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Short communication

Concurrent determination of topotecan and model permeability markers (atenolol, antipyrine, propranolol and furosemide) by reversed phase liquid chromatography: Utility in Caco-2 intestinal absorption studies

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Abstract

A simple, sensitive, specific and high-resolution reversed-phase liquid chromatographic method utilizing ultraviolet detection has been developed and validated for simultaneous determination of topotecan and four intestinal permeability markers (atenolol, antipyrine, propranolol and furosemide) as suggested by US-FDA. Chromatography was carried out on C-18 column with mobile phase comprising water (pH 3.0) and acetonitrile gradient pumped at a flow rate of 1 ml min^{-1}. The validation parameters included specificity, accuracy, precision, sensitivity and stability studies. Topotecan, an anti-cancer drug widely used in metastatic carcinoma, is a P-glycoprotein substrate having oral bioavailability of 30% with large inter-patient variability. The present method was successfully applied for demonstrating P-gp mediated transport of topotecan and its inhibition using verapamil in Caco-2 cell monolayer. The method can be used in identification of novel P-gp inhibitors for topotecan and estimating the contribution of P-gp in affecting oral bioavailability of topotecan. The other applications of method include its use in validation of Caco-2 monolayer assay for getting biowaiver based on Biopharmaceutic Classification System and its extrapolation to in situ and/or in vivo studies.

Keywords: Topotecan; P-glycoprotein; US-FDA listed intestinal permeability markers; Caco-2

1. Introduction

Improvement of oral drug bioavailability by temporary inhibition of drug transporters especially P-glycoprotein (P-gp) in the gut epithelium forms an attractive approach for various anti-cancer drugs including topotecan [1–4]. Topotecan is a water-soluble semi-synthetic derivative of camptothecin, and is reversibly hydrolysed to its carboxylate forms depending on pH [5]. Topotecan has oral bioavailability of 30–40% and there is little knowledge available for low plasma levels of the drug [6]. When co-administered with a combined P-gp and Breast Cancer Resistance Protein (BCRP) inhibitor, elacridar (GF 120918), there occurred a 7–8-fold increase in systemic exposure of topotecan. Also, genetic knock out of murine P-gp resulted in increase in intestinal absorption of topotecan. This triggered the research on exploring the possibilities of topotecan as a BCRP and/or P-gp substrate [7–9]. Caco-2 intestinal absorption model forms the most widely accepted in vitro method for permeability determination and P-gp substrate/inhibitor identification. However, inter-lab and inter batch variabilities exists in permeability estimation with Caco-2 due to discrepancies in culturing, handling and experimental procedures.

Present method was developed for analyzing cell monolayer permeability samples of topotecan done in combination with four permeability markers (atenolol, propranolol, antipyrine and furosemide). Simple/programmed fluorescence detection with complex sample preparation procedures have been published [6,10–14] for topotecan quantification in biological matrices

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like plasma, urine, bile and feces that are useful in pharmacokinetic studies. The novelty of the present work lies in utilizing a ultra-violet mode of detection with direct injection for topotecan without compromising on sensitivity and specificity. The assay can be used for screening and identification of novel compounds which act as P-gp inhibitors and can be used in bioavailability enhancement and multidrug resistance modulation of topotecan [15]. Verapamil (a known P-gp efflux inhibitor) is used as a positive control.

The four compounds other than topotecan, acted as internal permeability standards for measuring day-to-day comparability of Caco-2 system as well as, enabling monitoring of integrity and functional status of the intestinal membrane. Since the method also allows simultaneous determination of various other P-glycoprotein efflux substrates and US-FDA suggested permeability markers, it can be used for in-house validation of Caco-2 system to get waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a Biopharmaceutics Classification System. As stated in the guidance, for in vitro cell permeability assay validation, twenty model drugs are recommended by US-FDA [16]. Therefore, another application of present method lies in selecting group of non-interfering drugs and using them for Caco-2 permeability assay validation.

The method was validated for routine usage and its usefulness and reliability is demonstrated with a real time experiment with Caco-2 samples, each providing similar permeability values as reported [17]. Also, the method may be extrapolated to other in vitro (MDCK, MDCK-MDR), in situ (lumenal perfusate samples) or in vivo (plasma samples) models.

