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Short communication

Development and validation of RP-HPLC-UV method for simultaneous determination of buparvaquone, atenolol, propranolol, quinidine and verapamil: A tool for the standardization of rat in situ intestinal permeability studies

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Abstract

A simple, sensitive and specific reversed phase high performance liquid chromatographic (RP-HPLC) method with UV detection at 251 nm was developed for simultaneous quantitation of buparvaquone (BPQ), atenolol, propranolol, quinidine and verapamil. The method was applicable in rat in situ intestinal permeability study to assess intestinal permeability of BPQ, a promising lead compound for Leishmania donovani infections. The method was validated on a C-4 column with mobile phase comprising ammonium acetate buffer (0.02 M, pH 3.5) and acetonitrile in the ratio of 30:70 (v/v) at a flow rate of 1.0 ml/min. The retention times for atenolol, quinidine, propranolol, verapamil and BPQ were 4.30, 5.96, 6.55, 7.98 and 8.54 min, respectively. The calibration curves were linear (correlation coefficient $\geq$0.996) in the selected range of each analyte. The method is specific and sensitive with limit of quantitation of 15 μg/ml for atenolol, 0.8 μg/ml for quinidine, 5 μg/ml for propranolol, 10 μg/ml for verapamil and 200 ng/ml for BPQ. The validated method was found to be accurate and precise in the working calibration range. Stability studies were carried out at different storage conditions and all the analytes were found to be stable. This method is simple, reliable and can be routinely used for accurate permeability characterization.

Keywords: Buparvaquone; Atenolol; Propranolol; Quinidine; Verapamil; In situ intestinal permeability; P-glycoprotein; RP-HPLC

1. Introduction

The high cost of drug development has partially contributed to the high failure rate of drug candidates in clinical trials. The successful development of oral drug delivery formulation requires information on the intestinal permeability and possible role of membrane transporters, mainly P-glycoprotein (P-gp) on the total intestinal transport [1,2].

Several well established methods are available to determine permeability using in vitro and in situ absorption models such as adenocarcinoma cell line derived from human colonic epithelia cell monolayers (Caco-2), Madin-Darby Canine Kidney (MDCK) cells, immobilized artificial membrane (IAM) columns, parallel artificial membrane permeation assay (PAMPA), excised animal tissues in Ussing chambers and single pass intestinal perfusion (SPIP). These models are well correlated to reflect equivalent levels to the in vivo permeability and fraction of dose absorbed in humans. The determination of permeability of poorly soluble compounds is difficult in in vitro models, but it can be overcome by the use of protein (e.g. bovine serum albumin BSA) or by the addition of co-solvents (ethanol, dimethyl sulfoxide, etc.). A suitable cosolvent concentration can minimize the non-specific binding to the surfaces of the devices, intestinal membrane without affecting the integrity of the biological system [3,4].

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The rat in situ intestinal perfusion is a commonly used technique for the assessment of permeability of drugs and new chemical entities [5,6] and the functional role of P-gp on the total intestinal transport [7,8]. In this technique the term in situ refers to the methodology in which the animal's blood supply is kept intact, thus the rate of absorption determined by such method would be more consistent with the in vivo situation when compared to other in vitro techniques. Drug efflux pumps like P-gp and multidrug resistance associated proteins (MRPs) have received considerable attention with recognized functional role in the permeability and overall bioavailability of drugs [9]. In conducting in situ absorption experiments, FDA guidelines suggest the use of low, moderate and high permeable markers to characterize the permeability of drug substances and new chemical entities for classifying them according to the BCS (biopharmaceutical classification system). These markers are used in permeability studies to monitor the integrity and functional status of the intestinal membrane [10].

