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Abstract

Clinical evaluation of a colchicine specific antigen-binding fragment (Fab) in order to treat colchicine poisoning required the development of an accurate method allowing quantification of free and Fab-bound colchicine in plasma and urine, and free colchicine in tissues, to measure colchicine redistribution after Fab administration. Three methods have been developed for this purpose, and validated in plasma, urine and liver: total colchicine was determined after denaturation of Fab by dilution in water and heating; free colchicine was separated from Fab-bound colchicine by filtration with 30 KDa micro-filters; tissues were homogenized in a tissue mixer. Deuterated colchicine was used as internal standard. Samples were extracted by liquid-liquid extraction and analyzed with a LC-MS/MS. LOQ were 0.5 ng/mL in plasma and urine for free and total colchicine and 5 pg/mg in tissues. The methods were linear in the 0.5-100 ng/mL range in plasma and urine, and 5-300 pg/mg in tissues with determination coefficients > 0.99. Precision and accuracy of QC samples presented a CV < 9.4%. The methods require only 200 µl of sample and allow a high throughput due to short analytical run (2 min). These methods were successfully applied to a pig intoxicated with colchicine and treated with colchicine specific Fab fragments.

Keywords: colchicine; toxicology; mass spectrometry; immunoglobulin Fab fragments; antidote

1. Introduction

Colchicine is a tricyclic alkaloid extracted from *Colchicum autumnale*, used since historic times for acute gout [1]. Nowadays, colchicine is indicated in gouty arthritis, Behçet disease [2] and Mediterranean fever [3]. Colchicine is a potent inhibitor of tubulin polymerization and microtubules genesis, blocking cell mitosis and neutrophil phagocytosis.

Colchicine presents a variable oral bioavailability ranging from 18 to 79% (mean 45%) among healthy volunteers and elderly people [4]. After oral administration of 1 mg of colchicine, plasma peak concentration is observed between 0.5 and 1.5 hours [5]. Colchicine is well distributed in tissues, with a distribution half-life and volume respectively ranging from 0.9 to 1.4 hours and 2.3 to 8.5 L/Kg [4].

Among healthy subjects, the terminal half-life varies from 23 to 41 hours [4]. Less than 40% of a labeled oral dose of colchicine is excreted in urine [6].

Colchicine therapeutic dose is 0.015 mg/Kg, it can be toxic from 0.1 mg/Kg and lethal at 0.8 mg/Kg, exhibiting a narrow therapeutic index [7]. Intoxications are rare, but they are associated with high mortality [8–16]. The course of colchicine toxicity can be divided in three phases [17,18]. The first occurs within the first 24h after ingestion, with gastrointestinal symptomatology (mainly diarrhea). The second develops from 24 h to 72 h, and is manifested by multiorgan failure: disseminated intravascular coagulation (DIC), renal, liver, respiratory, bone marrow and cardiac failure. The third stage is characterized by organ recovery associated with a leukocytosis and alopecia. Currently, there is no treatment available for colchicine intoxication. Colchicine has been reported to be non-dialyzable due to a high distribution volume, and a low body clearance [19]. Colchicine presents a high dissociation constant (10^{-6} to 10^{-7} M at 37°C) with tubulin, with a dissociation half-life of the complex tubulin-colchicine ranging from 20 to 30 hours [20]. This observation explains the long colchicine half-life (20-60 h) among intoxicated patients which is closely related to the complex dissociation [21,22]. Following these observations, an immunotoxicotherapy was developed using colchicine specific fragment antigen-binding (Fab). This therapy was successfully applied in a murine model intoxicated with colchicine lethal doses [23]. In rabbits, after a non-lethal dose of colchicine, Fab alter colchicine disposition with an important redistribution from tissues to blood [24]. In 1995, a clinical case of colchicine intoxication was successfully treated with Fab [8]. Thereafter, in 1996, spongiform encephalopathy and mad cow disease have spread in Europe, and colchicine specific Fab production from ovine origin was stopped in France. Recently, a new colchicine specific Fab has been developed, with a high affinity for colchicine (Ka = 10^{11} M^{-1}) [25].

The evaluation of treatment efficacy required a specific analytical method allowing free and Fab-bound colchicine to be distinguished following colchicine specific Fab treatment. A previous method was developed by radioimmunoassay (RIA) with colchicine specific antiserum, but RIA is used less today due to environmental and economic issues [26]. We present here two rapid and sensitive mass spectrometric methods able to assess free and total (free + Fab-bound) colchicine in plasma and urine, and a third assay in organs.

2. Material and methods

2.1. Chemicals and reagents

The HPLC grade acetonitrile, MS grade water, isopropanol and hexane were provided by Merck (Darmstadt, Germany). Ammonium formate, formic acid and dichloromethane standards were supplied by Sigma Aldrich (Saint-Louis, USA). Colchicine was provided from LGC standards (Teddington, United Kingdom) and colchicine D-3 from Toronto Research Medical (Toronto, Canada). Potassium dihydrophosphate (KH_2PO_4) was purchased from VWR (Radnor, USA). Colchicine-specific Fab fragments were provided by Micropharm (Newcastle Emlyn, UK). Microcon centrifugal filters 30 KDa were purchased from Millipore (Merck, Darmstadt, Germany).

2.2. Standard and quality control solutions

Stock solutions of colchicine and colchicine D-3 (1 mg/mL) were prepared in methanol and stored for one year at – 20°C. Standards solutions were wrapped in aluminium foil to prevent photodegradation of colchicine. Working solutions of colchicine for calibration standards (CS) were prepared at three concentrations (1, 0.1 and 0.01 µg/mL) by dilution in MS grade water of the stock solution. Working solution of colchicine for quality control (QC) was prepared from another 1 mg/mL stock solution. A working internal standard solution (0.1 µg/mL) was prepared by dilution of colchicine D-3 stock solution with MS grade water.

