

**Development and validation of a reversed-phase high-performance liquid chromatographic method with solid-phase extraction (SPE) for the quantification of hydrochlorothiazide in *ex vivo* permeation studies**

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**Abstract**

Hydrochlorothiazide (HCT) is a diuretic used to treat hypertension. In order to study its intestinal permeation behavior applying an *ex vivo* methodology, a rapid, sensitive, and selective reversed-phase liquid chromatography (RP-HPLC) method coupled with UV detection (RP-HPLC UV) was developed for the analysis of HCT in TC199 culture medium used as mucosal and serosal solutions in the everted rat intestinal sac model. Also, analytical procedures for the quantification of HCT by RP-HPLC with UV detection required a sample preparation step by solid phase extraction (SPE). The method was validated in the concentration range of  $8.05 \times 10^{-7}$  M to  $3.22 \times 10^{-5}$  M for HCT. Chromatographic parameters, namely carry-over, lower limit of quantification ( $1.4491 \times 10^{-7}$  M), limit of detection ( $3.8325 \times 10^{-8}$  M), selectivity, inter- and intra-day precision, and extraction recovery were determined and found to be adequate for the intended purposes. The validated method was successfully used for permeability assays across rat intestinal epithelium applying the *ex vivo* everted rat gut sac methodology to study the permeation behavior of HCT.

*Key words:* hydrochlorothiazide, *ex vivo* permeation study, solid-phase extraction.

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## 1. Introduction

According to the Biopharmaceutics Classification System (BCS), hydrochlorothiazide (HCT) is a diuretic and class IV drug with low solubility and low permeability, exhibiting poor oral absorption. HCT is incompletely but fairly rapidly absorbed from the gastrointestinal tract, with a bioavailability ranging from ~65% to 80% (Beermamm & Groschinsky-Grind, 1977). This parameter was associated with a high variability, mainly attributed to its poor solubility, dissolution and permeability (Sanphui et al., 2015).

Our aim was to characterize *in vitro* the intestinal absorption characteristics of HCT using the everted rat gut sac model. This method is a well-established *in vitro* assay used to characterize the intestinal permeability of drugs (Balimane et al., 2000); however, a suitable analytical method to quantify drugs in the tissue culture medium used in this methodology is needed.

The everted gut sac method requires the tissue to be incubated in a tissue culture medium in the presence of the test drug, which in turn permeates through the enterocytes and is accumulated inside the sac. In order to obtain adequate permeation parameters, drug diffusion should be maintained under sink conditions, in which a low amount of drug diffuses from the mucosal (outer) to the serosal (inner) solution. The need to maintain sink conditions and the frequent low solubility of drug in the mucosal solutions result in very low concentration levels of permeated drugs. Therefore, sensitive and specific methods are required for the quantification of the analyte present in the serosal sides of the intestinal sac.

To the best of our knowledge, there are no validated HPLC methods in the literature for the quantification of HCT in complex TC199 tissue culture medium (TC199 medium).

In order to study the intestinal absorption characteristics of HCT by the everted gut sac methodology, a sensitive and accurate analytical method for the analysis of HCT in TC199 medium by reversed phase high performance liquid chromatography (HPLC) with ultraviolet (UV) detection was developed.

Because of the very low concentration levels of HCT and the complexity of the TC199 medium, pretreatment for sample clean-up was an indispensable procedure before instrumental analysis. The solid-phase extraction (SPE) was chosen as the extraction technique for the present study because of its simplicity, low cost, short extraction time, low volume of solvents required, its widespread availability in most laboratories, and its widely application to biological fluids (Wang et al., 2000). Therefore, we report herein the

development of a simple, sensitive, reproducible, and fast RP HPLC-UV method coupled with SPE sample pretreatment in order to measure HCT in TC199 medium used in intestinal permeability studies by the everted rat gut sac method.

## 2. Materials and methods

### 2.1 Chemicals and materials

Hydrochlorothiazide (HCT) was purchased from Parafarm® (Buenos Aires, Argentina) and used without further purification. The tissue culture medium (TC199) was obtained from Sigma-Aldrich (Buenos Aires, Argentina). HPLC-grade methanol was purchased from Todo Droga (Córdoba, Argentina). The water used in these studies was produced by a Milli-Q Water Purification System (Millipore® Bedford, USA). All other chemicals were reagent grade or higher.

### 2.2 Analytical method development

#### 2.2.1 Preparation of calibration standards and control sample

Stock solutions of HCT were prepared by dissolving an accurately weighed amount (0.005 g) of HCT in 25 mL of acetonitrile (ACN) with a final concentration of  $6.7 \times 10^{-4}$  M. Aliquots from this solution were taken to obtain three working standard solutions (WSS) prepared in water with a concentration of  $6.7 \times 10^{-5}$  M,  $6.7 \times 10^{-6}$  M, and  $6.7 \times 10^{-7}$  M, respectively. The calibration curve was prepared in TC199 medium with concentrations of: 0.081, 0.16, 0.24, 0.32, 0.64, 0.81, 1.2, 1.6, 2.4, and  $3.2 \times 10^{-5}$  M. An internal standard (IS) of caffeine (50 µL,  $7.7 \times 10^{-5}$  M) was used, which was added to samples before the extraction process by SPE, obtaining a fixed final concentration of  $1.9 \times 10^{-5}$  M. Control samples (QC samples) were prepared from WSS in TC199 medium with concentrations of 0.081, 0.81,  $3.2 \times 10^{-5}$  M, and the same fixed concentration of IS.

Calibration and QC samples were processed applying a solid phase extraction (SPE) procedure, which is explained later in section 2.3.4.