Buparvaquone (BPQ) is a second-generation hydroxynaphthoquinone and a therapeutic drug for theileriosis. BPQ has been found to be a promising lead compound in several in vitro studies justifying its use in the treatment of Leishmania donovani infections, with ED_{50} values between 0.12 and 0.005 μM. BPQ has very poor aqueous solubility (<0.03 μg/ml) and high lipophilicity. These unsuitable physicochemical properties are the most probable reason for the low in vivo activity of BPQ against leishmaniasis in oral drug delivery. These findings led to the synthesis of a number of water-soluble phosphate prodrugs such as BPQ-3-phosphate and 3-phosphonooxymethyl-BPQ whose aqueous solubility is more than 3.5 mg/ml over the pH range of 3.0–7.4. These prodrugs are sufficiently stable in the gastrointestinal tract before their absorption and rapidly hydrolyzed ($t_{1/2}$ = 1.2 and 3.8 min) to the parent compound in the presence of alkaline phosphatases [11–13].

Currently there are no published analytical methods for the estimation of BPQ in rat perfusates. This study reports a simple, selective and reproducible RP-HPLC-UV method with short run time of 12 min for the simultaneous determination of BPQ in the presence of permeability markers, propranolol (high permeability), atenolol (low permeability) which are FDA suggested markers and routinely used in permeability studies. The method was specific in the presence of P-gp modulators, quinidine (P-gp substrate) [8] and verapamil (P-gp Inhibitor). In addition, this method can be used to determine the concentration of BPQ when it's prodrugs such as BPQ-3-phosphate and 3-phosphonooxymethyl-BPQ are used in permeability studies. The validated HPLC-UV method was developed primarily for the characterization and classification of the permeability of BPQ in rat jejunum absorption model. Chemical structures of all analytes are represented in Fig. 1.

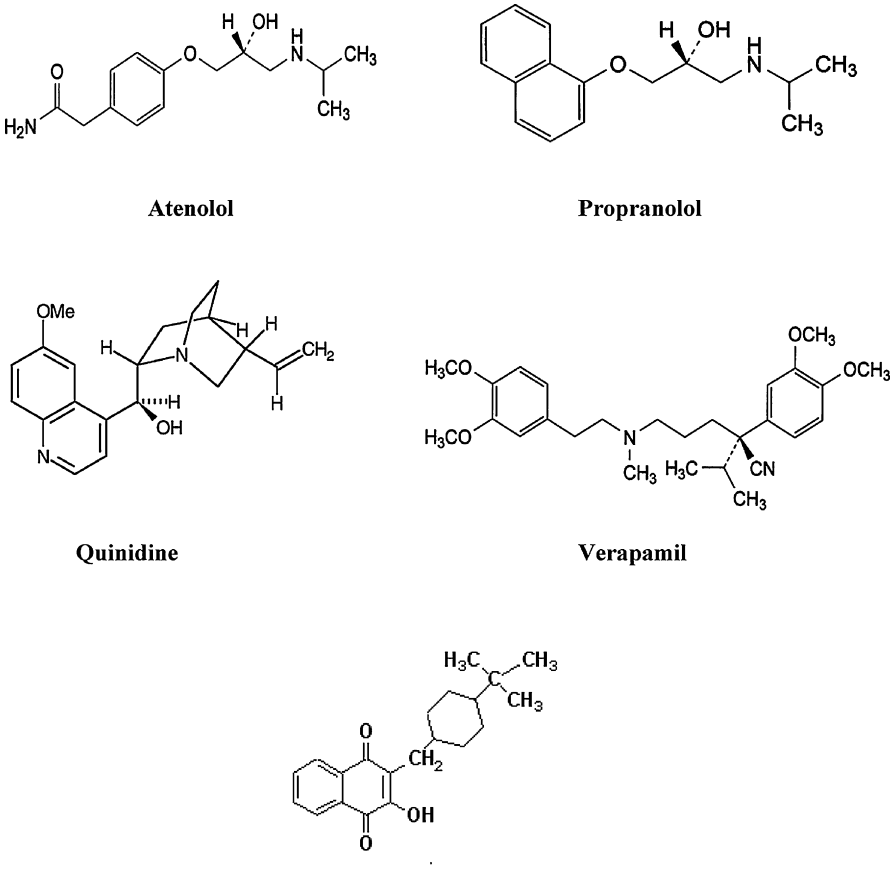

Fig. 1. Chemical structures of atenolol, propranolol, quinidine, verapamil and buparvaquone.

