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Simultaneous determination of nine model compounds in permeability samples using RP-HPLC: Application to prove the cassette administration principle in single pass intestinal perfusion study in rats☆

Wahajuddin[a,b,*,1], Sheelendra Pratap Singh[a,1], K.S.R. Raju[a], Asad Nafis[b], Girish Kumar Jain[a]

[a] Pharmacokinetics and Metabolism Division, CSIR - Central Drug Research Institute, Lucknow 226001, Uttar Pradesh, India
[b] Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research, Rae Bareli, India

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ABSTRACT

A simple, sensitive and specific reversed phase high performance liquid chromatographic (RP-HPLC) method for simultaneous determination of atenolol, paracetamol, hydrochlorothiazide, caffeine, cephalexin, metoprolol, propranolol, ketoprofen along with phenol red (a non-absorbable compound) in samples obtained from intestinal *in situ* single-pass perfusion studies, was developed and validated. Chromatography was carried out on RP18 column with mobile phase comprising of 10 mM phosphate buffer (pH 2.5) and methanol in gradient mode. The calibration curves were linear for all nine permeability model compounds ($r^2 > 0.999$) across the concentration range of 1.25–40 μg/ml. The coefficient of variation for intra and inter-day assay precision was between 0.04 and 3.08% and the accuracy was between 98.39 and 109.45%. Stability studies were carried out at different storage conditions and all the analytes were found to be stable. The method was successfully applied for analysing the permeability samples obtained from *in situ* single pass perfusion studies. The effective permeability (P_{eff}) values obtained upon cassette administration were in close proximity to the permeability values obtained upon single administration of model compounds. In conclusion, the developed RP-HPLC method can be used for high throughput cassette validation of rat *in situ* perfusion model for intestinal permeability assessment.

1. Introduction

The high failure rate of drug candidates in clinical trials leads to high cost of drug development. This development suffers from the serious drawback of very low bioavailability upon per-oral administration because of poor biopharmaceutical properties, namely solubility and permeability across gastrointestinal tract (GIT) epithelium. In the discovery stage, drug absorption studies can be performed only in laboratory animals/*in vitro* systems where the absorption process can be characterized both qualitatively and quantitatively. Solubility is easily quantifiable *in vitro* and can be manipulated by formulation strategies; however the same is not true in permeability which governs absorption of orally administered drugs. Hence, screening of the drug candidates for the permeability properties is imperative to select right candidate and to prevent the termination of the developing candidate at the later stage of drug development program. 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) and Single Pass Intestinal Perfusion (SPIP) assay. These models are well correlated to reflect equivalent levels to the *in vivo* permeability and fraction of dose absorbed in humans [1]. The rat *in situ* intestinal perfusion is a commonly used technique for the assessment of permeability of drugs and drug like molecules [2–4] and the functional role of P-gp on the total intestinal transport. 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 whilst comparing with other *in vitro* techniques. It provides experimental conditions closer to what is faced following oral administration. To demonstrate suitability of any permeability model, a rank-order relationship between

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* Corresponding author at: Pharmacokinetics and Metabolism Division, CSIR - Central Drug Research Institute, Lucknow 226001, Uttar Pradesh, India. Tel.: +91 522 2612411 18x4377; fax: +91 522 2623405.
E-mail addresses: wahajuddin@cdri.res.in, wahajuddin@gmail.com (Wahajuddin).

[1] These authors contributed equally to this work.