2.3. LC-MS/MS

Colchicine concentrations were measured using an UPLC system (Dionex Ultimate 3000, Thermo, USA) coupled with a tandem mass spectrometer (TSQ Quantiva, Thermo, USA). Analyte separation was carried out on a Hypersil GOLD Thermo column (100 x 2.1mm, 1.9 µm) maintained at 30°C. The device was completed with a pre-column (Thermo Hypersil GOLD 10 x 2.1mm, 5µm). Elution was in isocratic mode with a mobile phase composed by a mixture of 2 mM ammonium formate containing 0.1% formic acid and acetonitrile (40/60, v/v) pumped at 0.2 mL/min. A high rate of acetonitrile was necessary to achieve a short analytical run due to lipophilic properties of colchicine (LogP=1.8 with a

pka=1.85). The total run time for the analysis was 2 min. Analysis was performed on a tandem mass spectrometer (TSQ Quantiva, Thermo, USA) equipped with an electrospray ionization source set in positive mode. An ion-spray voltage of +3.5 kV was applied. The heated capillary temperature was set at 300°C. Nitrogen was employed as sheath and auxiliary gas at a pressure of 35 and 10 arbitrary units, respectively. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. Data were collected in selected reaction monitoring (SRM) mode. In order to determine the MS transitions, two solutions (analyte and IS) were infused into the mass spectrometer using a syringe pump. The most intense product ions resulting from these fragmentations were m/z 400.2 → m/z 295.1 (used for quantification) and → m/z 310.1 for colchicine and m/z 403.25 → m/z 295.1 for colchicine D-3 at collision energy of 37 for all transitions.

2.4. Sample preparation

2.4.1. Total colchicine

Drug-free human plasma samples were obtained from Etablissement Français du Sang, (Le Chesnay, France). Urine samples were collected from member of the laboratory personnel. To 200 µL of plasma or urine, 10 µL of internal standard were added. The determination of the total colchicine concentration required the denaturation of the Fab fragments after incubation in 5 volumes of water for plasma and 2 volumes for urine at 100°C, during 1 h according to the method described by Smith et al. for digoxin specific antibodies [27]. Mixture was then extracted by 3 mL *n*-hexane:dichloromethane:isopropanol (300:150:15, v:v:v) after adding 100 µL of phosphate buffer 2M (pH=8.4, adjusted by addition of sodium hydroxide). Samples were mixed for 10 min and then centrifuged at 3500 rpm for 10 min. The upper organic layer was decanted into another tube and evaporated to dryness under a nitrogen stream. Samples were reconstituted with 100 µL of mobile phase, vortex mixed for 10 s, and transferred into injection vials for analysis. In order to check the effectiveness of the method for degrading the Fab and releasing the colchicine, six plasma samples with 15 ng/mL of colchicine were spiked with an excess of Fab before heating.

2.4.2. Free colchicine

A 200 µL volume of plasma or urine was placed in a Microcon centrifugal filter 30 KDa and centrifuged for 20 min at 13000 rpm, 100 µL of the ultra filtrate were transferred into another tube and 10 µL of internal standard were added. Extraction was as described in section 2.4.1. In order to check the effectiveness of the method for separating Fab-bound and free colchicine, six plasma samples with 15 ng/mL of colchicine were spiked with an excess of Fab before filtration.

2.4.3. Organs

Drug-free human liver samples were provided from former forensic cases. Fifty to 150 mg of liver were weighted and diluted with 3 parts of desionized water (v/w). The mixture was then ground 1 hour using a Tissue Lyser LT (Qiagen, Hilden, Germany) 50 oscillations per second after adding a steel ball. Then 100 µL of phosphate buffer 2M (pH=8.4), 10 µL of IS and 1 mL of dichloromethane were added to 20 µL of homogenates (corresponding to 5 mg of tissue) and mixed 15 min at 50 oscillations by second. After 10 min of centrifugation at 13000 rpm, the upper organic layer was decanted into another tube and evaporated to dryness under a nitrogen stream. Samples were reconstituted with 100 µL of mobile phase, vortex mixed for 10 s, and transferred into injection vials for analysis.

2.5. Method validation procedure

2.5.1. Linearity

Calibration curves included a blank sample, a zero sample, and seven CS over the concentration [0.5-100 ng/mL] range for plasma and urine, and [5-300 pg/mg] for liver. Calibration curves over a period of one month were taken into account for the determination of the best fit. The best fit among linear and quadratic equations was determined using various weighting factors of the inverse concentration (e.g. $1/x$ and $1/x^2$). The equation showing the lowest and most constant percentage total bias from nominal CS values was considered as the best-fit model. The IS method was used for quantification: analytes/IS ratios were plotted against the spiked concentrations. Back-calculated concentrations of the CS had to be within 85-115% of the nominal concentrations.

2.5.2. Specificity, carry over

To investigate whether endogenous matrix constituents interfered with the assay, drug free matrix blank samples, zero samples and samples spiked at the LLOQ were analyzed according to the described procedure. Interferences with others drugs were investigated by analyzing a drug free matrix spiked with 1 µg/mL of analgesic and nonsteroidal anti-inflammatory drugs that could be used in acute gout treatment (acetaminophen, codeine, morphine, tramadol, salicylic acid, mefenamic acid, niflumic acid, nimesulide, ibuprofen, piroxicam, nabumetone, tiaprofenic acid, indomethacin, diclofenac, flurbiprofen, meloxicam, naproxen, sulindac, ketoprofen, rofecoxib, celecoxib). Assay specificity was defined by evidence of non-interference at retention times and ion channels identical to that of colchicine and IS in the blank samples. A blank sample was also analyzed immediately following the highest CS in each run to monitor the carry-over of analyte and IS.

2.5.3. Lower limit of quantification and detection

The LLOQ was defined as the lowest concentration for which an accuracy between 80% and 120% and a precision with a coefficient of variation of ± 20% or less that was obtained over six measurements. A QC was prepared in plasma, urine and in liver at the LLOQ and analyzed as the others QC levels. The limit of detection (LOD) was determined by analyzing serial dilutions in mobile phase of the LLOQ until obtaining the last concentration value with a signal to noise ratio >3.