#### 2.2.2 Chromatographic conditions

The HPLC system consisted of a Jasco chromatograph equipped with a quaternary pump (PU-2089s Plus) and a Jasco Multiple Wavelength detector (Jasco UV-2077 Plus) set at 270 nm. Chromatographic separations were performed on a C18 Restek (15 cm x 4.5 mm x 5 µm)

column thermostated at 30 °C. A Phenomenex Security Guard Fusion® RP (4 x 30 mm) guard column was also used. Chromatographic analyses were performed in the gradient mode, with the mobile phase consisting of water (pH = 3.6, adjusted with acetic acid): Methanol HPLC grade (MeOH<sub>HPLC</sub>) (80:20, v/v) at a flow rate of 1 mL/min from 0 to 5 minutes (min), which linearly changed to a 50:50 v/v mixture from 5 to 9 min and that was kept constant from 9 to 11 min. Finally, the solvent composition was linearly modified to its original rate (80:20, v/v) in 1 min. In addition to the time required for the injection cycle of the instrument, the injection time, and the needle wash cycle (3 min), the full equilibration time between runs was approximately 4 min to ensure the column re-equilibration prior to the following injection. 10 consecutive injections of HCT standards were performed to ensure complete system re-equilibration, and no significant changes were found in HCT retention times. The total run time was 12 min, with 15 µL injection volumes being applied.

### 2.2.3 Validation of the method

The HPLC-UV method for the quantification of HCT in TC199 medium was validated according to the FDA (Draft Guidance, 2013) and EMA (2011) guidelines on validation of bioanalytical methods in terms of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), extraction recovery, precision, and accuracy. Quantitative analysis of HCT in TC199 medium was performed using caffeine as internal standard (IS).

The calibration curves and validation studies were carried out by adding HCT WSS and IS to TC199 medium. Interference in the presence of any endogenous constituents was assessed by analysis of blank samples and TC199 medium spiked with HCT. The linearity of the method was evaluated using standard solutions of different concentrations of HCT prepared in TC199 medium within the range of  $8.05 \times 10^{-7}$  M and  $3.22 \times 10^{-5}$  M and a fixed level ( $1.9 \times 10^{-5}$  M) of IS. A ten-point calibration curve was constructed with the blank TC199 medium spiked with appropriate volumes of working standards. Additionally, the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS) were measured. The calibration curve for the analyte was generated by correlating the area ratio between HCT and IS against the ratio of concentrations between HCT and IS ( $Area_{HCT}/Area_{IS}$  vs  $[HCT]M/[IS]M$ ). The blank and zero samples were not taken into account to calculate the calibration curve parameters.

To assess precision and accuracy parameters, quality samples containing the blank TC199 medium spiked with the analyte and IS were prepared at three different concentration levels (FDA, 2013). These samples were replicated five times. Accuracy was reported as percent of

the nominal value of HCT. Precision was determined as %RSD of the replicate measurements. The procedure was further repeated on two consecutive days in order to determine inter-day variability.

The limit of detection (LOD) of the method was determined as the concentration of the analyte, which exhibited a peak area ratio 3 times higher than the baseline noise. The limit of quantification (LOQ) of the method was determined as the concentration of the analyte, which had a peak area ratio 10 times higher than the baseline noise (ICH, 2005). The lowest standard on the calibration curve was reported as the lower limit of quantification (LLOQ) (EMA, 2011; FDA, 2013).

The selectivity of the method was assessed by adding HCT and IS to the blank TC199 medium. The recovery includes the extraction efficiency of an analytical method. The recovery of the analyte does not need to be 100%, but the extent of recovery of an analyte and of the IS should be consistent, precise, and reproducible. Recovery experiments were performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards representing 100% recovery.

### 2.3 *Applicability of the method: intestinal permeation study*

#### 2.3.1 Animal treatments

Protocols involving experiments on laboratory animals were reviewed and approved by the Committee of Ethics for the Use of Animals in Experimental Protocols of the Faculty of Chemical Sciences, National University of Córdoba, Argentina (Res. No. 138/07). Healthy male Wistar rats were used at the age of 3 months (300 – 350 g). The animals were caged with free access to water and food pellets until 24 h prior to performing the assays, after which they were fasted and allowed to ingest water *ad libitum* until surgical procedures were carried out.

#### 2.3.2 Gut sac viability

In order to verify the integrity and viability of the gut sacs during the permeation experiments, glucose concentrations were measured both in the mucosal medium and in the sac contents using a modification of the method described by Lifschitz et al. 2009 (Lifschitz et al., 2009). The sacs were incubated in TC199 medium in the absence of HCT maintaining the same conditions as in the intestinal permeation assay. Samples from the mucosal and serosal solutions were collected every 10 min for 120 min, after which the glucose

concentration was quantified using a commercial assay (Wiener) as follows: 20  $\mu\text{L}$  of sample was incubated at 37  $^{\circ}\text{C}$  for 10 min with 2 mL of the following reagent solution: 50% of distilled water, 5% of 25 mmol/L 4-aminophenazone solution in Tris buffer (0.92 mol/L), 5% of 55 mmol/L phenol solution, and 0.3% of 1000 U/mL glucose oxidase in 120 U/mL peroxidase. The corresponding absorbance was measured using a spectrophotometer at 505 nm.

