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Journal of Chromatography B, 813 (2004) 347–352
JOURNAL OF CHROMATOGRAPHY B
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Short communication

# Simultaneous determination of digoxin and permeability markers in rat in situ intestinal perfusion samples by RP-HPLC

Manthena V.S. Varma, Namita Kapoor, Mahua Sarkar, Ramesh Panchagnula*

*Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S Nagar, Punjab 160062, India*

Received 31 July 2004; accepted 23 September 2004
Available online 27 October 2004

**Abstract**

A simple, sensitive and specific reversed-phase high performance liquid chromatographic (RP-HPLC) assay for simultaneous determination of digoxin and permeability markers, in samples obtained from intestinal in situ single-pass perfusion studies, was developed and validated. Chromatography was carried on C-18 column with mobile phase comprising of acetate buffer (pH 3.0), acetonitrile and methanol in the ratio of 50:25:25 (v/v/v), was pumped at a flow rate of 0.5 ml/min and UV detection was employed at 220 nm. The average retention times for phenolred, propranolol, frusemide and digoxin were 9.1, 10.7, 12.9 and 15.3 min, respectively. The calibration curves were linear ($R^2 > 0.998$) in the range for each analyte. The method is specific and sensitive with limit of quantification of 25 ng/ml for digoxin and frusemide and 10 ng/ml for phenolred and propranolol. The method is accurate and precise with recoveries of digoxin in the range of 95.2 and 103.2% and relative standard deviation (R.S.D.) <5%. We found that this method was simple and reliable in permeability determination and to estimate the contribution of P-glycoprotein in limiting intestinal absorption.


*Keywords:* Digoxin; Permeability markers; Rat in situ permeability studies; P-glycoprotein; RP-HPLC

## 1. Introduction

Permeability is an important, but still unpredictable determinant of absorption and it is informative to explore mechanisms contributing to permeability given the interest in development of structure-based computational models of this property. More recently, the role of efflux transporters in determining the permeability and overall bioavailability of drugs has gained considerable attention [1]. P-glycoprotein (P-gp), an energy dependent transmembrane drug efflux pump, is localized in wide range of tissues including enterocytes of GI tract [2]. An increasing number of drugs, including HIV protease inhibitors like indinavir, ritonavir, saquinavir and anti-cancer drugs like paclitaxel, docetaxel, etc. have been reported to be substrates for P-gp [3].

Digoxin is a P-gp substrate which has been used as a probe to (i) elucidate the quantitative contribution of P-gp-mediated efflux to intestinal absorption of drugs [4]; (ii) regional variability of P-gp expression [5]; and (iii) P-gp kinetic profiling [6]. Further digoxin is being increasingly used for understanding mechanistic aspects of intestinal P-gp.

In situ perfusion of intestinal segments of rodents (mice, rats and rabbits) is frequently used to study the permeability and absorption kinetics of drugs [7,8]. In vivo studies permit determination of absolute or relative bioavailability, but are also complex in terms of plasma assay and assessing rate-limiting process in drug absorption. Meaningful feedback to the drug discovery efforts generally requires a balance between the higher throughput of in vitro or in vivo studies. In vitro or in situ models are of particular utility when absorption is rate-limiting to systemic availability and permeability is rate-limiting to absorption. However, the main advantage of the in situ system compared to the other in vitro techniques is the presence of an intact blood and nerve supply in the ex-

* Corresponding author. Tel.: +91 172 2214 682/687; fax: +91 172 2214 692.
*E-mail address:* panchagnula@yahoo.com (R. Panchagnula).

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doi:10.1016/j.jchromb.2004.09.047

perimental animals. This methodology is found to be simple and highly accurate for predicting the intestinal absorption in humans [9]. The FDA guideline suggested internal standards and markers to characterize the permeability of drug substances for classifying them into biopharmaceutical classification system (BCS). Further these markers are useful in monitoring the integrity and functional status of the intestinal membrane and thus can be routinely used for accurate permeability characterization [10,11].