2.5.4. Accuracy and precision

Accuracy (measured value/nominal value) and precision (coefficient of variation) were determined for three QC levels (low, medium and high level) in each matrix. Each QC level was processed six times three different days over a period of one month. The values obtained were analyzed using analysis of variance (ANOVA), which separated the intra-day and inter-day standard deviation and consequently the corresponding coefficients of variation (CV). An accuracy within the range 85-115% of the nominal values and a precision with a CV of ±15% were required, except for the LLOQ.

2.5.5. Recovery and matrix effect

Three procedures (A, B and C) were performed on six different matrix sources for plasma, urine and liver at two concentrations (1.5-75 ng/mL for liquid matrices and 15-260 pg/mg for liver) in order to evaluate extraction yield and matrix effect (ME). (A) Analytes and the IS were spiked in the mobile phase and directly injected; (B) Analytes and the IS were spiked afterwards in extracted blank matrix samples and injected; and (C) Analytes and IS were spiked in plasma samples, the complete extraction procedure was carried through, and the samples were injected into the system. The mean chromatographic peaks obtained using the three procedures were compared. The ratios C/B, B/A determined the yield of extraction and the matrix effect, respectively.

2.5.6. Stability

Colchicine stability was investigated in plasma at four levels (QC levels and LOQ) after 24 hours at 20±5°C, and after 24 hours and 72 hours at 5±3°C (n=6). Long term stability was evaluated through 1 and 3 months at the same temperatures. Stability after three freeze/thaw cycles was also tested at the three QC levels (n=3). The stability of the vial inside the autosampler (8°C) was evaluated over 24 hours by reinjection of QC and LOQ samples. Samples were considered stable if colchicine variation was less than 15% from initial value.

2.6. Application

One Göttingen mini-pig, age one year, and obtained from the Ellegaard Göttingen Minipigs A/S (Dalmose, Denmark) was used for this pre-clinical study. The study was approved by the Edinburgh University's Ethical Review Committee and was licensed under the Animals (Scientific Procedures) Act 1986. The animal was weighed immediately before entering in the study (30 Kg). The animal was anesthetized before surgery, and remained anesthetized throughout the study under the care of the veterinary anesthetists. Central lines were inserted by "cut down" into the carotid artery and external jugular vein. Bladder catheterization was realized by mini laparotomy. Intra-venous fluids replacement with Hartmann's solution began at induction of anesthesia (10 mL/Kg over 30 min then 5 mL/Kg/hr). The flow rate was increased as required to support central venous pressure. Approximately 30 min before dosing, colchicine was dissolved in physiological saline to achieve selected dose (0.25 mg/Kg).

Solution was administered intravenously over one hour. Three hours after the end of colchicine administration, the animal was treated with an equimolar dose of Fab administered over 1 h. Arterial blood samples were taken at -10 min pre-treatment and 1 h, 5 h and 48 h post-dosing. Plasma was separated by centrifugation at 4.000 rpm for 10 min and transferred to a -80°C freezer for subsequent storage within 48 hours of sampling. Urine was collected pre-treatment and 3 h, 6 h and 48 h post-treatment and immediately frozen, urine volume being recorded. The animal was euthanized by IV overdose with pentobarbital at the end of the experiment (48 h). For post-mortem examination, heart, liver, one kidney and a sample of muscle and small intestine were removed from the animal and rinsed with saline. A sample was frozen at -80°C and stored for subsequent colchicine analysis.

3. Results

3.1. Validation

A chromatogram of a blank sample and plasma sample spiked with 0.5 ng/mL of colchicine (LOQ) are presented in figure 1. The retention times of colchicine and its IS was 1.55 min. No interference from constituents of drug-free human plasma and from all the others tested drugs at the retention times and the ion channels of colchicine and colchicine D-3 were observed. Mean carry-over was lower than 0.1 % for colchicine and colchicine D-3.

The calibration curves exhibited good linearity in the concentration range 0.5-100 ng/mL for plasma and urine, and 5-300 pg/mg for liver with 1/x weighting factor. Mean slopes, intercepts and determination coefficients are reported in table 1. Coefficient of variation (CV) and bias for the back-calculated concentrations of the calibration standards were all <15% and within 85-115% of the nominal concentrations, respectively (Table 1).

Matrix effect and extraction recovery for plasma and liver are presented in table 2.

LOQ and LOD were 0.5 ng/mL and 0.3 ng/mL for plasma and urine respectively, and 5 pg/mg and 3 pg/mg for liver, respectively. Intra-day, inter-day CV and bias for LOQ and QC samples were lower than 15%. Inter-day CV and bias are presented in table 3.

Plasma levels of colchicine were stable under the different tested conditions exhibiting a variation lower than 15%.

No free colchicine was found in plasma samples spiked with 15 ng/mL of colchicine and an excess of Fab before filtration. Conversely, the 15 ng/mL of colchicine were recovered following heating procedure, with a CV and a bias < 15%.

3.2. Application

Total and free colchicine obtained in plasma and urine are presented in table 4. Post-mortem colchicine levels measured in organs are presented in table 5.

4. Discussion

4.1. Validation

Colchicine assay using LC-MS/MS after Fab administration required a separation of free and Fab-bound colchicine, allowing an accurate estimation of colchicine redistribution from tissue to blood. To our knowledge, only one method devoted to measure free and total colchicine has been published [24]. Sabouraud et al. developed a radio immunoassay (RIA) with colchicine-specific antibodies; separation of both free and Fab-bound colchicine was realized using the method described for digoxin and digitoxin by Smith et al. [26,27]. Separation was carried out by equilibrium dialysis overnight for free colchicine. Total colchicine was estimated after Fab denaturation by dilution of plasma in 5 volumes and urine in 2 volumes of water heated to 100 °C for 1h. These authors estimated that 90-94% of bound-colchicine was transformed in free colchicine with their method. Today, due to environmental and safety issues, RIA assays in clinical laboratories are restricted, requiring the development of a novel method. Recently, a LC-MS/MS method was developed by Peake et al. for the determination of total colchicine in plasma and urine following Fab administration in rats [28]. However, the authors gave no information regarding the LOQ, and colchicine recovery following Fab lysis. We presented here a simple method, requiring a shorter preparation step for free colchicine thanks to microfiltration. The weight of Fab fragments is close to 50 KDa, Fab-bound colchicine was separated with 30 KDa

filter after centrifugation, allowing free colchicine (399 Da) to pass. Due to the loss of sample during this step, a larger volume than required (200 µL) was filtered, and the analysis was done using 100 µL of filtrate. Internal standard was necessarily added after filtration to avoid deuterated colchicine fixation to Fab.

