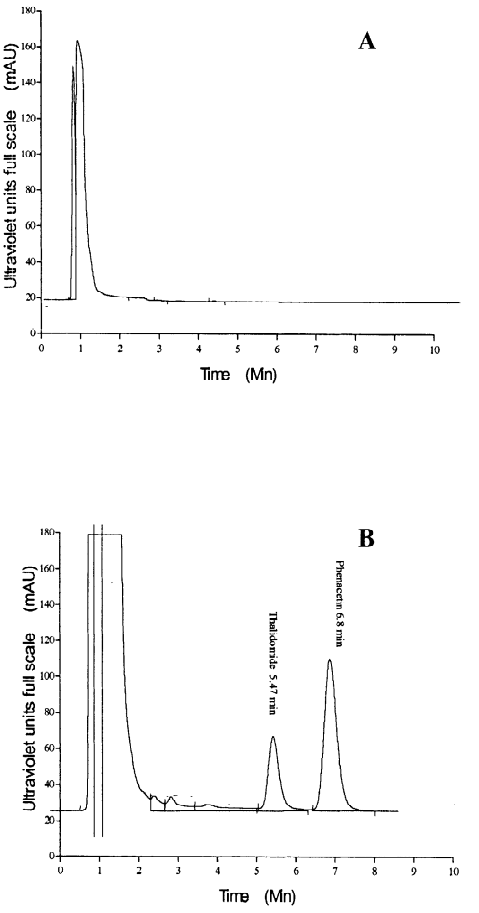


Fig. 2. Representative chromatographs of thalidomide and phenacetin in Hanks balanced salt solution (HBSS). Blank HBSS (A) and HBSS containing thalidomide (10 μ*M*) and phenacetin (10 μ*M*) (B).

These methods employed simple liquid–liquid extraction procedures as an alternative for solid-phase extraction, and resulted in an extraction efficiency of 92.6±8.5 and 90.3±9.1% for thalidomide at 1 and 10 μ*M* ($n=3$), respectively. No concentration dependence was observed. Matrix-specific interfering peaks that required modification of the mobile phase composition were not observed in any cases, including in the presence of drugs such as DMXAA, DACA, cytidine and diclofenac.

Calibration curves were linear over the concentration range of 0.025–1.0 and 1.0–25 μ*M* with the mean correlation coefficients >0.999 ($n=5$). The mean *y* intercepts were 0.003 (±0.001, $n=5$) and

0.08 ($\pm$0.04, $n=5$) for calibration curves over 0.025–1 μM and 1–50 μM respectively. The differences between the theoretical and actual concentration and the relative standard deviation were less than 15% at any QC concentrations. The results for precision and accuracy are shown in Table 1. The LOQ of the assay was 0.32 ng for a 50-μl aliquot of thalidomide in HBSS.

3.2. Stability of thalidomide in HBSS

As shown in Fig. 3, the degradation of thalidomide in HBSS was pH-dependent. Thalidomide in HBSS at pH 7.4 was unstable, with $<$20% remaining after 24 h, whereas no significant degradation was observed over 24 h when pH values decreased to $\leq$6.0. At pH 6.5, about 60% thalidomide remained after 24-h incubation. Within 1 h, $<$15% of thalidomide was hydrolyzed at pH 7.4.

3.3. Application to the transport of thalidomide by Caco-2 monolayers

The validation data for HPLC methods have demonstrated the applicability of the methods for the analysis of thalidomide in transport medium. After incubation with thalidomide at 10–300 μM loaded at either A or B side, the sample was collected from the other side for HPLC analysis. The study indicates

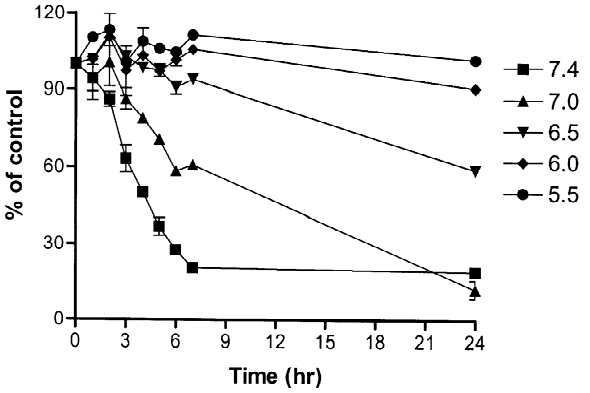


Fig. 3. Degradation of thalidomide in Hanks balanced salt solution (HBSS) at various pH values at 37 °C over 24 h.

