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Validation of phenol red versus gravimetric method for water reabsorption correction and study of gender differences in Doluisio's absorption technique

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ABSTRACT

The aim of the present study was to develop a method for water flux reabsorption measurement in Doluisio's Perfusion Technique based on the use of phenol red as a non-absorbable marker and to validate it by comparison with gravimetric procedure. The compounds selected for the study were metoprolol, atenolol, cimetidine and cefadroxil in order to include low, intermediate and high permeability drugs absorbed by passive diffusion and by carrier mediated mechanism. The intestinal permeabilities (P_{eff}) of the drugs were obtained in male and female Wistar rats and calculated using both methods of water flux correction. The absorption rate coefficients of all the assayed compounds did not show statistically significant differences between male and female rats consequently all the individual values were combined to compare between reabsorption methods. The absorption rate coefficients and permeability values did not show statistically significant differences between the two strategies of concentration correction. The apparent zero order water absorption coefficients were also similar in both correction procedures. In conclusion gravimetric and phenol red method for water reabsorption correction are accurate and interchangeable for permeability estimation in closed loop perfusion method.

1. Introduction

Oral route is the most convenient and common method of drug administration and more than 60% of marketed drugs are used as oral products. Drugs administered orally are absorbed into systemic circulation mainly from the small intestinal tract (Masaoka et al., 2006) thus a good estimation of human intestinal permeability is a crucial step for oral drug development or generic approval. Several methods in preclinical animal models have been proposed to predict human intestinal permeability. Rat intestinal permeability has demonstrated a good correlation with human values (Lennernas, 1997). The single-pass intestinal perfusion (SPIP) model and Doluisio's closed loop technique are the most used methods for rat permeability estimation. Both techniques have been widely used for drug permeability classification and human permeability predictions (Cao et al., 2006; Dahan et al., 2009; Escribano et al., 2012; Prieto et al., 2010).

Closed loop method experimental technique is simple and utilizes readily available laboratory equipment. In consists of creating a compartment in the small intestine of the rat, introducing the drug solution inside the loop and taking samples at predefined times in order to determine the remaining drug concentration in the intestinal lumen (Fig. 1).

The results (absorption rate coefficients and permeability values) are closely reproducible and correlated well with results obtained from humans or intact animals (Doluisio et al., 1969). Moreover, this technique is suitable to study the mechanism of drug absorption and to demonstrate whether a transporter is involved in drug permeation (Moll-Navarro et al., 1996).

However, the reduction in the volume of the perfused solutions at the end of the experiments is usually significant (up to 20%). This volume reduction may introduce errors in the absorption coefficient estimation and a correction becomes necessary to calculate accurately the absorption parameters. Usually, the water absorption is calculated by gravimetric method. Various methods that involve the co-perfusion of non-absorbed markers such as phenol red or 14C-PEG-3350 have been published in order to correct water flux through the segment more accurately in the rat single-pass intestinal perfusion model (Sutton et al., 2001; Zakeri-Milani

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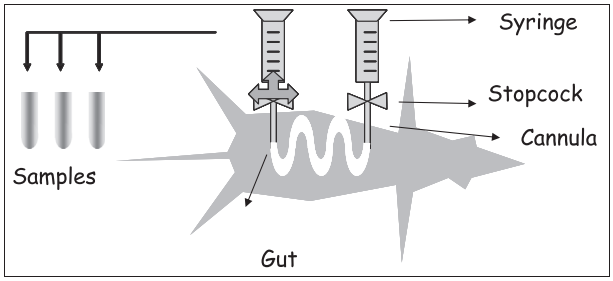

Fig. 1. Set up of the Doluisio's *in situ* absorption experiments in rats.

et al., 2005). On the other hand Doluisio's original method used a gravimetric water reabsorption correction and no attempt has been made to use a non-absorbable marker as an alternative procedure.

The aim of the present study was to develop and validate a phenol red method for water reabsorption correction in Doluisio's Perfusion Technique and to compare the permeability results obtained for metoprolol, atenolol, cimetidine and cefadroxil with both correction procedures in order to validate phenol red method results.