2. Material and methods

Atenolol and propranolol were given by S.M Pharmaceuticals (Sungai Petani, Malaysia). Quinidine and verapamil were purchased from Sigma–Aldrich (St. Louis, MO, USA). Buparvaquone was obtained from Glaxo SmithKline (UK). Acetonitrile and methanol of HPLC grade were obtained from J.T. Baker (Phillipsburg, USA). Ammonium acetate and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from BDH Laboratory (Poole, UK). NaCl and KCl were obtained from Prolabo (Paris, France). Na_2HPO_4 and NaH_2PO_4 were purchased from R & M Chemicals (Essex, UK), D-glucose was obtained from AJAX Chemicals (Perth, Australia) and sodium pentobitone was purchased from CIVA (Paris, France).

2.1. Instrument

The liquid chromatographic system consisted of a Perkin-Elmer PE 200 series pump, PE 200 series autosampler, PE 200 series degasser, PE 200 series UV/Visible detector and PE 200 series column oven (GenTech Scientific, NY, USA). All the parameters of HPLC were controlled by Total chrom software version 6.2.

2.2. Liquid chromatographic conditions

Chromatographic separations were obtained using a stainless steel column, Thermo Hypersil C-4, 5 μm, 250 mm × 4.6 mm (Thermo Hypersil-Keystone, USA) which was maintained at 35 °C. The analytical wavelength was set at 251 nm and samples of 50 μl were injected to HPLC system. The mobile phase was ammonium acetate (0.02 M: pH 3.5 adjusted with glacial acetic acid) and acetonitrile in the ratio of 30:70 (v/v) at a flow rate of 1.0 ml/min. The mobile phase was filtered through 0.45 μm filter (Sartorius, Germany) and degassed for 10 min by sonication.

2.3. Preparation of stock and working standard solutions

Primary standard stock solutions for atenolol, propranolol, quinidine, and verapamil were prepared separately in deionized water (Pure Lab UHQ ELGA, UK) with concentrations of 20, 10, 1, and 5 mg/ml, respectively. These solutions were further diluted with deionized water to obtain mixed working standard solutions of 300–2400, 100–1200, 16–160, and 200–2000 μg/ml for atenolol, propranolol, quinidine and verapamil, respectively. BPQ was prepared in DMSO with a concentration of 0.5 mg/ml and further diluted to obtain the working standards in the range of 4–30 μg/ml. Preliminary studies showed that all analytes were stable in solution.

2.4. Method validation

The chromatographic method was validated for specificity, linearity, precision and accuracy, recovery. The validation parameters were studied with rat blank perfusion solution. Calibration and quality control samples were prepared by spiking with an aliquot of 50 μl mixed working standard solution and 50 μl of BPQ working standard solution in blank perfusion solution. All samples were vortexed for 2 min and centrifuged at 12,000 rpm for 10 min. The clear supernatant was transferred into HPLC vials. Due to the simplicity of the procedure, no internal standard was used. Assay precision and accuracy was assessed by the calculation of inter-day and intra-day variability of quality control samples. Inter-day data was obtained by analysing the quality control samples on three consecutive days of assay, while intra-day precision data was obtained by analysing three sets of quality control samples in 1 day. The percentage recovery for all analytes were determined by comparing the mean area of analytes spiked in blank perfusate samples with the mean area of the drug standards prepared in perfusion buffer pH 7.2 (the perfusion solution consisted of 48 mM NaCl, 5.4 mM KCl, 2.8 mM Na_2HPO_4, 4 mM NaH_2PO_4 and 1 g/l D-glucose dissolved in deionized water). Stability experiments were performed under different conditions by simulating the same conditions occurring during study sample analysis. Experiments were performed to determine room temperature stability (after 6 h), freeze thaw stability (three cycles) and short-term stability at −20 °C for 30 days.