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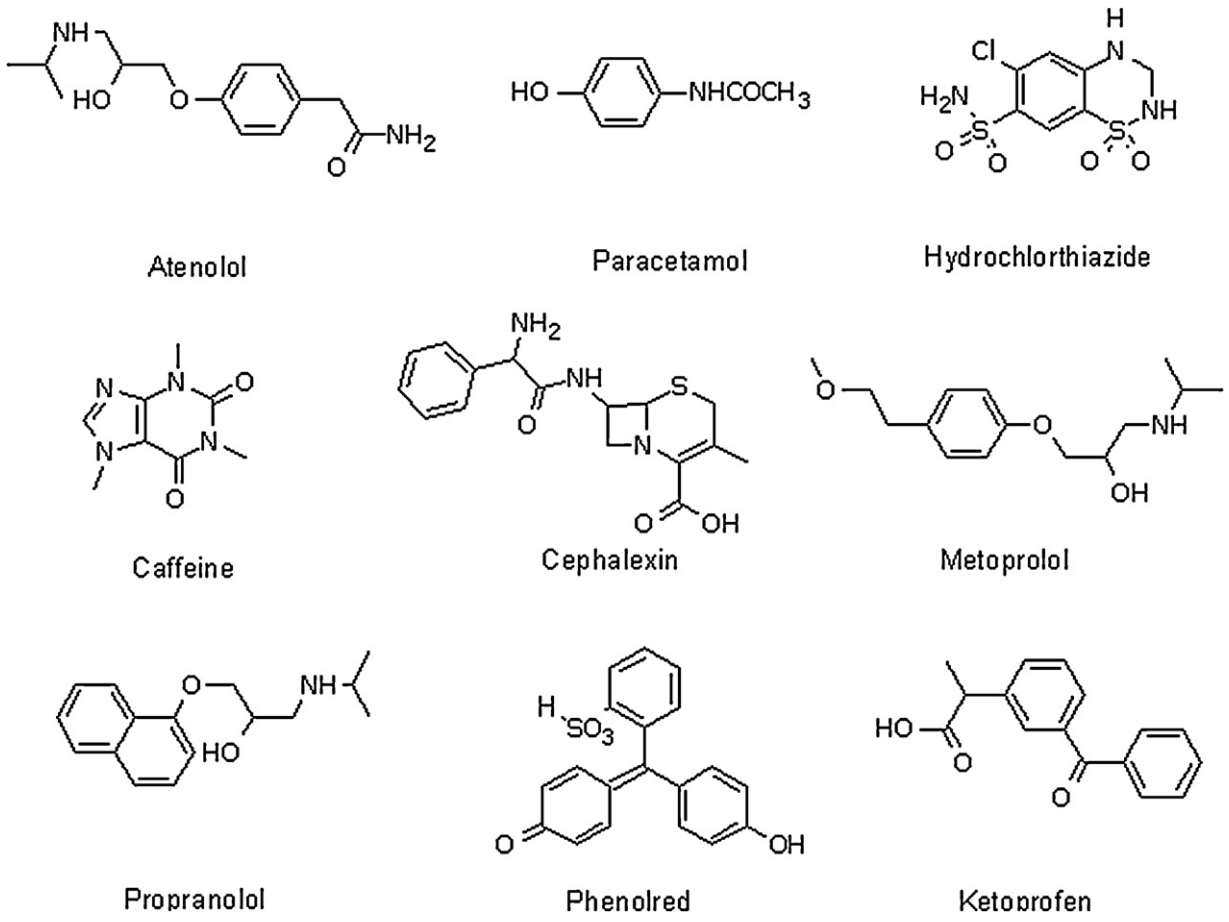

Fig. 1. Chemical structures of atenolol, paracetamol, hydrochlorothiazide, caffeine, cephalexin, metoprolol, propranolol, ketoprofen and phenol red.

permeability values and the extent of drug absorption in human subjects needs to be established using a sufficient number of model compounds. US-FDA guidelines [5] suggest the use of 20 model compounds as low (*e.g.*, <50% Fa), moderate (*e.g.*, 50–89% Fa), and high (≥90% Fa) permeability for classifying the permeability of new chemical entities (NCEs)/new drug candidates (NDCs) according to the Biopharmaceutical Classification System (BCS).

For carrying out permeability screening of (NCEs)/(NDCs), one has to optimize and validate the permeability assay using the model compounds recommended by regulatory bodies (for instance, US-FDA) under the in house laboratory conditions. This necessitates development of analytical method for the model compounds. The model compounds recommended for permeability classification vary greatly in their physicochemical properties which may lead to even development of multiple analytical methods resulting in consumption of efforts, time and resources. Till date only few analytical methods have been reported for simultaneous determination of model compounds [1,6,7]. The reported methods are for relatively very few marker compounds. However, whilst optimizing permeability assays for in house use and for more reliability, it is always preferable to have an analytical method with larger data set of marker compounds. To the best of our knowledge, there is no analytical method reported in the literature for simultaneous determination of at least two marker compounds from each class *viz.* low, medium and high permeability for more effective validation of the permeability model and more reliable permeability classification of new chemical entities.

Therefore, a simple and sensitive RP-HPLC–PDA method is being reported hereby for simultaneous analysis of atenolol, paracetamol, hydrochlorothiazide, caffeine, cephalexin, metoprolol, propranolol, ketoprofen and phenol red. These markers cover the entire range of high, low, intermediate and virtually zero (non-absorbable) permeability model compounds. The method was validated for routine use; and its usefulness and reliability for standardization of rat *in situ* single pass intestinal perfusion model is demonstrated. We have also established the applicability of the cassette dosing principle and its practice to intestinal perfusion studies for the first time. This method will enable the simultaneous *in situ* permeability determination of most reliable permeability model compounds in single experiment which will result in saving time, efforts and cost involved in developing different method for different permeability model compounds.

2. Materials and methods

2.1. Chemicals and reagents

Atenolol, propranolol, paracetamol, hydrochlorothiazide, ketoprofen, cephalexin, caffeine and metoprolol were purchased from Sigma Aldrich Ltd. (St. Louis, USA). Chemical structures of all permeability markers are shown in Fig. 1. Phenol red and acetonitrile of HPLC grade were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, India). Potassium dihydrogen ortho-phosphate (KH_2PO_4), orthophosphoric acid and potassium hydroxide were purchased from Sigma Aldrich Ltd. (St. Louis, USA). Sodium dihydrogen ortho-phosphate ($NaH_2PO_4{\cdot}2H_2O$) and anhydrous di-sodium hydrogen ortho-phosphate (Na_2HPO_4) were purchased from Glaxo Laboratories Limited (Mumbai, India). Sodium chloride (NaCl) was purchased from Ranbaxy Laboratories Limited (Punjab, India). Milli-Q pure water was obtained from a Millipore Elix water