2. Experimental methods

2.1. Reagents and chemicals

Topotecan (>95% purity) originated from Dabur Pharma Limited (U.P, India). Atenolol, antipyrine, propranolol, furosemide and verapamil were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile of HPLC grade was obtained from J.T Baker (USA). *O*-phosphoric acid and DMSO were from Merck Ltd. (India). Dulbecco's modified Eagle's medium (DMEM) was from Caisson Labs. (North Logan, UT, USA), fetal bovine serum (FBS) from Cansera (Rexdale, Ontario, Canada), trypsin-ethylenediaminetetraacetic acid from Amresco (Solon, Ohio, USA), nonessential amino acids from Sigma-Aldrich (St. Louis, MO, USA), penicillin and streptomycin were from HyClone (Logan, UT, USA). All other chemical and reagents were of analytical or HPLC grade as appropriate.

Phosphate buffer saline, pH 7.4 contained sodium chloride (8.0 g l^{-1}), potassium chloride (0.2 g l^{-1}), disodium hydrogen phosphate (1.15 g l^{-1}) and potassium dihydrogen phosphate (0.2 g l^{-1}). The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Millipore, Bedford, MA, USA). Transport buffer refers to 9.80 g of Hank's balanced Salt Solution (HBSS), 0.37 g of sodium bicarbonate, 3.50 g of glucose, 5.69 g of *N*-[2-monohydroxyethyl] piperazine-N9-[4-butanesulfonic acid] (HEPES), 1.16 g of sodium chloride and made up to 1 l with Milli-Q water and pH was adjusted to 7.4 using 1 M NaOH or 1 M HCl. Transport buffer was sterilized by filtering through a 0.2 μm filter.

2.2. Cell-culture

The Caco-2 cells (HTB-37) originating from a human colorectal carcinoma was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage number 17. Cells were cultured in DMEM supplemented with 20% FBS, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 100 U ml^{-1} penicillin and streptomycin. Cells were maintained by serial passaging in T-75 tissue culture flasks (Nalge Nunc Int., NY, USA) when reached 70–80% confluence at a split ratio of 1–5 to 1–7. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C and given fresh medium every 3–4 days. Viable cells were counted using the trypan blue exclusion method. The cells were negative for mycoplasma infection.

For transport studies, cells were seeded in 6-well plates at a density of 3.0×10^5 cells/insert in 3.0-μm pore-size 25 mm i.d. polycarbonate tissue culture inserts (Nalge Nunc Int., NY, USA). The culture medium (1.5 ml in the insert and 2.6 ml in the well) was replaced every 3 days for first 7 days and every 2 days thereafter. Cells were used for transport experiments at passage 21–27 at 21–25 days after seeding. The trans-epithelial electric resistance (TEER), expressed in Ω cm^2, was measured using a Milli-cell-ERS apparatus (Millipore, Bedford, MA, USA) at room temperature. In addition to routine TEER measurements, the paracellular transport marker lucifer yellow was used to confirm the integrity of Caco-2 monolayers. Lucifer yellow was quantified by spectrofluorimetry at excitation and emission wavelengths of 485 nm and 530 nm, respectively, using Varioskan Instrument (Thermo Electron Corp, Waltham, MA, USA) controlled by Skan-It software version 2.2.1. The monolayers used for the transport experiments had TEER values greater than 400 Ω cm^2 and the leakage rate of lucifer yellow was less than 1% per hour.

2.3. Bi-directional transport and inhibition studies

On the day of experiment before performing the transport experiments, the cells monolayers were washed twice with phosphate buffer saline pH 7.4 to remove traces of culture media. After washing, the plates were incubated at 37 °C for 30 min and TEER was measured. TEER values measured for the inserts with cells were corrected by subtracting the values with bare filter inserts. Transport buffer on both sides was then removed gently by aspiration. For the measurement of apical-to-basolateral transport (A–B), 1.5 ml of transport buffer containing drug solution (topotecan, atenolol, propranolol, antipyrine and furosemide) was added to the apical side and 2.6 ml of blank transport medium to basolateral compartment. For the investigation of basolateral-to-apical transport, drug was placed in the basolateral side and blank media on the other side. Drug containing compartment is referred to as donor compartment whereas samples with-

drawing compartment is the receptor/acceptor compartment. Samples of 200 μl were withdrawn from acceptor compartment at respective time points of 30, 60, 90 and 120 min. The volume drawn was replenished with the blank transport buffer every time. For inhibition studies, verapamil at 200 μM was added to the transport buffer in both apical and basolateral sides. Vehicle was used for the control inserts [18,19].