that the transport rate across the monolayers from A to B side was similar to that from B to A side (Fig. 4A). The transport of thalidomide appeared to be linear within 1 h. The P_{app} values of thalidomide at 10–300 μM from the A to B and from B to A side was 2–6$\times 10^{-5}$ cm/s, with a marked decrease in P_{app} values from A to B at increased thalidomide concentration (Fig. 4B). The transport of thalidomide was sodium- and temperature-dependent, as replacement of extracellular sodium chloride or reducing temperature resulted in significant decreases in the P_{app} values. The flux of thalidomide was significantly influenced by the apical pH. Sodium azide and

Table 1
Accuracy and precision of the HPLC methods for the analysis of thalidomide in Hanks Balanced Salt Solution (HBSS, pH 7.4)

Theoretical concentration (μM)	Measured concentration (μM) (mean$\pm$SD)	% Recovery of theoretical concentration	CV (%)	No. of samples
Intra-assay				
0.025	0.027$\pm$0.001	110.02	3.70	4
0.1	0.098$\pm$0.005	98.02	4.83	4
0.5	0.448$\pm$0.003	89.68	0.63	4
2.5	2.418$\pm$0.019	96.71	0.79	4
25	24.61$\pm$0.227	98.42	0.92	4
250	252.1$\pm$0.617	100.80	1.56	4
Inter-assay				
0.025	0.028$\pm$0.001	112.97	4.45	4
0.1	0.098$\pm$0.005	97.76	5.00	4
0.5	0.452$\pm$0.007	90.45	1.45	4
2.5	2.391$\pm$0.030	95.63	1.25	4
25	24.50$\pm$0.341	97.99	1.39	4
250	249.4$\pm$2.170	99.76	5.41	4

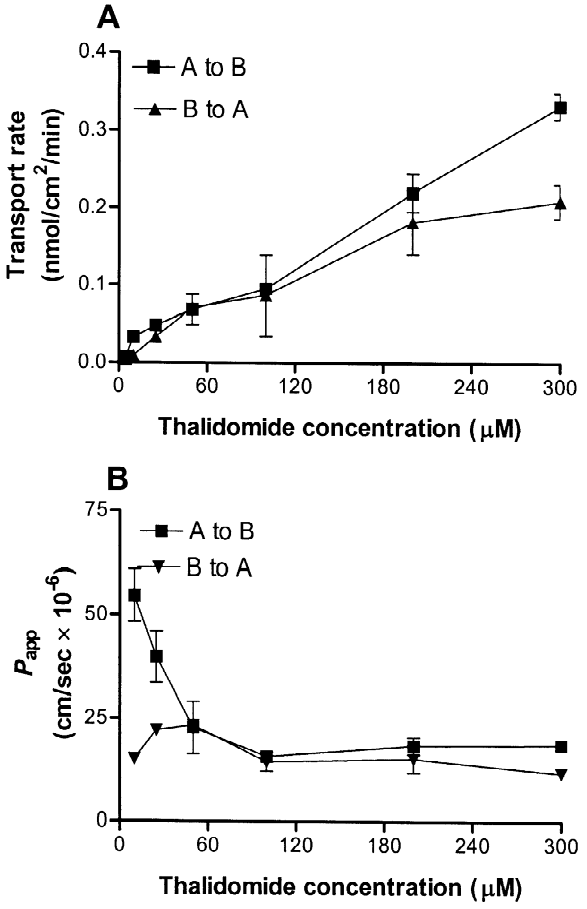


Fig. 4. (A) The transport rate of thalidomide (100 μ*M*) from apical (A) to basolateral (B) side and from B to A side by Caco-2 monolayers; (B) the permeability coefficients (P_{app}) for thalidomide from A to B side and from B to A side by Caco-2 monolayers.