### 2.3.3 *Ex vivo* intestinal absorption study of HCT by the everted rat gut sac model

The intestinal permeation of HCT was studied by applying the everted rat gut sac technique (Quevedo & Briñón, 2009; Quevedo et al., 2011; Onnainty et al., 2016; Raposo, 2016). The medium was TC199, pH 7.4, and with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  gas bubbling at 37  $^{\circ}\text{C}$ . After an overnight fast, the rats were anesthetized by intraperitoneal administration of urethane (1000 mg/kg) and a 2 cm incision was made in the midline abdominal cavity, isolating a segment of 10 cm of proximal jejunum (just 15 cm distal to the ligament of Treitz). After the tissues were dissected, the animals were immediately sacrificed by applying  $\text{CO}_2$ . Intestinal segments were flushed with 30 mL of oxygenated TC199 medium (37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ ) and gently everted over a glass rod (2.5 mm diameter). A glass cannula was inserted into the intestinal segment and tied at the top and bottom, with 10 cm of tissue being exposed to the drug permeation. Then, a 10 g weight was tied to the bottom of the segment and the device with the tissue was submerged in 45 mL of warm pH 7.4 oxygenated TC199 medium (5:95  $\text{CO}_2$ : $\text{O}_2$ , 37  $^{\circ}\text{C}$ ) containing HCT (8000  $\mu\text{g}/\text{mL}$ ) dissolved in 500  $\mu\text{L}$  of dimethyl sulfoxide. These conditions ensured tissue viability during the whole assay sampling interval, a feature that was determined by means of the glucose concentration test described in section 2.3.2.

To perform the permeation assay, 1 mL of pH 7.4 oxygenated TC199 medium (5:95  $\text{CO}_2$ : $\text{O}_2$ , 37  $^{\circ}\text{C}$ ) was placed in the intestinal sac, withdrawn at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 min, and replaced by 1 mL of drug-free oxygenated TC199 medium. Between each permeation sample, 1 mL of pH 7.4 oxygenated TC199 (37  $^{\circ}\text{C}$ ) was used to rinse the cannula, with this aliquot being added to the corresponding permeation sample. The collected aliquots were subjected to SPE described in the sample preparation technique (Section 2.3.4) and HPLC analysis protocol.

Permeation assays were performed in triplicate. After the 2 h permeation study, apparent permeability coefficient ( $P_{\text{app}}$  cm/min) was calculated applying the equation 2:

$$P_{app} = \frac{F}{SAC_o} \quad (1)$$

$$SA = 2\pi rh$$

where  $SA$  is the surface area of the barrier membrane ( $\text{cm}^2$ ),  $C_o$  is the initial drug concentration ( $\mu\text{g/mL}$ ) in the mucosal compartment,  $r$  is the intestinal segment mean radius (0.40 cm), and  $h$  is the length of intestinal segment (10 cm). All data are expressed as means  $\pm$  SD.

#### 2.3.4 Sample preparation for *ex-vivo* samples

In order to isolate the analyte from the TC199 medium, a solid phase extraction (SPE) protocol was developed and fully validated. Strata-X® (60 mg, Phenomenex®) SPE cartridges were used, and the following protocol was applied: (a) cartridges were preconditioned by sequentially applying 2 mL of ( $\text{MeOH}_{\text{HPLC}}$ ) which was followed (b) by an equilibration step containing 2 mL of pH 2 (100 mM) phosphate buffer. The sample intended for extraction was prepared by adding the IS solution to the corresponding sampled aliquots prior to extraction, which were homogenized, and then (c) applied to the preconditioned cartridge at a flow rate of 1 drop/s. Afterwards, (d) the cartridge was dried under vacuum for 1 min, (e) the analyte elution was accomplished by applying 1 mL of (ACN), and (f) the eluted sample was concentrated under a  $\text{N}_2$  stream at 40 °C and re-suspended in 400  $\mu\text{L}$  of a  $\text{MeOH}_{\text{HPLC}}$ :  $\text{H}_2\text{O}$  50:50 mixture.

Extraction recovery was evaluated by performing SPE in triplicate on the standard solution containing HCT at three concentration levels of the analyte and IS. Extraction recovery for the analyte was determined using the following equation:

$$r = \frac{100C}{C_o} \quad (2)$$

where  $C_o$  is the concentration of the compound in the initial solution and  $C$  is the calculated concentration.

### 3. Results and discussion

#### 3.1 Method validation

The chromatographic method was validated according to the ICH, FDA, and EMA guidelines on validation of bioanalytical methods.

### 3.1.1 Selectivity

Selectivity, which describes the ability of a method to differentiate the analyte of interest and IS from other components of the sample, was determined by comparing representative chromatograms of blank TC199 medium samples with those of blank TC199 medium samples spiked with HCT and IS (Figure 1). Possible interference by substances present in the transport medium was tested by comparison of the chromatograms. Under the chromatographic conditions used for the analysis of HCT no other interfering peaks were observed in the blank culture medium and the method was found to be specific for HCT at the corresponding wavelength (270 nm). The absence of interfering components co-eluting with HCT and IS indicated that the method was highly selective. The retention times for HCT and IS (caffeine) were of 2.8 min and 6.3 min, respectively. The HCT and IS chromatographic run times allowed the analysis of a large number of samples in a short time. Thus, the method proved to be simple and highly selective.

### 3.1.2 Standard curves and linearity

The calibration curve was linear over the concentration range of  $8.05 \times 10^{-7}$  M to  $3.22 \times 10^{-5}$  M. Linearity or concentration-response of an analytical method was evaluated by calculating the regression equation and the correlation coefficient ( $R^2$ ) by the method of least squares (Figure 2). The  $R^2$  of 0.9985 ( $\pm 0.0771$ ) indicated a good linearity of the proposed range. Moreover, the linearity of calibration data was also evaluated by graphical assessment, which includes linearity, residuals, and relative response factor plots (Figure 2) (Raposo, 2016). The visual evaluation of the pattern of the residual plot (Figure 2b) showed a random behavior in a constant range without a systematic pattern, indicating a correct linearity. Also, the linearity plot (Figure 2c) indicated the existence of linearity over the full range of calibration as none of the sensitivity points (relative response factors) intersected or were beyond the  $\pm 5\%$  tolerance limits. The typical equation for the calibration curve was as follows:  $y = (3.4471 \pm 0.0063) x + (0.0441 \pm 0.0324)$  ( $n = 10$ ), where “x” represents the ratios of HCT and IS concentration in TC199 medium and “y” represents the ratios of HCT and IS peak area.