Experiment was performed in shaker incubator at 37 °C and 50–60 rpm. The samples collected from each time point were stored at −20 °C until determined by HPLC with UV detection. All experiments were conducted in triplicates.

2.4. HPLC method development

2.4.1. Instrumentation

The liquid chromatographic system LC-2010C_{HT} series (Shimadzu, Nakagyo-ku, Kyoto, Japan) consisting of gradient flow Control Pump, on-line Solvent Degasser, Autosampler, Diode Array Detector and Column Oven. All the parameters of HPLC were controlled by LC solutions software version 1.21 SP1.

2.4.2. Liquid chromatographic conditions

Chromatographic separations were achieved using YMC, C-18, ODS-A RP column (250 mm × 4.6 mm, 4 μm) stainless steel column, which was maintained at 35 °C. The samples of 50 μl were injected into HPLC system.

The mobile phase consisted of 100% acetonitrile (mobile phase A) and Milli-Q water, adjusted to pH 3.0 with 20% o-phosphoric acid (mobile phase B) at a flow rate of 1.0 ml min^{-1}. The column were initially equilibrated at 98% mobile phase B. After injection, the concentration of mobile phase B was reduced to 70% over 8 min, then further decreased to 55% in 14 min and finally to 40% in 16 min. After 16 min, the system was returned to original conditions (mobile phase B at 98%) and equilibrated for 4 min before the next injection. The mobile phase was filtered through 0.45 μm filter (Sartorius, Germany) and deaerated for 15 min by sonication.

2.5. Validation procedures

2.5.1. Preparation of stock and working standard solutions

Primary standard stock solutions for atenolol, propranolol, antipyrine and furosemide were prepared separately in DMSO at a concentration of 50 mM. These solutions were further diluted in blank transport buffer to obtain mixed working standard solutions of 2–100 μM. Topotecan was prepared in DMSO at 25 mM concentration and further diluted to obtain the working standards in the range of 1–50 μM.

2.5.2. System suitability test

Before proceeding for the system suitability evaluation, the column was equilibrated sufficiently to get a stable baseline. System suitability solution containing the analytes was injected and chromatograms were recorded.

2.5.3. Method validation

The chromatographic method was further validated for specificity, linearity, sensitivity, selectivity, precision and accuracy. All validation runs were performed in triplicates on three consecutive days to assess inter-day and intra-day variation. Six-point calibration curves were constructed for all drugs over the selected concentration range.

Blank Caco-2 monolayer samples ($n = 5$) were injected for determination of specificity. Precision was measured at three concentrations, i.e. low, medium and high using three determinations per concentration. Precision is further subdivided into intra-day and inter-day precision, which measures precision within a single day and on two different days. Limit of detection (LOD) and limit of quantitation (LOQ) was also determined, taking into consideration of a signal-to-baseline noise ratio of 3 and 10, respectively. Quality control (QC) samples for each analyte were prepared in triplicates, at three different concentrations, viz. low (LQC), medium (MQC) and high (HQC), from weighing independent of those used for preparing calibration curves. The assay for selectivity was done by examining, if any interference is there in presence of various permeability markers and P-gp modulators.

2.5.4. Short-term room temperature stability

Stability experiments were done under conditions, simulating the actual conditions during study sample analysis. Six aliquots of each of the low and high concentrations (LQC and HQC) was kept at room temperature for 12 h and analyzed.

2.6. Data treatment and statistical analysis

Data from three-four independent experiments are presented as mean ± S.D. Results of bi-directional transport and inhibition studies are expressed as permeability coefficient (nm s^{-1}), which was calculated using the equation:

$$P_{app} = \frac{dQ}{dT} \times C \times A$$

where, dQ/dT is the rate of appearance of compound in the receptor chamber in nmoles. s^{-1}, C is the substrate concentration in the donor chamber in micromolar and A is the cross-sectional area of the cell monolyer in cm^2. Student's unpaired *t*-test with $P < 0.05$ was regarded as significant. Statistical significance was assessed by GraphPad prism software version 4.0.