3. Results and discussion

3.1. Method development

In order to develop and validate a simple and sensitive RP-HPLC method that is suitable for permeability studies of BPQ in rat in situ absorption model, the following parameters were optimized. Since a fixed wavelength UV detector was used, the optimum wavelength for detecting all the analytes with adequate sensitivity was ascertained and found to be 251 nm. Buffers with different pH (0.02 M-ammonium acetate buffer pH at 3.0, 3.5, 4.0, 5.0 and 7.0) and acetonitrile were selected for the initial separation studies. At pH 3.0 the resolution between quinidine and propranolol was found to be poor but the other analytes were well separated with good peak shapes. At pH 4.0, 5.0 and 7.0 tailing of the BPQ peak occurred and poor resolution was observed between quinidine and propranolol. The best peak shapes and resolution were obtained with 0.02 M ammonium acetate pH 3.5.

Optimization of composition of the mobile phase for better resolution of all analytes was done with 0.02 M ammonium acetate (pH 3.5) and acetonitrile. Experiments were carried out with 60, 65, 70 and 75% of acetonitrile in the mobile phase. The best peak shape and maximum separation was achieved with mobile phase composition of 30:70 (v/v). The best separation, peak symmetry and reproducibility were obtained on C4 Hypersil column. When experiments were performed with C18 and C8 columns all the analytes were eluted at early retention except for BPQ due to the presence of high organic content in the optimized mobile phase. When experiments were performed with methanol instead of acetonitrile as the organic solvent in the mobile phase, quinidine, propranolol and verapamil were unresolved and reten-