Fab lysis was carried out using the method of Smith et al. with a good recovery since colchicine is not thermolabile [27]. When the analysis was done without heating, in the presence of Fab, we observed a total disappearance of deuterated colchicine and a loss of non-deuterated colchicine due to Fab fixation. Consequently, Fab lysis and colchicine release was assessed by deuterated colchicine recovery and by recovering the total colchicine of a spiked plasma with 15 ng/mL and an excess of Fab, showing that our lysis was complete and better than that obtained in the Sabouraud study (24).

Despite a low sample volume (200µL) and a high sensitivity required (therapeutic plasma concentrations: 0.3-2.4 ng/mL), the LOQ of our method was 0.5 ng/mL with a LOD at 0.3 ng/mL [29]. For plasma dilution in 5 volumes of water compared to filtration, extraction recovery fell from 73-85% to 31-33% respectively. This observation is partially explained by an identical volume of solvent used for LLE despite a larger sample volume. In the same way, urine dilution in 2 volumes of water compared to filtration induces a decrease in extraction recovery from 78-90% to 65-69% respectively. However, this methodology was conserved for the validation since the LOQ was still acceptable despite this poor recovery. Three compositions of organic solvent were tested for LLE. Hexane (H), dichoromethane (D) and hexane:dichoromethane:isopropanol (300:150:15, v:v:v) (HDI) [30]. H presented a low colchicine recovery. D and HDI presented equivalent recoveries, but a higher matrix effect was observed in plasma with D. HDI was chosen for plasma and urine LLE.

Few methods are described in literature for colchicine tissue analysis. A first procedure was developed for post-mortem organs by Rochdi et al. using RIA [22]. Later, Kintz et al. presented a novel method developed with HPLC/UV with a sensitivity similar to our (LOQ = 5 pg/mg) [15]. However, this procedure required a large sample (0.75g), a high volume of organic solvent (10 mL dichloromethane) and a longer analytical run (7.6 min). Recently, Peake et al. designed a study to evaluate colchicine specific Fab efficacy in a murine model of colchicine intoxication, with a LC-MS/MS procedure for colchicine measurements in plasma, urine and organs [28]. In this paper, samples homogenates were

diluted in methanol (1 part of sample plus 3 parts methanol) and directly injected in the system, but few information are given regarding the validation criteria of the method (limit of quantification, matrix effect, colchicine recovery). The procedure presented here required a low volume of sample (20µL of organ homogenates corresponding to 5mg of tissue), a low volume of organic solvent (1 mL of dichloromethane) and a short analytical run (2 min) with a good sensitivity. For the LLE in liver, contrary to plasma, a lower matrix effect was observed with D rather than HDI with equivalent recoveries, so D was chosen for liver extraction. LLE was initially performed by simple turning of the tube of homogenates leading to a low extraction yield. A dynamic extraction using the same procedure as liver homogenates production was then tested (50 oscillations by second after adding a steel ball) allowing achievement of an acceptable recovery (58-73%).

4.2. Application

The method was successfully applied to an animal intoxicated with colchicine and treated with a colchicine specific Fab. Concentrations observed for total colchicine were over our LOQ throughout the experiment after colchicine administration. Before Fab administration but after colchicine administration (T1h), free and total colchicine levels were similar both in plasma and urine, all the circulating colchicine being free. After Fab administration (T5h), colchicine was completely bound to Fab, free colchicine being under our LOQ both in plasma and in urine, showing the efficacy of the Fab to bind colchicine and that colchicine is also in the Fab-bound form in urine. After 48h, colchicine is present in both forms (free and Fab-bound) in particular in urine probably because of the Fab metabolism which alter Fab affinity for colchicine, as previously shown for digoxin-specific Fab [31]. Post-mortem organ analyses found similar concentrations between organs, ranging from 73 to 114 pg/mg. Interpretation remains difficult since there is no data for colchicine levels in organs among pigs. Nevertheless, these data appeared low but probably because elimination of colchicine was already significant at 48h as shown by low concentrations in plasma and urine at that time.

5. Conclusion

Two LC-MS/MS methods have been developed for the determination of free and Fab-bound colchicine in pig plasma and urine, and validated in both matrices. The methods require only 200 µl of sample, and allow a high throughput due to a short analytical run (2 min). A third method was developed for tissue analysis and validated in liver. All methods were successfully applied in real samples. This method will allow the pharmacokinetics /pharmacodynamics relationship studies on the efficacy of the Fab fragments in colchicine poisoning.

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Legend

Fig 1. Colchicine ion chromatogram of a plasma blank sample (A); plasma sample spiked with colchicine at a concentration of 0.5 ng/mL (B); a real plasma sample (T5h after Fab infusion) with free colchicine < 0.5 ng/mL (C) and total colchicine at 110 ng/mL (D).

Table 1. Mean slope, intercept, determination coefficient and calibration standard (CS) range (minimum and maximum), CV and bias for the different methods.

Table 2. Mean matrix effect ± coefficient of variation (%) and extraction recovery in plasma and liver.

Table 3. Intra-day and inter-day coefficient of variation and mean bias observed for the limit of quantification (n=6) and quality controls (n=6).

Table 4. Total and free colchicine levels (ng/mL) in plasma and urine measured during the experiment.

Table 5. Post-mortem colchicine levels found in organs.