We present a simple and reliable RP-HPLC method for routine analysis of digoxin from in situ permeability studies in rats, in the presence of high and low permeability markers. This method utilizes isocratic-mode with UV detection, which is more feasible and less time-consuming than any other method of detection. Although a number of methods are available for quantification of digoxin in various biological matrices [12–15], the uniqueness of the present method is the quantification of digoxin in the presence of phenolred, frusemide and propranolol, which are FDA suggested markers routinely used in permeability studies [11]. Phenolred (integrity marker) is transported across the intestine through paracellular pathway, while frusemide (low permeable) and propranolol (high permeable) are transcellularly transported, thus these markers indicate the integrity of membrane and the tight junctions and also provide basis for permeability characterization. Further this method is specific in the presence of various P-gp modulators and thus facilitates studying various aspects of P-gp-mediated efflux, taking digoxin as the P-gp probe. Hence, the proposed method was further validated for its application in intestinal permeability studies.

## 2. Materials and methods

### 2.1. Chemicals

Digoxin, frusemide, phenolred and propranolol were kindly provided by Burroughs Wellcome Ltd., India; Matrix Labs., India; HiMedia Lab., India; and Baroda Pharma Limited, India, respectively. Quinidine and verapamil HCl were purchased from Sigma–Aldrich Co., USA. All the solvents used were of HPLC grade (J.T. Baker, Mexico) and reagents and chemicals were of analytical grade.

### 2.2. Instrument

For analysis, Waters HPLC system (Milford, MA, USA) consisting of 600 E multisolvent delivery system, 717 plus autosampler and 2487 dual λ absorbance detector was used. MILLENNIUM$^{32}$ software (version 3.05.01) was used for data acquisition and processing. Other instruments include Elgastat (Elga Ltd., Bucks, UK), electronic balance AG 245 (Greifensee, Switzerzerland), Branson 3210 sonicator (The Hague, The Netherlands), Millipore syringe filtration assembly (Bangalore, India), Brand autopipettes from E. Merck (Mumbai, India) and microlitre syringes from Hamilton (Bonaduz, Switzerland).

### 2.3. Methodology

#### 2.3.1. Chromatography

The column used for chromatographic separations was Symmetry C18 WAT054275 (Waters, USA) and guard column of Symmetry C-18 WAT054225 (Waters, USA). Mobile phase was pumped in isocratic-mode at a flow rate of 0.5 ml/min at ambient temperature. The analytical wavelength was set at 220 nm and samples of 20 μl were automatically injected. The chromatographic separations were accomplished using mobile phase, consisting of 10 mM acetate buffer (pH adjusted to 3.0 with glacial acetic acid), methanol and acetonitrile (50:25:25, v/v/v), filtered through 0.45 μm filter (Millipore) and deaerated in ultrasonic bath (Branson 3510).

#### 2.3.2. Preparation of standard solutions

Primary stock solution of digoxin and other markers were prepared in ultra pure water to obtain a concentration of 2 mg/ml. Primary standard solution was diluted to 100 μg/ml that served as the secondary stock, which was further diluted in blank in situ perfusion samples (obtained after perfusion of blank perfusion buffer, see Section 2.5) to obtain working standards in the selected range.

### 2.4. Method validation

The chromatographic method was validated to determine the linearity, sensitivity, precision and accuracy for each analyte [16]. The calibration curves and validation studies were performed with blank perfusion solution collected from in situ single-pass perfusion (see Section 2.5). Interference in the presence of any endogenous constituents of perfusion solution was assessed by analysis of blank perfusion samples. Further retention times of P-gp modulators, quinidine and verapamil were measured. A six-point calibration curve was prepared within each range by spiking blank perfusion solution with appropriate volumes of working standards. Calibration curves for each analyte were generated in two ranges to avoid uneven distribution of concentration in demonstrating the linearity and to improve the accuracy. Quality control standards were run with each batch of samples.