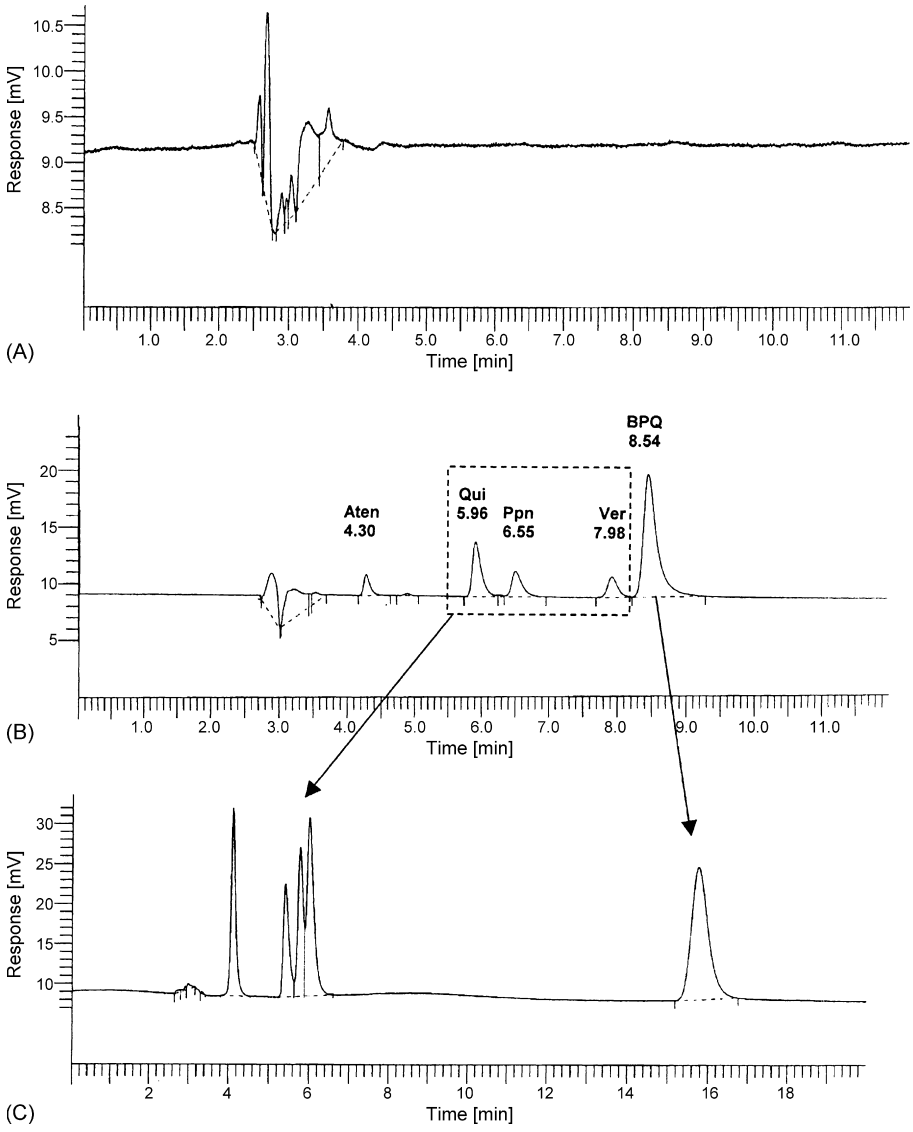

Fig. 2. Representative chromatograms of (A) blank perfusion sample (B) standards spike in blank perfusion sample, the peaks of interest are atenolol (Aten), quinidine (Quin), propranolol (Ppn), verapamil (Ver) and BPQ. (C) Methanol as organic solvent in the mobile phase.

tion time of BPQ increased from 8.5 to 15.7 min (Fig. 2C). Peak tailing was observed for verapamil and BPQ when the flow rate was 0.9 ml/min using optimized mobile phase conditions. However, a flow rate of 1.0 ml/min yielded optimum separation. The effect of column temperature was studied at room temperature and elevated temperatures. The column temperature was optimized at 35 °C, which gave the best resolution. With optimized chromatographic condition, the average retention times were 4.30, 5.96, 6.55, 7.98 and 8.54 min, for atenolol, quinidine, propranolol, verapamil and BPQ, respectively (Fig. 2B).

The method was also developed for non-absorbable marker phenol red, which is usually used to correct the net water flux (NWF) in the rat in situ experiment. The retention time of phenol red was 2.35 min by using the optimized chromatographic condition. When the method was checked for specificity there was an interfering peak from an endogenous compound observed at the retention time of phenol red in a few rat perfusates. NWF can also be obtained by using a gravimetric method instead of using phenol red and radiolabelled poly ethylene glycol (PEG) 4000 methods [14–17].

3.2. Specificity

Specificity experiment was carried out using five different blank rat perfusion samples. Chromatogram obtained from blank perfusion sample is represented in Fig. 2A. There were no interfering peaks of endogenous compounds observed at the retention time of the analytes.

3.3. Linearity

A six-point linearity curve was constructed for three consecutive days for each analyte. Samples were quantified using the concentration–peak area relationships and were calculated by the simple regression analysis $y = mx + c$. The minimum correlation coefficient of the calibration curves for five analytes

Table 1
Linearity parameters for BPQ and permeability markers

Analyte	Range (μg/ml)	Slope[a]	Intercept[a]	Correlation coefficient[a]
Atenolol	15–120	2503.0 ± 28.05	11905.0 ± 1257.09	0.9971
Propranolol	5.0–60	6913.3 ± 94.24	2263.67 ± 1439.00	0.9990
Quinidine	0.8–8.0	26873.0 ± 699.17	4158.13 ± 866.96	0.9977
Verapamil	10–100	4989.5 ± 10.00	7406 ± 439.15	0.9999
BPQ	0.2–1.2	50.44 ± 11.78	1180 ± 1273.03	0.9964

[a] Values are mean ± S.D. of three calibrations.

was more than 0.996 and regression parameters are listed in Table 1.