Highlights :

- Determination of free and Fab-bound colchicine following Fab fragments administration.

- Three LC-MS/MS methods were designed and validated in plasma, urine and organs.

- The methods require only 200 µl of sample, and allow a high throughput due to a 2 min analytical run.

- These methods were applied to a pig treated with colchicine specific Fab fragments.

LC-MS/MS quantification of free and Fab-bound colchicine in plasma, urine and organs following colchicine administration and colchicine-specific Fab fragments treatment in Göttingen minipigs

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Abstract

Clinical evaluation of a colchicine specific antigen-binding fragment (Fab) in order to treat colchicine poisoning required the development of an accurate method allowing quantification of free and Fab-bound colchicine in plasma and urine, and free colchicine in tissues, to measure colchicine redistribution after Fab administration. Three methods have been developed for this purpose, and validated in plasma, urine and liver: total colchicine was determined after denaturation of Fab by dilution in water and heating; free colchicine was separated from Fab-bound colchicine by filtration with 30 KDa micro-filters; tissues were homogenized in a tissue mixer. Deuterated colchicine was used as internal standard. Samples were extracted by liquid-liquid extraction and analyzed with a LC-MS/MS. LOQ were 0.5 ng/mL in plasma and urine for free and total colchicine and 5 pg/mg in tissues. The methods were linear in the 0.5-100 ng/mL range in plasma and urine, and 5-300 pg/mg in tissues with determination coefficients > 0.99. Precision and accuracy of QC samples presented a CV < 9.4%. The methods require only 200 µl of sample and allow a high throughput due to short analytical run (2 min). These methods were successfully applied to a pig intoxicated with colchicine and treated with colchicine specific Fab fragments.

Keywords: colchicine; toxicology; mass spectrometry; immunoglobulin Fab fragments; antidote

1. Introduction

Colchicine is a tricyclic alkaloid extracted from *Colchicum autumnale*, used since historic times for acute gout [1]. Nowadays, colchicine is indicated in gouty arthritis, Behçet disease [2] and Mediterranean fever [3]. Colchicine is a potent inhibitor of tubulin polymerization and microtubules genesis, blocking cell mitosis and neutrophil phagocytosis.

Colchicine presents a variable oral bioavailability ranging from 18 to 79% (mean 45%) among healthy volunteers and elderly people [4]. After oral administration of 1 mg of colchicine, plasma peak concentration is observed between 0.5 and 1.5 hours [5]. Colchicine is well distributed in tissues, with a distribution half-life and volume respectively ranging from 0.9 to 1.4 hours and 2.3 to 8.5 L/Kg [4].

Among healthy subjects, the terminal half-life varies from 23 to 41 hours [4]. Less than 40% of a labeled oral dose of colchicine is excreted in urine [6].

Colchicine therapeutic dose is 0.015 mg/Kg, it can be toxic from 0.1 mg/Kg and lethal at 0.8 mg/Kg, exhibiting a narrow therapeutic index [7]. Intoxications are rare, but they are associated with high mortality [8–16]. The course of colchicine toxicity can be divided in three phases [17,18]. The first occurs within the first 24h after ingestion, with gastrointestinal symptomatology (mainly diarrhea). The second develops from 24 h to 72 h, and is manifested by multiorgan failure: disseminated intravascular coagulation (DIC), renal, liver, respiratory, bone marrow and cardiac failure. The third stage is characterized by organ recovery associated with a leukocytosis and alopecia. Currently, there is no treatment available for colchicine intoxication. Colchicine has been reported to be non-dialyzable due to a high distribution volume, and a low body clearance [19]. Colchicine presents a high dissociation constant (10^{-6} to 10^{-7} M at 37°C) with tubulin, with a dissociation half-life of the complex tubulin-colchicine ranging from 20 to 30 hours [20]. This observation explains the long colchicine half-life (20-60 h) among intoxicated patients which is closely related to the complex dissociation [21,22]. Following these observations, an immunotoxicotherapy was developed using colchicine specific fragment antigen-binding (Fab). This therapy was successfully applied in a murine model intoxicated with colchicine lethal doses [23]. In rabbits, after a non-lethal dose of colchicine, Fab alter colchicine disposition with an important redistribution from tissues to blood [24]. In 1995, a clinical case of colchicine intoxication was successfully treated with Fab [8]. Thereafter, in 1996, spongiform encephalopathy and mad cow disease have spread in Europe, and colchicine specific Fab production from ovine origin was stopped in France. Recently, a new colchicine specific Fab has been developed, with a high affinity for colchicine (Ka = 10^{11} M^{-1}) [25].

The evaluation of treatment efficacy required a specific analytical method allowing free and Fab-bound colchicine to be distinguished following colchicine specific Fab treatment. A previous method was developed by radioimmunoassay (RIA) with colchicine specific antiserum, but RIA is used less today due to environmental and economic issues [26]. We present here two rapid and sensitive mass spectrometric methods able to assess free and total (free + Fab-bound) colchicine in plasma and urine, and a third assay in organs.

2. Material and methods

2.1. Chemicals and reagents

The HPLC grade acetonitrile, MS grade water, isopropanol and hexane were provided by Merck (Darmstadt, Germany). Ammonium formate, formic acid and dichloromethane standards were supplied by Sigma Aldrich (Saint-Louis, USA). Colchicine was provided from LGC standards (Teddington, United Kingdom) and colchicine D-3 from Toronto Research Medical (Toronto, Canada). Potassium dihydrophosphate (KH_2PO_4) was purchased from VWR (Radnor, USA). Colchicine-specific Fab fragments were provided by Micropharm (Newcastle Emlyn, UK). Microcon centrifugal filters 30 KDa were purchased from Millipore (Merck, Darmstadt, Germany).