### 3.1.3 Sensitivity

A gradient elution mode was used to improve the sensitivity of the method in a short run-time. Different combinations of methanol and water were tested in order to provide separation efficiency and good peak resolution. From these combinations, an adequate



analyte peak shape and chromatographic resolution were achieved with water (pH = 3.6, adjusted with acetic acid):MeOH<sub>HPLC</sub> (80:20, v/v) at a flow rate of 1 mL/min from 0 to 5 min, which linearly changed to a 50:50 v/v mixture from 5 to 9 min, and that was kept constant from 9 to 11 min. The LOD was determined as  $3.8325 \times 10^{-8}$  M, the LOQ was  $1.4491 \times 10^{-7}$ , and the LLOQ was  $8.05 \times 10^{-7}$  M (Table 1).

#### 3.1.4 Precision and accuracy

The accuracy of the method, which depends on the closeness between observed and true concentration values, was determined using three samples from the low ( $8.05 \times 10^{-7}$  M), medium ( $8.05 \times 10^{-6}$  M) and high ( $3.22 \times 10^{-5}$  M) concentration levels (QC samples).

Accuracy and intra-day precision were established by analyzing blank TC199 medium aliquots of each QC sample on the same day. Accuracy and inter-day precision were determined by analyzing each QC sample in quintuplicate on two different days.

Result data for intra- and inter-day precision and accuracy obtained from blank QC TC199 medium aliquots at three different levels of analyte are shown in Table 1. The intra- and inter-day precision (%RSD values) did not exceed 6%. Therefore, these results were within the accepted limits for bioanalytical method validation, which should not exceed 15% of the actual value, except at the LLOQ which should not exceed 20%. Intra- and inter-day recoveries varied from 88.35 % to 100.28 % and 87.47 % to 98.76 %, respectively, with a coefficient of variation < 7%.

These results suggest that the method assessed in this study is highly accurate, precise, and reproducible.

#### 3.2 Solid phase extraction method (SPE)

Because of the presence of potentially interfering compounds in the TC199 matrix, such as salts and phenol red, which could in turn decrease analyte recoveries and affect its separation and analysis, an appropriate SPE protocol on the samples drawn from the permeability study was developed.

There are no currently available methods for sample preparation and quantification of HCT in a TC199 matrix used in *in vitro* intestinal permeability studies.

Several cartridges for separation of HCT from endogenous interferences in human plasma have been used for SPE of HCT. Zendelovska et al. (2004) tested two solid phase extractions (C18 and RP-selected B) and obtained satisfactory results for the recovery of HCT and

caffeine (IS) with RP-select B cartridges. Shang et al. (2011), however, developed an HPLC with on-line SPE and DAD detection for the simultaneous determination of nitrendipine and HCT in rat plasma. Sample preparation by SPE to remove interfering biomatrix substances was carried out testing several SPE columns (CAPCELL PAK MF Ph-1, CAPCELL PAK MF SCX, and CAPCELL PAK MF C8 columns). The best results for HCT were obtained on the CAPCELL PAK MF C8 column. SPE for the simultaneous analysis of HCT and Lisinopril (LIS) in human plasma on Waters Oasis HLB cartridges has been reported by Shah et al. (2016). The best results were achieved by preconditioning and washing the cartridges with 5.0 mM ammonium formate (pH 3.0) and eluting drugs with a mixture of acetonitrile and 5.0 mM ammonium formate (pH 4.5 adjusted with 0.1% formic acid 85:15 v/v).

In this study, an outline SPE procedure was used, which is shown in Figure 3. Here, a reversed phase functionalized polymeric sorbent (Strata-X cartridges) was used for the first time for the HCT extraction from a biological matrix (TC199 culture medium). This kind of cartridges contains a surface-modified styrene divinylbenzene polymer which gives a SPE sorbent through H-bonding and  $\pi$ - $\pi$  bonding capabilities for enhanced retention of polar and aromatic analytes.

According to the conditions for using SPE cartridges and the chemical properties of HCT, ACN was selected since it can be quickly removed by evaporation and its UV cut-off wavelength is appropriate for UV detection. Considering that HCT has two ionizable protons (and hence two pKa values) (Raposo, 2016), an aqueous solution of pH  $\sim$ 2 was selected as the loading and washing solvent.

Table 1 shows the extraction recovery of HCT and IS from the blank TC199 medium at low ( $8.05 \times 10^{-7}$  M), medium ( $8.05 \times 10^{-6}$  M), and high ( $3.22 \times 10^{-5}$  M) concentration levels by SPE extraction. The mean ( $\pm$ SD) extraction recovery for IS was  $96.02 \pm 1.57$  % ( $n=10$ ). The use of an IS in the extraction procedure is crucial to account for variability in extraction efficiency. In the present study, caffeine was chosen as the IS because of its structural analogy and similar chemical characteristics and properties to those of HCT. It also displayed appropriate chromatographic retention with adequate peak resolution of HCT (Figure 1).

### 3.3 Application of the method

This method was successfully applied to the *ex vivo* everted rat gut sac permeability assay to assess the oral transport (permeability) characteristics of HCT.