2. Materials and methods

2.1. Chemicals

Metoprolol hydrochloride, atenolol, cimetidine, cefadroxil and phenol red solution (%0.5 in DPBS) were purchased from Sigma (St. Louis, USA). Sodium chloride, potassium chloride, sodium dihydrogen phosphate, di-sodium hydrogen phosphate and calcium chloride anhydrous were obtained from Panreac (Panreac Quimica, Barcelona, España). All other chemicals were HPLC or analytical grade and used without further purification.

2.2. Chromatographic conditions

Intestinal samples were analyzed by HPLC. Details of HPLC conditions are given in Table 1. A Novapack C18 (Waters®) cartridge-type column (5 μM particle size × 200 mm) was used. Chromatographic methods were previously validated over the concentration range of the samples. Accuracy was calculated by means of the percentage of error associated with measuring ±8 standards, analyzed at least three times. It was demonstrated to be less than 5% regardless of the concentration of analyte. Precision (within and between days) was calculated as the coefficient variation over the same standards. It was shown to be less than 10%. Linearity was established over the range of concentrations present in the samples ($r^2 > 0.999$). All the samples were above the limit of quantitation (LOQ).

2.3. Stability tests

The suitability of sample storage at room and low temperature was evaluated. One set of samples were maintained at room temperature for 24 h and another was subjected to three cycles of freezing at −4 °C and thawing to room temperature (22 ± 2 °C). Both experimental studies were carried out at three concentrations (10, 30 and 50 μM) in duplicate. Peak areas of the compounds in test solutions and freshly prepared standard solutions were compared.

2.4. Absorption studies

2.4.1. In situ perfusion rat technique

The animal study was approved by the Scientific Committee of the Faculty of Pharmacy (project reference DI-MBS-001-12) and followed the guidelines described in the EC Directive 86/609, the Council of the Europe Convention ETS 123 and Spanish national laws governing the use of animals in research (Real Decreto 223/1988, BOE 67, 18-3-98:8509-8511).

In situ perfusion of the whole small intestine (“closed loop”-Doluisio's Technique) adapted as described by Bermejo et al. (1999), Ferrando et al. (1999), Martin-Villodre et al. (1986), Ruiz-Garcia et al. (1999) was used to characterize the absorption rate coefficient and the permeability value of the compounds at 100 μM. Male and female Wistar rats were weighted after an overnight fast with access to water. Rats were anesthetized using a mixture of diazepam (Valium, Roche) (1.67 mg/kg), ketamine (Ketolar; Parke-Davis) (50 mg/kg) and atropine (Atropina; Braun) (1 mg/kg) and placed on heated surface to be maintained at 37 °C. A midline abdominal incision was made and the bile duct was closed before the perfusion in order to prevent enterohepatic recycling and the presence of bile salts in lumen. Studies employed the entire small intestine. Briefly, the method consists of creating a compartment in the small intestine with the aid of two syringes and two three-way stopcock valves. Drug solutions are placed in the intestinal segment with the aid of the syringes and samples of the intestinal fluid are withdrawn at predefined times to analyze the remaining drug concentration. Test solutions were prepared immediately before use. Sampling of the perfusate into glass tubes was carried out at fixed times, after 5 min at intervals of 5 min up to 30 min. All samples were analyzed by HPLC.*In situ* perfusion experiments were performed in eight groups of rats ($n = 3$), four groups of males and four of females with the drugs at 100 μM and phenol red at 100 μg/mL. Metoprolol, cimetidine, atenolol and cefadroxil were

Table 1
Details of chromatographic conditions.

Compound	Detector	Mobile phase	Injection volume (IV)-flow (F)	λ
Metoprolol	Fluorescence	Water: 60 Methanol: 20 Acetonitrile: 20	IV: 10 μL F: 1 mL/min	λem: 307 λex: 231
Atenolol	Fluorescence	Water: 90 Methanol: 5 Acetonitrile: 5	IV: 25 μL F: 1 mL/min	λem: 307 λex: 231
Cimetidine	UV	Methanol: 15 Acetonitrile: 85	IV: 50 μL F: 1 mL/min	210 nm
Cefadroxil	UV	Water: 65 Acetonitrile: 35	IV: 15 μL F: 1 mL/min	363 nm
Phenol red	UV	Water: 60 Acetonitrile: 40	IV: 50 μL F: 1 mL/min	262 nm