3.4. Accuracy and precision

The results are given in Table 2. Both inter-day and intra-day accuracy given by relative error (%RE) of quality control samples were ≤6.20% while inter-day and intra-day precision given by relative standard deviation (%R.S.D.) of quality control samples were ≤9.33% for each analyte.

3.5. Recovery

The percentage recoveries of all the analytes were carried out for the low, medium and high quality control samples. The overall mean recoveries calculated for atenolol, quinidine, propranolol, verapamil and BPQ were 96.82, 97.81, 97.93, 100.19 and 93.01%, respectively.

3.6. Stability

Stability studies were investigated at low and high quality control levels. All analytes were found to be stable in blank perfusion sample for 6 h at room temperature and at repeated freeze and thaw conditions (three cycles). The samples were stored at −20 °C for short-term stability experiment. The results summarized in Table 3 show that all the analytes were stable for 30 days.

3.7. Application of method

In situ permeability studies were performed using established methods adopted from literature [8,17]. The validated analytical method was applied for rat in situ intestinal perfusion study to assess intestinal permeability of buparvaquone in the presence of the permeability markers atenolol and propranolol. This study was carried out in male Sprague–Dawley rats ($n = 6$). Prior to the surgical procedure, the rats were fasted overnight (16–20 h) with water ad libitum. The rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.; Nembutal®, CIVA, France). The intestine of the rats was exposed by a midline abdominal incision using an electrical cautery knife (Rimmer Brothers, London, UK) and a 12–15 cm segment of the proximal rat jejunum was located, gently rinsed with phosphate buffer saline and cannulated with polyethylene tube (PE 100, Protex, Kent, UK) and finally coupled with a automated perfusion assembly (Perfusor Secura, B. Braun, Germany). Perfusion buffer pH 7.2 was infused for 10 min by a syringe pump followed

Table 2
Intra- and inter-day precision and accuracy of atenolol, propranolol, quinidine, verapamil and BPQ

Analyte	Spiked concentration (μg/ml)	Intra-day[a]			Inter-day[b]		
		Mean calculated concentration (μg/ml)	%RE	%R.S.D.	Mean calculated concentration (μg/ml)	%RE	%R.S.D.
Atenolol	50	50.74	1.47	4.25	51.71	3.41	3.10
	75	74.81	−0.26	2.20	76.27	1.70	3.09
	100	101.50	1.50	2.18	99.99	−0.01	3.10
Propranolol	15	15.03	0.20	3.78	14.78	−1.47	3.31
	25	25.46	1.83	0.75	25.06	0.24	1.31
	50	51.47	2.94	0.68	51.05	2.11	1.53
Quinidine	2	1.99	−0.50	4.93	1.93	−3.50	0.89
	5	4.77	−4.60	1.09	4.76	−4.80	1.48
	7	6.78	−3.14	1.19	6.80	−2.85	1.29
Verapamil	30	30.24	0.79	0.26	29.88	−0.39	2.62
	50	50.26	0.52	0.58	49.19	−1.62	2.31
	70	70.01	0.01	0.53	69.78	−0.31	1.50
BPQ	0.3	0.30	1.66	4.22	0.30	0.99	3.46
	0.5	0.46	−6.20	1.06	0.47	−5.80	2.55
	0.9	0.87	−2.66	3.43	0.91	1.77	9.33

[a] Intra-day accuracy and precision was determined with five replicates for each concentration.
[b] Inter-day accuracy and precision was determined with 15 replicates (day 1, $n = 5$; day 2, $n = 5$; day 3, $n = 5$) for each concentration.