Figure 4 shows that, in the absence of HCT, the ratios of the glucose contents in the serosal and mucosal sides of sacs were increased with the incubation time up to 120 min, which indicated that the tissue of the gut sacs was viable over the incubation time periods in the *ex vivo* permeability assays.

Results from studies on intestinal absorption kinetics of HCT showed that the cumulative absorbed dose of HCT increased linearly with the increase of incubation time up to 120 min (Figure 5), with a calculated apparent permeability ( $P_{app}$ ) coefficient for HCT of  $1.38 \pm 0.05 \times 10^{-4}$  cm/min. From the absorption profile of HCT, it can be inferred that HCT would be transported across the small intestinal epithelium by the transcellular route into the sac fluid of the serosal side.

#### **4. Conclusions**

A rapid and reliable RP-HPLC method coupled with UV detection for determination of HCT from *ex vivo* rat intestinal permeability studies was developed and fully validated. It was found that the resulting analytical method was very sensitive, accurate, selective, and appropriate to analyze the drug in the presence of TC199 tissue culture medium. Also, the developed method proved to be an efficient tool to assess sample cleanup, which was obtained from the mucosal and serosal solutions of gut sacs. SPE procedures avoided analyte and matrix component interference, thus improving the resolution and sensibility of the method. This methodology has been successfully applied to elucidate the intestinal absorption behavior of HCT.