3. Results and discussion

The optimum wavelength for detecting all the analytes with adequate sensitivity was ascertained and found to be 220 nm. The retention times of atenolol, topotecan (carboxylate and lactone forms), antipyrine, propranolol and furosemide were 6.8, 8.5, 9.1, 11.7, 12.2 and 17.4 min, respectively. The tailing factors of all the peaks were found to be not more than 2.0.

Table 1
Regression analysis[a] of calibration curves for all analytes in the specified concentration range

Analyte	Range (μM)	Slope[b] ($m \pm$ S.D)	Intercept[b] ($c \pm$ S.D.)	Correlation coefficient[c] (r)
Topotecan	1–50	157929 ± 3763.41	−14750.13 ± 2620.56	0.9997
Atenolol	2–100	25872.71 ± 402.08	6511.22 ± 906.57	0.9999
Antipyrine	2–100	27565.13 ± 896.41	10981.99 ± 1500.56	0.9989
Propranolol	2–100	99389.37 ± 1024.48	77211.32 ± 6165.22	0.9980
Furosemide	2–100	82781.21 ± 477.69	34807.67 ± 6953.07	0.9996

[a] Linear regression analysis with a regression equation of $y = mx \pm c$, in which x is the concentration in μM and y is the peak area.
[b] Values are mean ± S.D. of three calibrations.
[c] r is the correlation coefficient obtained from six-point calibration curve. The concentrations across the range were evenly distributed.

3.1. Specificity

Injection of blank transport buffer onto HPLC column showed no peak on the chromatogram. Fig. 1 shows the representative chromatogram of a sample containing atenolol, topotecan (lactone and carboxylate forms), antipyrine, propranolol and furosemide. The retention time of verapamil, a known P-gp inhibitor, likely to be used during the study was also measured by injecting drug solution onto the HPLC column. No interfering peaks of endogenous substances at the retention times of analytes and P-gp inhibitor verapamil, indicated specificity of the method.

3.2. Range and linearity

Six-point calibration curves were constructed for all the analytes for three consecutive days at specified concentrations. The concentration range was selected on the basis of anticipated drug concentrations in the permeability studies. The linearity of the assay procedure was determined by calculation of regression line using the method of unweighed least square analysis. Samples were quantified from the resulting concentration–peak area relationships from regression equation of the calibration curve. The regression parameters are listed in Table 1.

3.3. Accuracy and precision

The percent recovery of method was found to be 100 ± 10% and the coefficient of variation (C.V) for precision determined at each concentration level did not exceed 5%, indicating method is accurate and precise. The values are shown in Table 2.

3.4. Sensitivity and selectivity

Caffeine, colchicine, digoxin, doxorubicin, hydrochlorothiazide, phenyl alanine, quinidine, rhodamine, tamoxifen, verapamil and vinblasine had run time of 9.4, 14.1, 15.6, 12.0, 10.9, 6.6, 9.5, 12.4, 18.9, 13.3 and 11.2 min, respec-

Table 2
Accuracy and precision data (interday and intra-day) of chromatographic method for analysis of topotecan, atenolol, propranolol, antipyrine and furosemide in transport buffer (pH 7.4)

Analyte	Theoretical concentration[a] (μM)	Intraday			Interday		
		Measured concentration (μM)	% Recovery	C.V	Measured concentration (μM)	% Recovery	C.V.
Topotecan[b]	40	41.23	97.02	1.28	40.57	98.60	0.64
	15	14.87	100.87	4.78	15.49	96.84	2.54
	2	2.15	93.02	3.58	1.96	102.04	3.22
Atenolol	75	74.86	100.19	0.98	76.89	97.54	4.09
	25	26.91	92.90	2.45	25.41	98.39	3.25
	5	5.46	91.58	2.93	5.23	95.60	3.47
Antipyrine	75	75.37	99.51	0.76	75.64	99.15	0.97
	25	25.81	96.86	0.58	25.63	97.54	1.29
	5	4.98	100.40	0.97	5.14	97.28	1.87
Propranolol	75	76.48	98.06	4.97	74.53	100.63	0.93
	25	25.48	98.12	2.59	25.69	97.31	1.67
	5	4.76	105.04	1.46	5.18	96.53	0.94
Furosemide	75	75.47	99.38	3.43	74.86	100.19	2.36
	25	24.79	100.85	4.22	26.42	94.63	3.76
	5	5.12	97.66	1.19	4.94	101.21	2.48

Intraday and interday accuracy and precision was determined with triplicates for each concentration. C.V. (coefficient of variation) = (S.D./mean) × 100.
[a] Selected concentrations represent the low, medium and high (LQC, MQC and HQC, respectively) concentration lying within standard curve.
[b] Topotecan was calculated as the total of the lactone plus carboxylate forms in transport buffer, pH 7.4.