For precision and accuracy, five replicates of blank perfusion solution spiked with all analytes at four different concentration levels were used. Precision was determined as %R.S.D. of the replicate measurements. Limit of quantification (LOQ) was determined by serial dilution of working standards, while the LOD was taken as one-third of LOQ [16].

### 2.5. In situ single-pass intestinal permeability and P-gp inhibition studies

#### 2.5.1. Animals and legal prerequisites

Sprague–Dawley rats (270–350 g) were used to perform in situ single-pass perfusion. Anesthesia, surgical and perfu-

sion procedures were justified in detail and were approved by the Institutional Animal Ethics Committee (IAEC, NIPER). The study complied with local and federal requirements for animal studies.

### 2.5.2. Methodology

The surgical procedure and the in situ single-pass perfusion experiments were performed according to the methods described previously [9,17]. Rats were fasted for 16–18 h, prior to study, with tap water ad libidum. After anesthesia via intraperitoneal administration of thiopental sodium (50 mg/kg), rats were placed on a heating pad to maintain body temperature at 37 °C. The intestine of the rats was exposed by a midline abdominal incision and a 12–15 cm ileum segment (5 cm above the ileoceceal junction) was isolated and cannulated at both ends with glass tubing. The segment was rinsed with phosphate buffer saline (10 ml) and the perfusion solution maintained at 37 °C was pumped at a flow rate of 0.1 ml/min using syringe pump (Harvard Apparatus PHD 2000 pump, MA, USA). The perfusion solution (pH 7.4) consisted of NaCl 48 mM, KCl 5.4 mM, $Na_2HPO_4$ 2.8 mM, $NaH_2PO_4$ 4 mM and D-glucose 1 g/l; and contained drugs with and without quinidine (200 μM) as P-gp inhibitor. Final concentrations used in the studies were 50 μM, phenolred; 100 μM, propranolol; 50 μM, frusemide and 20 μM, digoxin.

A two-step perfusion procedure was followed to determine the permeability of compounds with and without P-gp inhibitor [17]. This included sampling every 5 min for a 30 min perfusion period with one perfusion solution after 20 min equilibration, and then switching to second perfusion solution and similarly sampling every 5 min for 30 min perfusion period after 20 min equilibration. Out of four rats ($n = 4$) used for each compound, two rats were first perfused with only test compounds solution and then switched to perfusion solution containing both the test compounds and P-gp inhibitor, while other two rats were first perfused with solution containing both the test compounds and P-gp inhibitor and then switched to perfusion solution containing only compounds. Equilibration of 20 min prior to sampling was found to be sufficient for both washout and to reach an initial steady-state [17]. Water flux was quantified by weight and volume measurements [17,18]. Water flux is the ratio of the volume of perfusion solution exiting from the intestine to the volume of perfusion solution entering the intestine during each sampling period. Volume was determined from weighing of the collected solutions and density measurements [18].

### 2.5.3. Data treatment and statistics

Permeabilities (without and with P-gp inhibitor) are 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 (5 min).

$$P_{\text{eff,control}} \quad \text{(or)} \quad P_{\text{eff,inh}} = \frac{Q\left[\left(\frac{C_{\text{in}}}{C_{\text{out}}}\right) - 1\right]}{2\Pi rl} \qquad (1)$$

where $Q$ is the flow rate, $C_{\text{in}}$ and $C_{\text{out}}$ are the respective inlet and outlet concentration, $r$ is radius of intestine (0.21 cm) and $l$ is length of intestine measured after completion of perfusion [9,19,20].

Values were indicated as mean ± S.D. for permeability in four independent rats. Statistical difference between the permeabilities of the drugs without and with P-gp inhibitor was evaluated by one-way ANOVA (SigmaStat, version 2.03, SPSS Inc., IL, USA) at $P < 0.05$ and $P < 0.01$.