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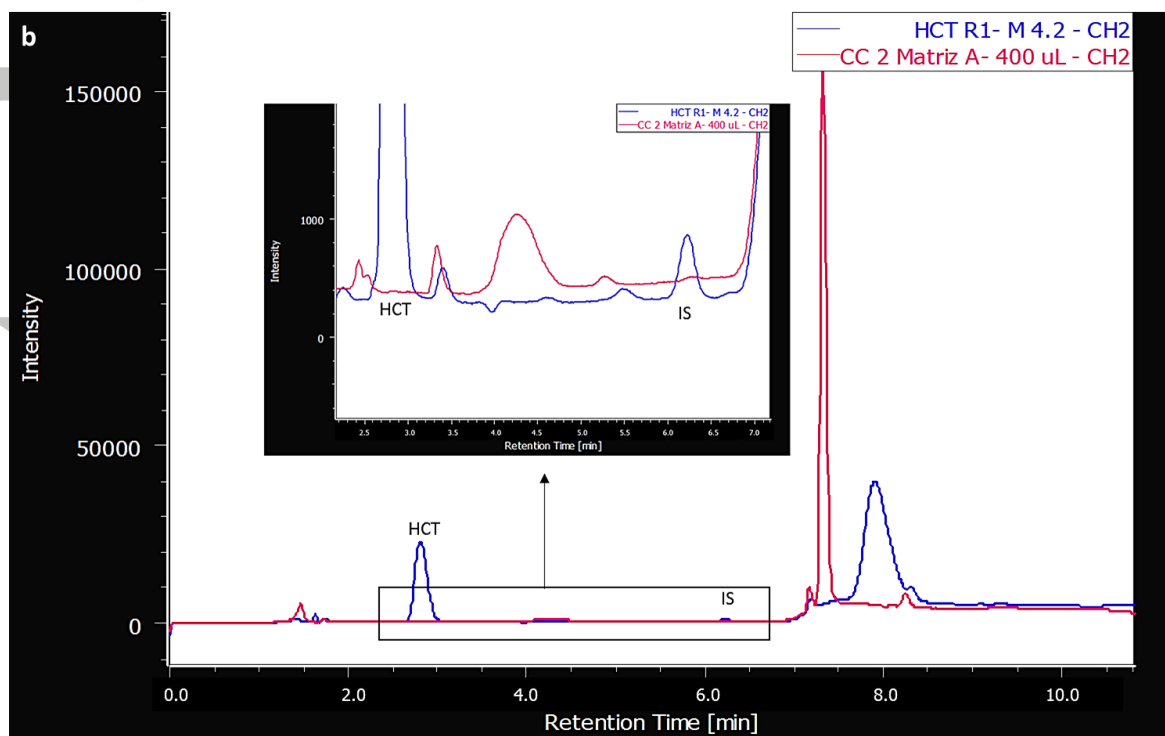
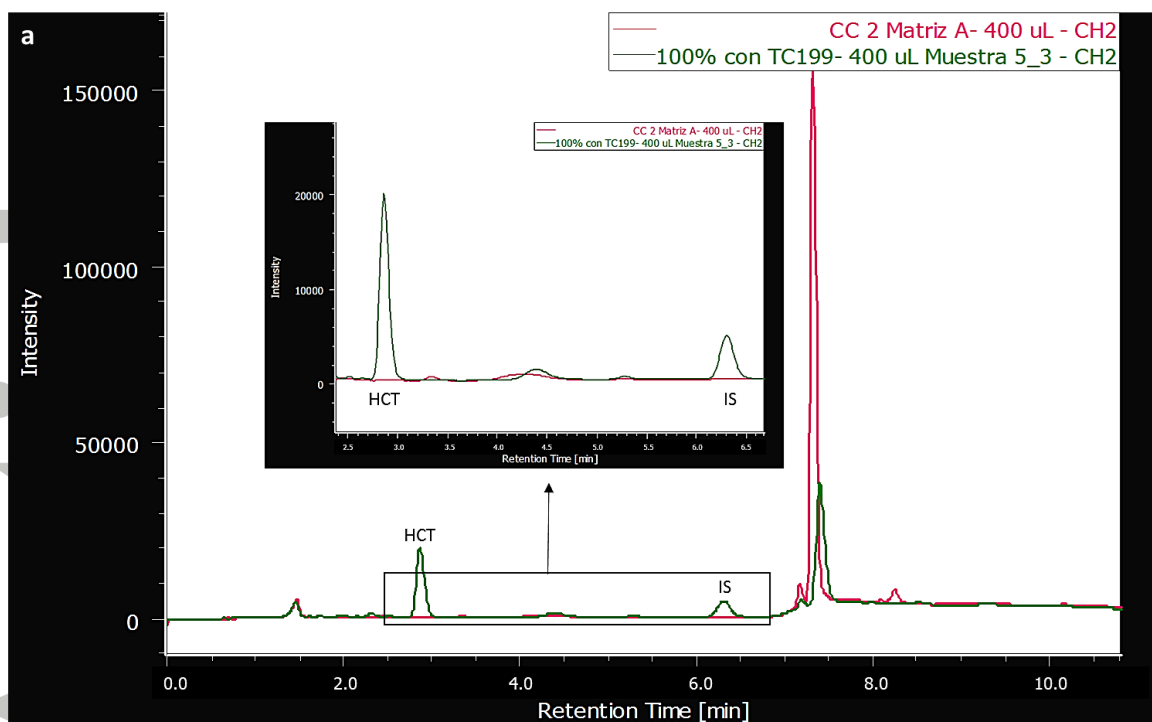
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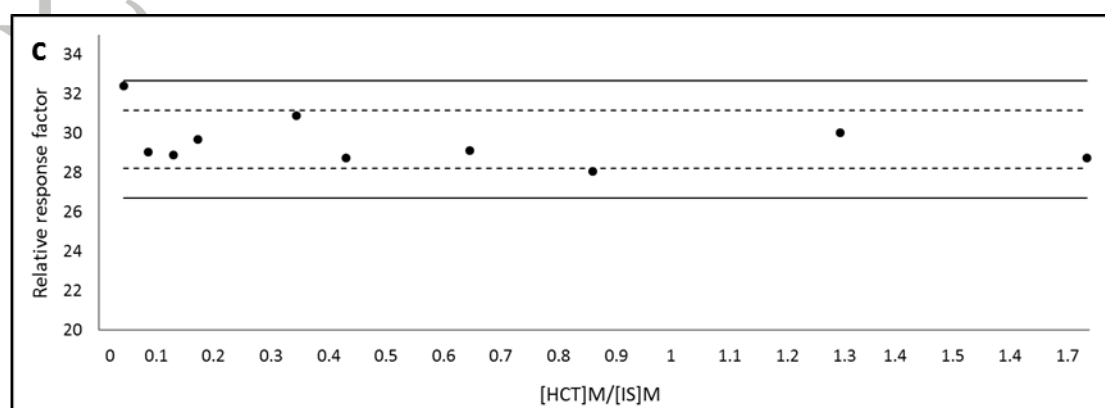
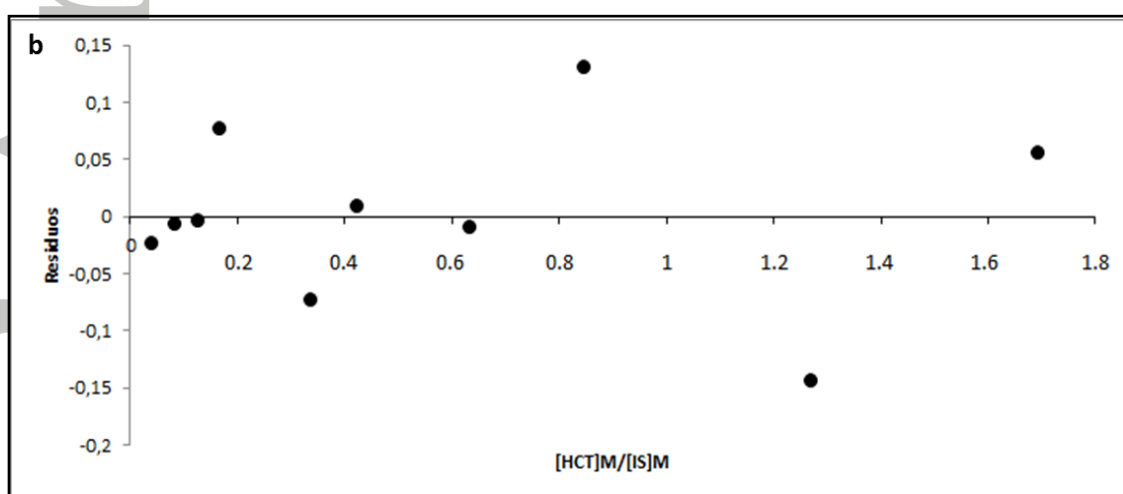
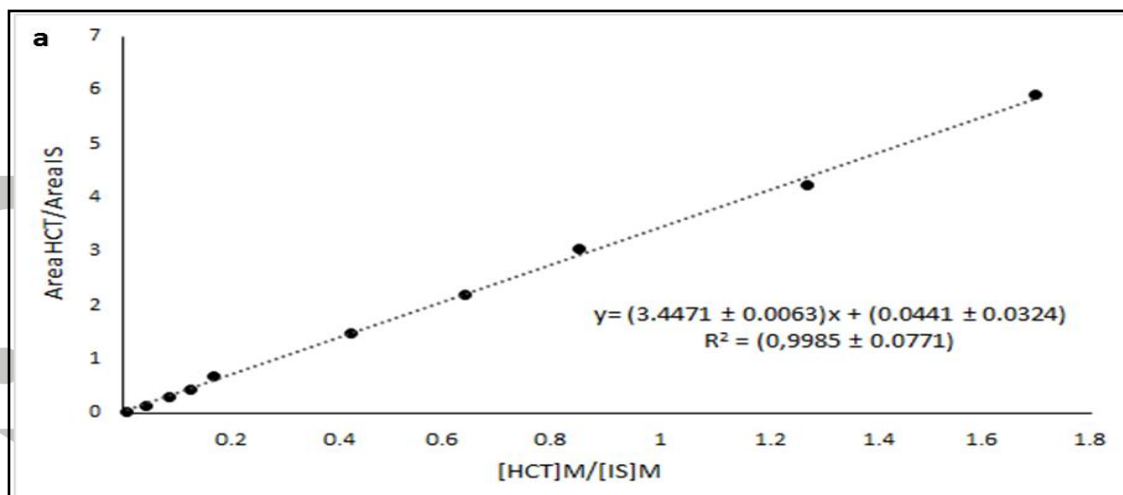
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**Table 1.** Method validation parameter, recovery extraction percentage from SPE and intestinal permeability results (mean  $\pm$  SD; n =10).