Table 3
Stability studies for BPQ and permeability markers

	Spiked concentration (μg/ml)	Bench top[a]		Freeze and thaw[b]		Short term[c]	
		Mean (±S.D) obtained concentration (μg/ml)[d]	%R.E	Mean (±S.D) obtained concentration (μg/ml)[d]	%R.E	Mean (±S.D) obtained concentration (μg/ml)[d]	%R.E
Atenolol	50	50.96 ± 0.90	1.91	52.20 ± 0.80	4.40	52.21 ± 0.69	4.41
	100	99.85 ± 2.07	−0.15	97.69 ± 2.78	−2.31	98.72 ± 1.85	1.28
Propranolol	15	14.67 ± 0.55	−2.19	15.16 ± 0.44	1.10	15.49 ± 0.45	3.28
	50	49.09 ± 0.32	−1.82	49.23 ± 0.56	−1.53	48.88 ± 0.35	−2.24
Quinidine	2	1.90 ± 0.04	−5.00	1.94 ± 0.02	−3.00	1.84 ± 0.02	−8.00
	7	6.73 ± 0.03	−3.86	6.87 ± 0.02	−1.86	6.48 ± 0.03	−7.43
Verapamil	30	29.71 ± 0.25	−0.97	30.06 ± 0.20	0.20	29.40 ± 0.37	−2.00
	70	69.54 ± 2.0	−0.66	70.86 ± 0.17	1.23	67.23 ± 1.61	−3.96
BPQ	0.3	0.29 ± 8.84	−0.87	0.28 ± 32.03	−5.31	0.29 ± 15.04	−1.33
	0.9	0.86 ± 16.03	−3.76	0.82 ± 23.71	−8.09	0.89 ± 77.52	−0.18

[a] After 6 h at room temperature.
[b] After three freeze and thaw cycles at −20 °C.
[c] At −20 °C for 30 days.
[d] Values are mean ± S.D. for five replicates.

by 1.2 μg/ml of BPQ, 106.52 μg/ml of atenolol and 29.58 μg/ml of propranolol at a flow rate of 0.2 ml/min. The perfusate was collected at every 5 min intervals for duration of 90 min. All animal study protocols were carried out in accordance with the international guidelines adopted by the Animal Ethics Committee of Universiti Sains Malaysia, who also approved the study protocol. The outlet samples were processed and analysed for BPQ, atenolol and propranolol. At the end of the experiment the animal was euthanized by a single intravenous lethal bolus dose of anesthetics (pentobarbitone sodium) and length of the intestinal segment was measured. The permeability coefficient (P_{eff}) of buparvaquone and permeability markers were calculated after correcting the outlet concentration for water flux on the basis of the ratio of volume of perfusion solution collected and infused for each sampling point by using Eq. (1). Samples were kept frozen at −20 °C until analysed.

$$P_{eff} = \frac{Q\,[(C_{in}/C_{out}) - 1]}{2\pi r L} \quad (1)$$

where Q is the flow rate (0.2 ml min^{-1}), C_{in} and C_{out} the respective inlet and outlet concentrations, r the radius of intestine (0.21 cm) and L is the length of the intestine measured after completion of perfusion. The permeability coefficient of BPQ, atenolol and propranolol were found to be 0.912×10^{-4}, 0.126×10^{-4} and 0.716×10^{-4} cm/s, respectively.

4. Conclusion

The RP-HPLC method developed and validated for the simultaneous determination of buparvaquone, atenolol, propranolol, quinidine and verapamil has acceptable precision, accuracy and linearity with short run time for rat in situ intestinal permeability studies as well as for studies on the role of P-gp carrier efflux activity in total intestinal transport. This technique eliminated endogenous interferences from the biological matrix and permitted analysis involving easy sample preparation. Since BPQ-3-phosphate and 3-phosphonooxymethyl-BPQ prodrugs are rapidly hydrolyzed to BPQ in the presence of alkaline phosphatases, the method can be used to determine BPQ when the prodrugs are used in permeability studies.

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