## 3. Results and discussion

### 3.1. Chromatography and specificity

Retention times of phenolred, propranolol, frusemide and digoxin were 9.1, 10.7, 12.9 and 15.3 min, respectively (Fig. 1). No other interfering peaks were observed in the blank perfusion solution and neither of the P-gp modulators, quinidine and verapamil, interfered with the analytes (Fig. 1). The proposed method was specific to all the analytes, especially in the presence of quinidine which was used as P-gp modulator in the present study. Furthermore, the duration of the chromatographic run was 18 min, which permitted the analysis of a large number of samples in a short period of time.

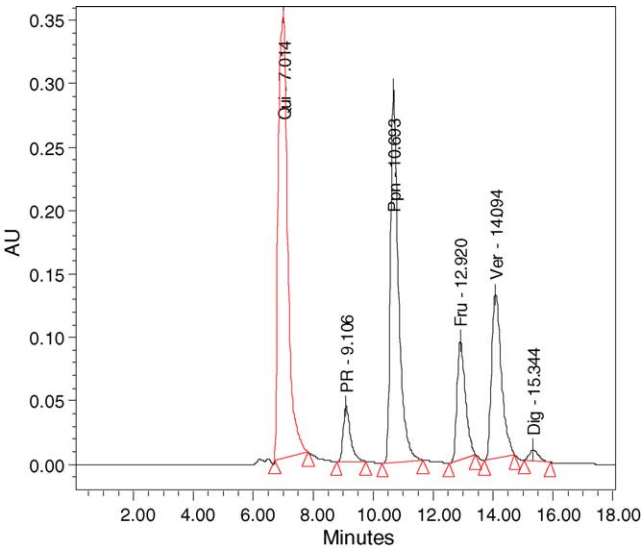


Fig. 1. Representative overlaid chromatograms of blank (dotted line) and in situ perfusion samples containing phenolred (PR), propranolol (Ppn), frusemide (Fru), digoxin (Dig) and P-gp modulators quinidine (Qui) and verapamil (Ver). The peaks are annotated with their respective names and retention times. Chromatograms were obtained from the blank sample and the sample containing Qui 200 μM, PR 50 μM, Ppn 100 μM, Fru 50 μM, Ver 200 μM and Dig 20 μM, collected after in situ perfusion through rat intestine. For chromatographic conditions refer Section 2.3.

Table 1
Regression statistics of digoxin and intestinal permeability markers

| Analyte | Range (μg/ml)[a] | Slope[b] | Intercept[b] | Correlation coefficient[b] |
|---|---|---|---|---|
| Phenolred | 0.025–1 | 289372 (±6273.4) | +18427 (±10632.1) | 0.9990 (±0.0010) |
| | 1–6 | 304415 (±6288.3) | −11264 (±22142.3) | 0.9997 (±0.0007) |
| Propranolol | 0.025–1 | 2082303 (±36146.0) | −159964 (±4623.3) | 0.9983 (±0.0005) |
| | 1–6 | 2372288 (±41445.7) | −19540 (±6652.3) | 0.9987 (±0.0007) |
| Frusemide | 0.05–0.5 | 806294 (±12125.6) | +5534 (±1112.6) | 0.9991 (±0.0011) |
| | 0.5–3 | 889322 (±1936.2) | −37443 (±56326.5) | 0.9987 (±0.0003) |
| Digoxin | 0.05–0.5 | 382825 (±5856.5) | −7531 (±6642.9) | 0.9995 (±0.0003) |
| | 0.5–3 | 409510 (±4190.2) | −42947 (±3129.4) | 0.9999 (±0.0006) |

[a] Six concentrations for each range were evenly distributed.
[b] Values are mean ± S.D. of three calibration curves.

### 3.2. Validation

#### 3.2.1. Range and linearity

The six-point calibration curves that were constructed for each assay batch was linear over the two ranges for each analyte ($R^2 > 0.998$) (Table 1). Two calibration curves for each analyte were generated to cover the whole range avoiding the biasness in the regression analysis. This provided more accurate recoveries at different levels of the ranges.