2.2. Standard and quality control solutions

Stock solutions of colchicine and colchicine D-3 (1 mg/mL) were prepared in methanol and stored for one year at – 20°C. Standards solutions were wrapped in aluminium foil to prevent photodegradation of colchicine. Working solutions of colchicine for calibration standards (CS) were prepared at three concentrations (1, 0.1 and 0.01 µg/mL) by dilution in MS grade water of the stock solution. Working solution of colchicine for quality control (QC) was prepared from another 1 mg/mL stock solution. A working internal standard solution (0.1 µg/mL) was prepared by dilution of colchicine D-3 stock solution with MS grade water.

2.3. LC-MS/MS

Colchicine concentrations were measured using an UPLC system (Dionex Ultimate 3000, Thermo, USA) coupled with a tandem mass spectrometer (TSQ Quantiva, Thermo, USA). Analyte separation was carried out on a Hypersil GOLD Thermo column (100 x 2.1mm, 1.9 µm) maintained at 30°C. The device was completed with a pre-column (Thermo Hypersil GOLD 10 x 2.1mm, 5µm). Elution was in isocratic mode with a mobile phase composed by a mixture of 2 mM ammonium formate containing 0.1% formic acid and acetonitrile (40/60, v/v) pumped at 0.2 mL/min. A high rate of acetonitrile was necessary to achieve a short analytical run due to lipophilic properties of colchicine (LogP=1.8 with a

pka=1.85). The total run time for the analysis was 2 min. Analysis was performed on a tandem mass spectrometer (TSQ Quantiva, Thermo, USA) equipped with an electrospray ionization source set in positive mode. An ion-spray voltage of +3.5 kV was applied. The heated capillary temperature was set at 300°C. Nitrogen was employed as sheath and auxiliary gas at a pressure of 35 and 10 arbitrary units, respectively. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. Data were collected in selected reaction monitoring (SRM) mode. In order to determine the MS transitions, two solutions (analyte and IS) were infused into the mass spectrometer using a syringe pump. The most intense product ions resulting from these fragmentations were m/z 400.2 → m/z 295.1 (used for quantification) and → m/z 310.1 for colchicine and m/z 403.25 → m/z 295.1 for colchicine D-3 at collision energy of 37 for all transitions.

2.4. Sample preparation

2.4.1. Total colchicine

Drug-free human plasma samples were obtained from Etablissement Français du Sang, (Le Chesnay, France). Urine samples were collected from member of the laboratory personnel. To 200 µL of plasma or urine, 10 µL of internal standard were added. The determination of the total colchicine concentration required the denaturation of the Fab fragments after incubation in 5 volumes of water for plasma and 2 volumes for urine at 100°C, during 1 h according to the method described by Smith et al. for digoxin specific antibodies [27]. Mixture was then extracted by 3 mL *n*-hexane:dichloromethane:isopropanol (300:150:15, v:v:v) after adding 100 µL of phosphate buffer 2M (pH=8.4, adjusted by addition of sodium hydroxide). Samples were mixed for 10 min and then centrifuged at 3500 rpm for 10 min. The upper organic layer was decanted into another tube and evaporated to dryness under a nitrogen stream. Samples were reconstituted with 100 µL of mobile phase, vortex mixed for 10 s, and transferred into injection vials for analysis. In order to check the effectiveness of the method for degrading the Fab and releasing the colchicine, six plasma samples with 15 ng/mL of colchicine were spiked with an excess of Fab before heating.

2.4.2. Free colchicine

A 200 µL volume of plasma or urine was placed in a Microcon centrifugal filter 30 KDa and centrifuged for 20 min at 13000 rpm, 100 µL of the ultra filtrate were transferred into another tube and 10 µL of internal standard were added. Extraction was as described in section 2.4.1. In order to check the effectiveness of the method for separating Fab-bound and free colchicine, six plasma samples with 15 ng/mL of colchicine were spiked with an excess of Fab before filtration.

2.4.3. Organs

Drug-free human liver samples were provided from former forensic cases. Fifty to 150 mg of liver were weighted and diluted with 3 parts of desionized water (v/w). The mixture was then ground 1 hour using a Tissue Lyser LT (Qiagen, Hilden, Germany) 50 oscillations per second after adding a steel ball. Then 100 µL of phosphate buffer 2M (pH=8.4), 10 µL of IS and 1 mL of dichloromethane were added to 20 µL of homogenates (corresponding to 5 mg of tissue) and mixed 15 min at 50 oscillations by second. After 10 min of centrifugation at 13000 rpm, the upper organic layer was decanted into another tube and evaporated to dryness under a nitrogen stream. Samples were reconstituted with 100 µL of mobile phase, vortex mixed for 10 s, and transferred into injection vials for analysis.

2.5. Method validation procedure

2.5.1. Linearity

Calibration curves included a blank sample, a zero sample, and seven CS over the concentration [0.5-100 ng/mL] range for plasma and urine, and [5-300 pg/mg] for liver. Calibration curves over a period of one month were taken into account for the determination of the best fit. The best fit among linear and quadratic equations was determined using various weighting factors of the inverse concentration (e.g. $1/x$ and $1/x^2$). The equation showing the lowest and most constant percentage total bias from nominal CS values was considered as the best-fit model. The IS method was used for quantification: analytes/IS ratios were plotted against the spiked concentrations. Back-calculated concentrations of the CS had to be within 85-115% of the nominal concentrations.

2.5.2. Specificity, carry over

To investigate whether endogenous matrix constituents interfered with the assay, drug free matrix blank samples, zero samples and samples spiked at the LLOQ were analyzed according to the described procedure. Interferences with others drugs were investigated by analyzing a drug free matrix spiked with 1 µg/mL of analgesic and nonsteroidal anti-inflammatory drugs that could be used in acute gout treatment (acetaminophen, codeine, morphine, tramadol, salicylic acid, mefenamic acid, niflumic acid, nimesulide, ibuprofen, piroxicam, nabumetone, tiaprofenic acid, indomethacin, diclofenac, flurbiprofen, meloxicam, naproxen, sulindac, ketoprofen, rofecoxib, celecoxib). Assay specificity was defined by evidence of non-interference at retention times and ion channels identical to that of colchicine and IS in the blank samples. A blank sample was also analyzed immediately following the highest CS in each run to monitor the carry-over of analyte and IS.