Parameter	QC concentration level	Result
<i>Regression equation</i>		3.4471 ( $\pm$ 0.0063)X – 0.0441 ( $\pm$ 0.00324)
Range		1.6107x10 <sup>-7</sup> M to 3.2215x10 <sup>-5</sup> M
Slope ( $\pm$ SD)		3.4471 $\pm$ 0.0063
Intercept ( $\pm$ SD)		0.0441 $\pm$ 0.00324
LOD (3:1 signal to noise ratio) $\mu$ g/mL		3.8325x10 <sup>-8</sup> M
LOQ (10:1 signal to noise ratio) $\mu$ g/mL		1.4491x10 <sup>-7</sup> M
LLOQ $\mu$ g/mL		8.05x10 <sup>-7</sup> M
<i>Accuracy (% recovery)</i>		
Intraday accuracy	L (8.05x10 <sup>-7</sup> M)	88.35 $\pm$ 5.25
	M (8.05x10 <sup>-6</sup> M)	98.15 $\pm$ 4.13
	H (3.2x10 <sup>-5</sup> M)	100.28 $\pm$ 2.25
Inter-day accuracy	L (8.05x10 <sup>-7</sup> M)	87.47 $\pm$ 1.26
	M (8.05x10 <sup>-6</sup> M)	96.07 $\pm$ 3.71
	H (3.2x10 <sup>-5</sup> M)	98.76 $\pm$ 6.87
<i>Precision (%RSD)</i>		
Intraday precision	L (8.05x10 <sup>-7</sup> M)	5.93 $\pm$ 0.01
	M (8.05x10 <sup>-6</sup> M)	4.08 $\pm$ 0.06
	H (3.2x10 <sup>-5</sup> M)	2.19 $\pm$ 0.13
Inter-day precision	L (8.05x10 <sup>-7</sup> M)	4.42 $\pm$ 0.01
	M (8.05x10 <sup>-6</sup> M)	6.28 $\pm$ 0.09
	H (3.2x10 <sup>-5</sup> M)	8.35 $\pm$ 0.49
<i>Repeatability</i>	M (8.05x10 <sup>-6</sup> M)	3.95 $\pm$ 0.05
<i>Recovery extraction % (<math>\pm</math>SD) from SPE</i>		
	L (3.4x10 <sup>-6</sup> M)	81.68 $\pm$ 2.99
	M (1.7x10 <sup>-5</sup> M)	104.49 $\pm$ 2.65
	H (8.4x10 <sup>-5</sup> M)	118.92 $\pm$ 2.31
<i>Intestinal permeability parameters for HCT</i>		
Flux ( $\mu$ g/ml)		0.6199 $\pm$ 0.0085
P <sub>app</sub> (cm/min)		(1.3803 $\pm$ 0.0563) x 10 <sup>-4</sup>

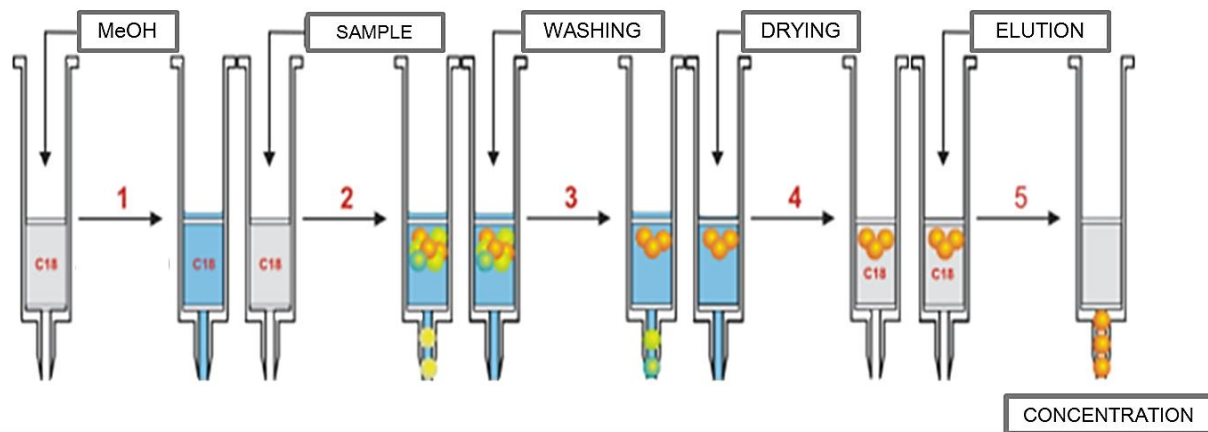


**Figure 1:** a) Blank TC199 medium samples spiked with HCT and IS using the SPE technique. b) Representative intestinal sample using the SPE technique from *ex vivo* rat everted sac study containing HCT and IS.