#### 3.2.2. Accuracy and precision

The data are summarized in Table 2. Both the intra- and inter-day R.S.D. of quality control standards were less than 6% over the selected range (Table 2). Quality control standards prepared in blank perfusion solution are dilutions from weightings independent of those used for preparing calibration curves. Accuracy of digoxin ranged from 98.6 to 106.5%, while for other drugs percent recovery was always between 95.2 and 103.2%. Overall, data showed the reproducibility and the precision of the method for each analyte (Table 2) [21].

#### 3.2.3. Sensitivity

The lower end of the range indicates that the method is quit sensitive even at nanograms concentration (see Table 1). LOD and LOQ decide about the sensitivity of the method. LOD is the lowest detectable concentration of the analyte by the method whereas LOQ is the minimum quantifiable concentration. On serial dilution and analysis by the method, LOQ was found to be 25 ng/ml for digoxin and frusemide and 10 ng/ml for phenolred and propranolol. At these concentrations the mean response (peak area) of corresponding analyte is 10 times more than the standard deviation of six determinations [16]. This data shows the high sensitivity of the method and thus shows the advantage of sensitive method involving no complex sample preparation procedures, over the existing methods.

Table 2
Estimated intra- and inter-day precision and accuracy of the RP-HPLC method[a]

| Analyte | Concentration (μg/ml) | Intra-day ($n = 5$) | | Inter-day ($n = 15$)[b] |
|---|---|---|---|---|
| | | Accuracy (%) | Precision (%R.S.D.) | Precision (%R.S.D.) |
| Phenolred | 0.1 | 95.2 | 4.6 | 5.4 |
| | 0.6 | 97.8 | 4.1 | 2.6 |
| | 2.0 | 102.6 | 1.9 | 2.1 |
| | 4.8 | 98.9 | 2.6 | 1.9 |
| Propranolol | 0.1 | 99.1 | 2.4 | 2.6 |
| | 0.6 | 98.6 | 1.6 | 2.4 |
| | 2.0 | 103.2 | 1.1 | 2.6 |
| | 4.8 | 103.0 | 2.1 | 0.8 |
| Frusemide | 0.05 | 93.2 | 4.5 | 3.8 |
| | 0.3 | 100.2 | 1.1 | 2.3 |
| | 1.0 | 100.1 | 0.5 | 3.2 |
| | 2.4 | 98.2 | 1.4 | 1.5 |
| Digoxin | 0.05 | 106.5 | 3.7 | 4.8 |
| | 0.3 | 101.4 | 2.1 | 3.9 |
| | 1.0 | 98.6 | 0.3 | 2.5 |
| | 2.4 | 99.4 | 0.6 | 2.4 |

[a] Accuracy and precision were determined with quality control samples.
[b] Five replicates each were analyzed on three consequent days.

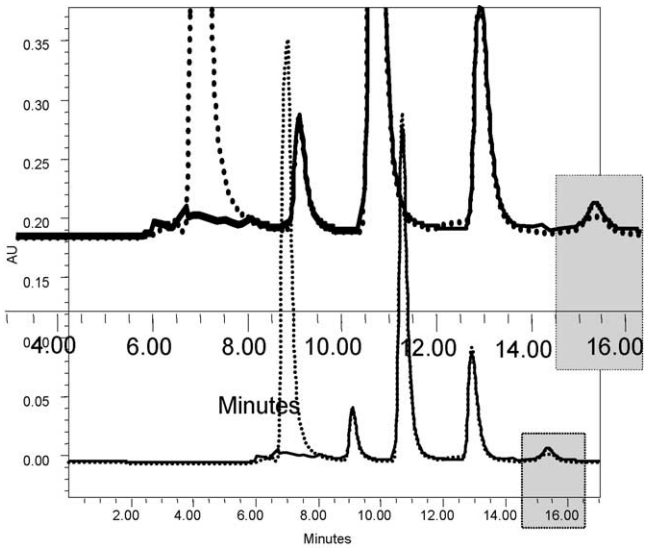


Fig. 2. Representative chromatograms of perfusion samples obtained during permeability determination of digoxin and markers in the absence (solid line) and presence (dotted line) of P-gp modulator, quinidine (200 μM). Inset is the magnification of digoxin peak demonstrating the sensitivity of the method.