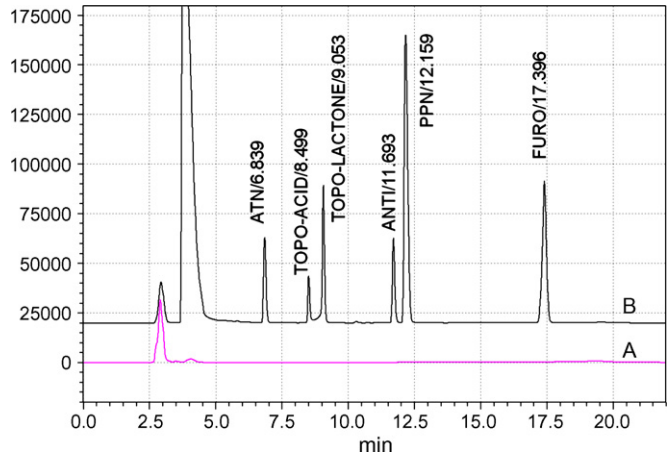

Fig. 1. Representative stacked chromatograms of (A) blank Caco-2 permeability sample (B) simultaneous HPLC separation of standards spiked in blank Caco-2 permeability sample containing atenolol (ATN), topotecan as ring-opened carboxylate form (TOPO-ACID), topotecan as lactone form (TOPO-LACTONE), antipyrine (ANTI), propranolol (PPN) and furosemide (FURO). The peaks are annotated with their names and respective retention times. Chromatographic conditions are mentioned in Section 2.4.

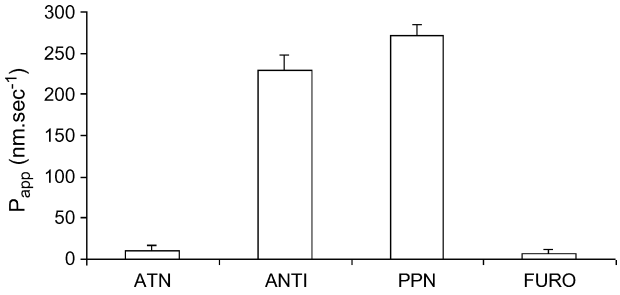

Fig. 2. Absorptive permeability coefficients of permeability markers, atenolol (ATN), antipyrine (ANTI), propranolol (PPN) and furosemide (FURO) determined at 100 μM concentration, in the Caco-2 cell monolayer assay. Atenolol and furosemide belong to low permeability class whereas anipyrine and propranolol have high permeability. Values are mean of three measurements ± S.D.; Assay conditions are mentioned in Section 2.3.

tively. Cyclosporine A, docetaxel, paclitaxel and testosterone did not elute in the due chromatographic run of 22 min. The limit of quantification (LOQ) was defined as the lowest drug concentration that could be determined with acceptable precision (i.e. C.V. ≤ 15%) and accuracy (i.e. recovery 100 ± 5%). The limit of detection (LOD) is defined as the amount, which could be detected with a signal to noise ratio of 3. In the present study, the LOQ for atenolol, and antipyrine was 0.4 μM and for furosemide and propranolol was 0.1 μM. The detection limits were found to be maximum 0.002 μM. LOD and LOQ for both lactone and carboxylate forms was 0.002 and 0.08 μM, respectively.

3.5. Stability

There was no significant decrease of topotecan as total of lactone and carboxylate forms under tested conditions. Percent change between mean concentration of minimum of six determination at each concentration of stability samples against the mean of back calculated value of freshly prepared sample (stability control) at the appropriate concentrations was not more than ±15%.