2.5.3. Lower limit of quantification and detection

The LLOQ was defined as the lowest concentration for which an accuracy between 80% and 120% and a precision with a coefficient of variation of ± 20% or less that was obtained over six measurements. A QC was prepared in plasma, urine and in liver at the LLOQ and analyzed as the others QC levels. The limit of detection (LOD) was determined by analyzing serial dilutions in mobile phase of the LLOQ until obtaining the last concentration value with a signal to noise ratio >3.

2.5.4. Accuracy and precision

Accuracy (measured value/nominal value) and precision (coefficient of variation) were determined for three QC levels (low, medium and high level) in each matrix. Each QC level was processed six times three different days over a period of one month. The values obtained were analyzed using analysis of variance (ANOVA), which separated the intra-day and inter-day standard deviation and consequently the corresponding coefficients of variation (CV). An accuracy within the range 85-115% of the nominal values and a precision with a CV of ±15% were required, except for the LLOQ.

2.5.5. Recovery and matrix effect

Three procedures (A, B and C) were performed on six different matrix sources for plasma, urine and liver at two concentrations (1.5-75 ng/mL for liquid matrices and 15-260 pg/mg for liver) in order to evaluate extraction yield and matrix effect (ME). (A) Analytes and the IS were spiked in the mobile phase and directly injected; (B) Analytes and the IS were spiked afterwards in extracted blank matrix samples and injected; and (C) Analytes and IS were spiked in plasma samples, the complete extraction procedure was carried through, and the samples were injected into the system. The mean chromatographic peaks obtained using the three procedures were compared. The ratios C/B, B/A determined the yield of extraction and the matrix effect, respectively.

2.5.6. Stability

Colchicine stability was investigated in plasma at four levels (QC levels and LOQ) after 24 hours at 20±5°C, and after 24 hours and 72 hours at 5±3°C (n=6). Long term stability was evaluated through 1 and 3 months at the same temperatures. Stability after three freeze/thaw cycles was also tested at the three QC levels (n=3). The stability of the vial inside the autosampler (8°C) was evaluated over 24 hours by reinjection of QC and LOQ samples. Samples were considered stable if colchicine variation was less than 15% from initial value.

2.6. Application

One Göttingen mini-pig, age one year, and obtained from the Ellegaard Göttingen Minipigs A/S (Dalmose, Denmark) was used for this pre-clinical study. The study was approved by the Edinburgh University's Ethical Review Committee and was licensed under the Animals (Scientific Procedures) Act 1986. The animal was weighed immediately before entering in the study (30 Kg). The animal was anesthetized before surgery, and remained anesthetized throughout the study under the care of the veterinary anesthetists. Central lines were inserted by "cut down" into the carotid artery and external jugular vein. Bladder catheterization was realized by mini laparotomy. Intra-venous fluids replacement with Hartmann's solution began at induction of anesthesia (10 mL/Kg over 30 min then 5 mL/Kg/hr). The flow rate was increased as required to support central venous pressure. Approximately 30 min before dosing, colchicine was dissolved in physiological saline to achieve selected dose (0.25 mg/Kg).

Solution was administered intravenously over one hour. Three hours after the end of colchicine administration, the animal was treated with an equimolar dose of Fab administered over 1 h. Arterial blood samples were taken at -10 min pre-treatment and 1 h, 5 h and 48 h post-dosing. Plasma was separated by centrifugation at 4.000 rpm for 10 min and transferred to a -80°C freezer for subsequent storage within 48 hours of sampling. Urine was collected pre-treatment and 3 h, 6 h and 48 h post-treatment and immediately frozen, urine volume being recorded. The animal was euthanized by IV overdose with pentobarbital at the end of the experiment (48 h). For post-mortem examination, heart, liver, one kidney and a sample of muscle and small intestine were removed from the animal and rinsed with saline. A sample was frozen at -80°C and stored for subsequent colchicine analysis.

3. Results

3.1. Validation

A chromatogram of a blank sample and plasma sample spiked with 0.5 ng/mL of colchicine (LOQ) are presented in figure 1. The retention times of colchicine and its IS was 1.55 min. No interference from constituents of drug-free human plasma and from all the others tested drugs at the retention times and the ion channels of colchicine and colchicine D-3 were observed. Mean carry-over was lower than 0.1 % for colchicine and colchicine D-3.

The calibration curves exhibited good linearity in the concentration range 0.5-100 ng/mL for plasma and urine, and 5-300 pg/mg for liver with 1/x weighting factor. Mean slopes, intercepts and determination coefficients are reported in table 1. Coefficient of variation (CV) and bias for the back-calculated concentrations of the calibration standards were all <15% and within 85-115% of the nominal concentrations, respectively (Table 1).

Matrix effect and extraction recovery for plasma and liver are presented in table 2.

LOQ and LOD were 0.5 ng/mL and 0.3 ng/mL for plasma and urine respectively, and 5 pg/mg and 3 pg/mg for liver, respectively. Intra-day, inter-day CV and bias for LOQ and QC samples were lower than 15%. Inter-day CV and bias are presented in table 3.

Plasma levels of colchicine were stable under the different tested conditions exhibiting a variation lower than 15%.

No free colchicine was found in plasma samples spiked with 15 ng/mL of colchicine and an excess of Fab before filtration. Conversely, the 15 ng/mL of colchicine were recovered following heating procedure, with a CV and a bias < 15%.

3.2. Application

Total and free colchicine obtained in plasma and urine are presented in table 4. Post-mortem colchicine levels measured in organs are presented in table 5.