### 3.3. Application of method to determine P-gp mediated efflux

Intestinal permeability was determined in rat ileum using in situ single-pass perfusion technique and the samples were analyzed by the proposed method. Fig. 2 further confirms the sensitivity of the proposed method for application to this specific purpose. It is obvious from chromatographs of the perfusate samples collected after in situ perfusion that the amount of digoxin remaining (area of peak) after perfusion is considerably lower when co-perfused with quinidine (200 μM). The permeability ($P_{eff,control}$) of digoxin was found to be $0.04 \pm 0.01 \times 10^{-4}$ cm/s (Fig. 3). Propranolol showed highest permeability while phenolred and frusemide showed low permeability. Based on permeability data, digoxin can be classified into low permeability class drug as per BCS [9]. Inhibition of P-gp by quinidine (200 μM) significantly

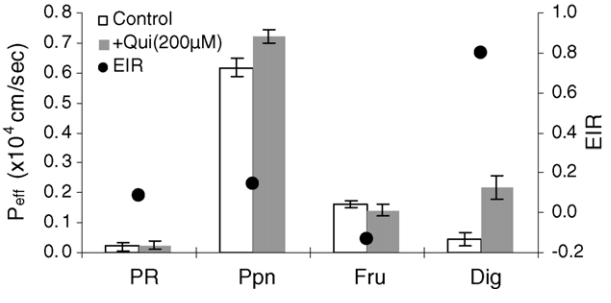


Fig. 3. Intestinal permeability of digoxin and markers in the absence and presence of P-gp modulator, quinidine (200 μM). secondary *Y*-axis shows EIR of each compound. Values are the mean ± S.D. of four determinations. EIR = $1 - (P_{eff,control}/P_{eff,inh})$. $^{**}P < 0.01$, statistically significant difference with reference to control of the same compound (one-way ANOVA).

increased the permeability ($P_{eff,inh}$) of digoxin ($P < 0.01$) while no change in the permeability was found for phenolred, frusemide and propranolol, indicating digoxin as a substrate to P-gp. The average water uptake during perfusion without and with quinidine was $6.23 \pm 4.39$ and $4.52 \pm 3.64$%, respectively. In the present case, phenolred, propranolol and frusemide were used as permeability markers so as to check the integrity of the intestinal membrane on different occasions and to obtain accurate permeability data for digoxin.

To evaluate the quantitative functional role of P-gp, the intestinal efflux inhibition ratio (EIR), the ratio of permeability due to P-gp-mediated efflux activity ($P_{P\text{-}gp}$) and the passive permeability ($P_{PD}$), was calculated. $P_{P\text{-}gp}$ was obtained by subtracting $P_{eff,control}$ from $P_{eff,inh}$ while $P_{PD}$ is equal to $P_{eff,inh}$. Assuming complete P-gp inhibition by quinidine (200 μM), EIR for digoxin was found to be 0.82 (Fig. 3), indicating that 82% of passive transport of digoxin is attenuated by P-gp-mediated transport. This data substantiates the reports that digoxin is a strong substrate to P-gp [22].

## 4. Conclusions

The proposed RP-HPLC method provides a sensitive and reliable determination of digoxin from in situ intestinal permeability samples with an additional advantage of simultaneous quantification of US FDA suggested permeability markers. This technique eliminated endogenous interferences from the biological matrix, provided high and precise recuperations, and permitted analysis involving no complex sample preparation procedures and with a short run time. The method is sensitive for each analyte as indicated by its quantification limits. Further the P-gp modulators elute in the same run time without interfering with any of the analytes. Finally, this method was used for routine quantification in permeability studies and for elucidating the functional role of P-gp in intestinal drug absorption.

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