3.6. Utility of method in determining permeability of standard drugs and P-gp mediated efflux of topotecan in presence of P-gp inhibitor, verapamil

Validated HPLC method was used for Caco-2 monolayer transport assay for permeability determination of topotecan (10 μM) and four model permeability markers (100 μM) with and without verapamil (200 μM) added to both compartments. The drug mixture was added to either apical or basolateral compartments and 200 μl sample were collected from opposite compartment for HPLC analysis.

Fig. 2 shows P_{app} values of atenolol, antipyrine, propranolol and furosemide. Atenolol and furosemide have P_{app} values of <10 nm s^{-1} suggesting to have low permeability, whereas both antipyrine and propranolol are high permeability markers (>200 nm s^{-1}). The study indicated that the transport rate of

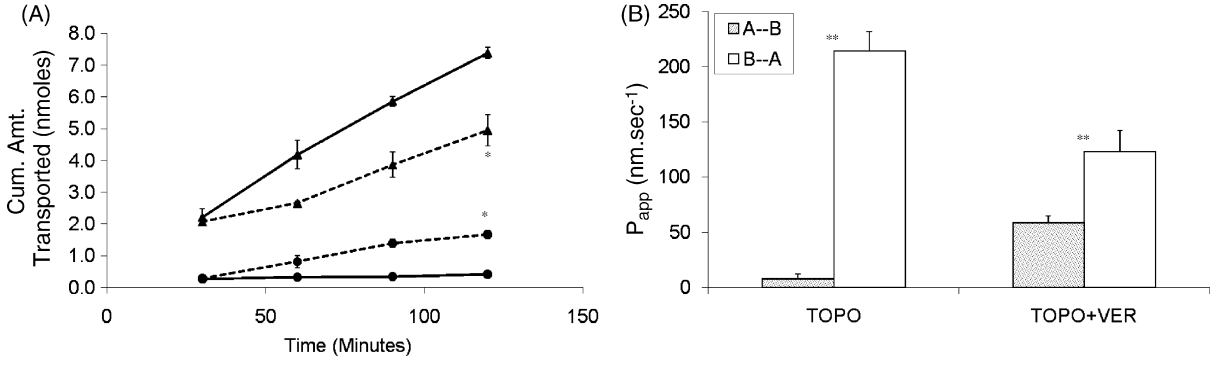

Fig. 3. (A) Absorptive (A–B) and secretory (B–A) transport of topotecan (TOPO), with and without verapamil (VER), in the Caco-2 system as a function of time. Results are expressed as the cumulative amount transported (nmol) ± S.D. of three determinations. (B) Effect of verapamil (200 μM) on permeability (Papp) of topotecan (10 μM) across Caco-2 monolayers from apical-to-basolateral (A–B) and basolateral to apical (B–A) direction. Results are expressed as P_{app} in nm s^{-1} ± S.D. of three determinations. The method allowed elution of verapamil at 13.3 min. The compound mixture was loaded on either apical or basolateral side and incubated at 37 °C. At indicated time, samples were collected from receiving side and determined by HPLC. $^{*}P < 0.05$, $^{**}P < 0.01$; statistically significant difference from control permeability in the presence of P-gp efflux inhibitor, verapamil.

permeability markers from A → B side was similar to that from B → A side. Whereas for topotecan, the transport in B → A direction was much higher (27-fold) than in A → B direction. The P_{app} values for the same are indicated in Fig. 3. This transport was significantly decreased by addition of verapamil (a known P-gp inhibitor) suggesting involvement of P-gp [20]. The cumulative amount transported in secretory direction decreased by 67% by the inhibitory effect of verapamil. The secretory transport of permeability markers was not significantly affected by verapamil, as expected. This is because permeability of these compounds is controlled by passive transcellular diffusion.

4. Conclusions

The reversed phase HPLC method using UV detection was developed and validated for simultaneous determination of topotecan, atenolol, antipyrine, propranolol and furosemide, has acceptable accuracy, precision and linearity. It was used successfully to demonstrate P-gp mediated transport of topotecan and its inhibition using verapamil in Caco-2 cell monolayer. The method has good resolution and allows analysis of four other compounds in a short run time of 22 min. The present assay is simple in terms of utilizing UV detection, mobile phase preparation (without salts, ion-pairing agent) and direct sample injection. The application of method includes its use in routine validation of Caco-2 and determination of P-gp functionality (as substrate and inhibitor).

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