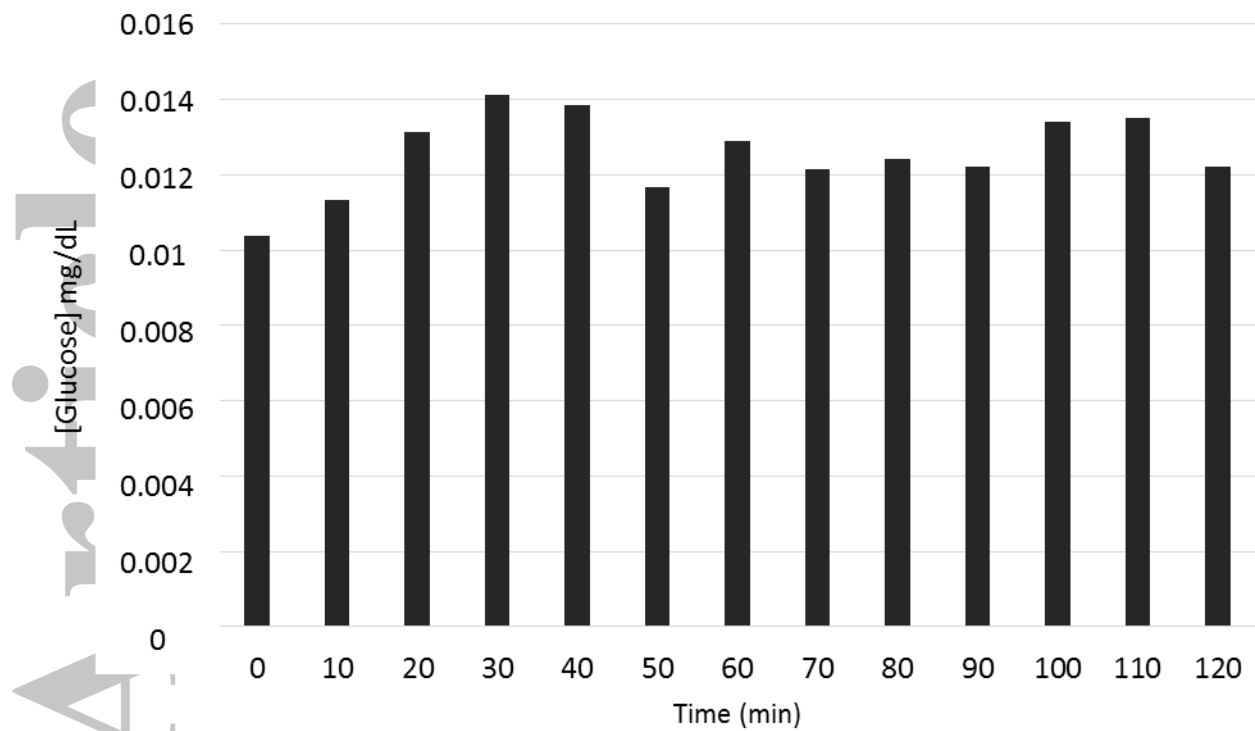


**Figure 2:** a) Calibration plot. b) Residuals plot. c) Linearity plot.

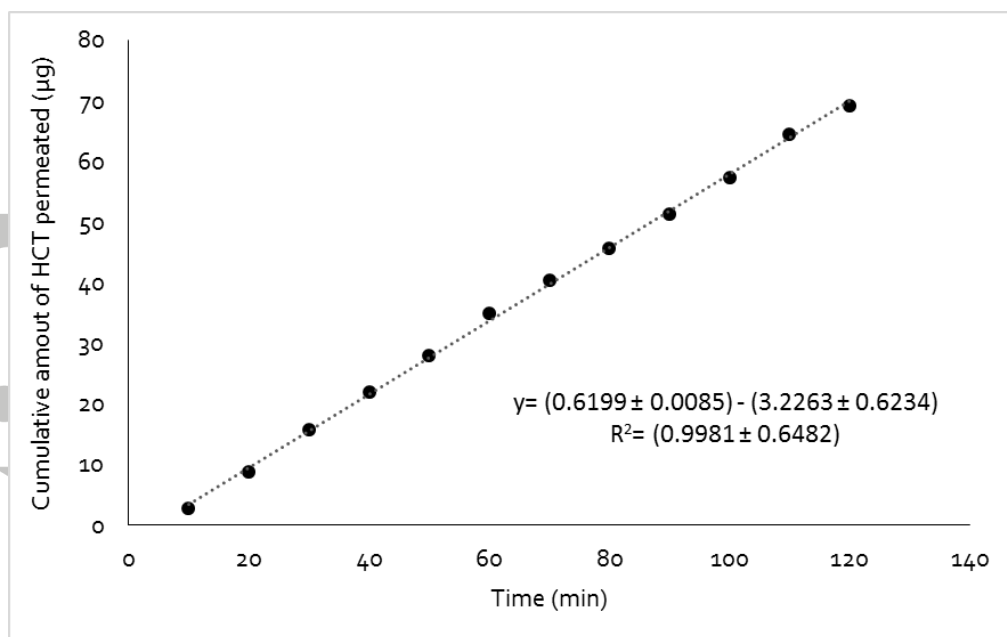




**Figure 3:** Solid phase extraction scheme.



**Figure 4:** Viability of the everted rat gut sac over the time period of incubation in the *in vitro* permeability studies.



**Figure 5:** *In vitro* permeability profiles of HCT. Cumulative amount of HCT permeated (µg) vs time (min).

Accepted