2,4-dinitrophenol (both ATP inhibitors) inhibited the transport of thalidomide. 5-Fluorouracil, cytidine, diprodomole, papaverine, and glutamic acid also significantly inhibited the transport of thalidomide by 25%. However, the P-glycoprotein inhibitor verapamil and other nucleosides and nucleotides such as thymidine and guanine had no effect.

4. Discussion

We developed and validated simple HPLC methods for the determination of thalidomide in the transport buffer for Caco-2 cell monolayers. These methods have the advantage of being fast and efficient, with the retention time of thalidomide and I.S. being shorter than those reported previously [50,53,55]. Therefore, the risk of degradation of thalidomide can be reduced dramatically during sample analysis. The LOQ value (0.32 ng) appeared to be much lower than those reported by Torano et al. (4.44 ng) [68] and Heney et al. (2.0 ng) [53]. Lower LOQ was important for transport studies of thalidomide, as the low concentration range over 0.025–0.1 μ*M* was often encountered. However, a drawback of our HLPC methods reported here was lack of chiral separation. In vivo study indicates that *R*- and *S*-thalidomide had faster oral absorption than racemate when given separately [35]. This may be due to the fact that the enantiomers have higher solubility compared with that of the racemate. It is unclear whether there is a difference in the permeability by Caco-2 monolayers between *R*- and *S*-thalidomide.

Since thalidomide is unstable at physiological pH, we chose the 30-min incubation time point for the calculation of P_{app} values. Appropriate handling of biological samples containing thalidomide is crucial to avoid degradation. This is particularly important for plasma samples from patient studies, as thalidomide degradation due to unsuitable sample handling may lead to marked inter-individual variation in the pharmacokinetic parameters. Since the degradation of thalidomide (mainly non-enzymatic hydrolysis) is dependent on pH and temperature only [35,36,40], the half-life of thalidomide in plasma, blood or other biological samples at pH 7.4 should be similar. The reported large inter-individual and inter-laboratory variations in the pharmacokinetic parameters of thalidomide in humans (in particular in patients) are considered mainly due to inappropriate sample handling. In this study, lowering pH and quick chilling of the samples by ice-cold organic mixtures with 2% acetic acids (pH 4.5) was used to prevent hydrolysis of thalidomide in transport buffer.

The results of transport studies indicated that thalidomide underwent a rapid transport by Caco-2 monolayers, dependent on energy, temperature and sodium ion for A to B flux. The high P_{app} values of thalidomide from A to B side suggests that thalidomide might have high oral bioavailability

[69]. The P_{app} values of thalidomide decreased dramatically at concentrations >50 μM, which might partly explain the delayed and variable oral absorption at high doses in humans [51]. Since 5-fluorouracil, cytidine and glutamic acid inhibited the transport of thalidomide, and these nucleobases exhibited inhibitory effects on the transport of nucleoside or nucleobase transporter substrates such as nitrofurantoin [70], thus nucleoside transporters might be involved in the transport of thalidomide. However, further study is needed to investigate the mechanism for the intestinal absorption of thalidomide and identify the transporters involved.

In conclusion, the HPLC methods developed for the quantification of thalidomide in HBSS are sensitive and reliable. The validated methods were used to characterize the transport of thalidomide by Caco-2 monolayers, and found that the transport of thalidomide was rapid, and might involve an energy-dependent mechanism.

5. Nomenclature

A	apical
B	basolateral
CV	coefficient of variation
DACA	*N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DMXAA	5,6-dimethylxanthenone-4-acetic acid
EDTA	ethylenediaminetetraacetic acid
HBSS	Hanks balanced salt solution
HEPES	*N*-[2-hydroxyethyl]piperazine-*N′*-[4-butanesulfonic acid]
I.S.	internal standard
LOQ	limit of quantification
MES	2-[*N*-morpholino]ethanesulfonic acid
P_{app}	apparent permeability coefficient
QC	quality control
TEER	transepithelial electric resistance

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