4. Discussion

4.1. Validation

Colchicine assay using LC-MS/MS after Fab administration required a separation of free and Fab-bound colchicine, allowing an accurate estimation of colchicine redistribution from tissue to blood. To our knowledge, only one method devoted to measure free and total colchicine has been published [24]. Sabouraud et al. developed a radio immunoassay (RIA) with colchicine-specific antibodies; separation of both free and Fab-bound colchicine was realized using the method described for digoxin and digitoxin by Smith et al. [26,27]. Separation was carried out by equilibrium dialysis overnight for free colchicine. Total colchicine was estimated after Fab denaturation by dilution of plasma in 5 volumes and urine in 2 volumes of water heated to 100 °C for 1h. These authors estimated that 90-94% of bound-colchicine was transformed in free colchicine with their method. Today, due to environmental and safety issues, RIA assays in clinical laboratories are restricted, requiring the development of a novel method. Recently, a LC-MS/MS method was developed by Peake et al. for the determination of total colchicine in plasma and urine following Fab administration in rats [28]. However, the authors gave no information regarding the LOQ, and colchicine recovery following Fab lysis. We presented here a simple method, requiring a shorter preparation step for free colchicine thanks to microfiltration. The weight of Fab fragments is close to 50 KDa, Fab-bound colchicine was separated with 30 KDa

filter after centrifugation, allowing free colchicine (399 Da) to pass. Due to the loss of sample during this step, a larger volume than required (200 µL) was filtered, and the analysis was done using 100 µL of filtrate. Internal standard was necessarily added after filtration to avoid deuterated colchicine fixation to Fab.

Fab lysis was carried out using the method of Smith et al. with a good recovery since colchicine is not thermolabile [27]. When the analysis was done without heating, in the presence of Fab, we observed a total disappearance of deuterated colchicine and a loss of non-deuterated colchicine due to Fab fixation. Consequently, Fab lysis and colchicine release was assessed by deuterated colchicine recovery and by recovering the total colchicine of a spiked plasma with 15 ng/mL and an excess of Fab, showing that our lysis was complete and better than that obtained in the Sabouraud study (24).

Despite a low sample volume (200µL) and a high sensitivity required (therapeutic plasma concentrations: 0.3-2.4 ng/mL), the LOQ of our method was 0.5 ng/mL with a LOD at 0.3 ng/mL [29]. For plasma dilution in 5 volumes of water compared to filtration, extraction recovery fell from 73-85% to 31-33% respectively. This observation is partially explained by an identical volume of solvent used for LLE despite a larger sample volume. In the same way, urine dilution in 2 volumes of water compared to filtration induces a decrease in extraction recovery from 78-90% to 65-69% respectively. However, this methodology was conserved for the validation since the LOQ was still acceptable despite this poor recovery. Three compositions of organic solvent were tested for LLE. Hexane (H), dichoromethane (D) and hexane:dichoromethane:isopropanol (300:150:15, v:v:v) (HDI) [30]. H presented a low colchicine recovery. D and HDI presented equivalent recoveries, but a higher matrix effect was observed in plasma with D. HDI was chosen for plasma and urine LLE.

Few methods are described in literature for colchicine tissue analysis. A first procedure was developed for post-mortem organs by Rochdi et al. using RIA [22]. Later, Kintz et al. presented a novel method developed with HPLC/UV with a sensitivity similar to our (LOQ = 5 pg/mg) [15]. However, this procedure required a large sample (0.75g), a high volume of organic solvent (10 mL dichloromethane) and a longer analytical run (7.6 min). Recently, Peake et al. designed a study to evaluate colchicine specific Fab efficacy in a murine model of colchicine intoxication, with a LC-MS/MS procedure for colchicine measurements in plasma, urine and organs [28]. In this paper, samples homogenates were

diluted in methanol (1 part of sample plus 3 parts methanol) and directly injected in the system, but few information are given regarding the validation criteria of the method (limit of quantification, matrix effect, colchicine recovery). The procedure presented here required a low volume of sample (20µL of organ homogenates corresponding to 5mg of tissue), a low volume of organic solvent (1 mL of dichloromethane) and a short analytical run (2 min) with a good sensitivity. For the LLE in liver, contrary to plasma, a lower matrix effect was observed with D rather than HDI with equivalent recoveries, so D was chosen for liver extraction. LLE was initially performed by simple turning of the tube of homogenates leading to a low extraction yield. A dynamic extraction using the same procedure as liver homogenates production was then tested (50 oscillations by second after adding a steel ball) allowing achievement of an acceptable recovery (58-73%).

4.2. Application

The method was successfully applied to an animal intoxicated with colchicine and treated with a colchicine specific Fab. Concentrations observed for total colchicine were over our LOQ throughout the experiment after colchicine administration. Before Fab administration but after colchicine administration (T1h), free and total colchicine levels were similar both in plasma and urine, all the circulating colchicine being free. After Fab administration (T5h), colchicine was completely bound to Fab, free colchicine being under our LOQ both in plasma and in urine, showing the efficacy of the Fab to bind colchicine and that colchicine is also in the Fab-bound form in urine. After 48h, colchicine is present in both forms (free and Fab-bound) in particular in urine probably because of the Fab metabolism which alter Fab affinity for colchicine, as previously shown for digoxin-specific Fab [31]. Post-mortem organ analyses found similar concentrations between organs, ranging from 73 to 114 pg/mg. Interpretation remains difficult since there is no data for colchicine levels in organs among pigs. Nevertheless, these data appeared low but probably because elimination of colchicine was already significant at 48h as shown by low concentrations in plasma and urine at that time.

5. Conclusion

Two LC-MS/MS methods have been developed for the determination of free and Fab-bound colchicine in pig plasma and urine, and validated in both matrices. The methods require only 200 µl of sample, and allow a high throughput due to a short analytical run (2 min). A third method was developed for tissue analysis and validated in liver. All methods were successfully applied in real samples. This method will allow the pharmacokinetics /pharmacodynamics relationship studies on the efficacy of the Fab fragments in colchicine poisoning.

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Legend

Fig 1. Colchicine ion chromatogram of a plasma blank sample (A); plasma sample spiked with colchicine at a concentration of 0.5 ng/mL (B); a real plasma sample (T5h after Fab infusion) with free colchicine < 0.5 ng/mL (C) and total colchicine at 110 ng/mL (D).

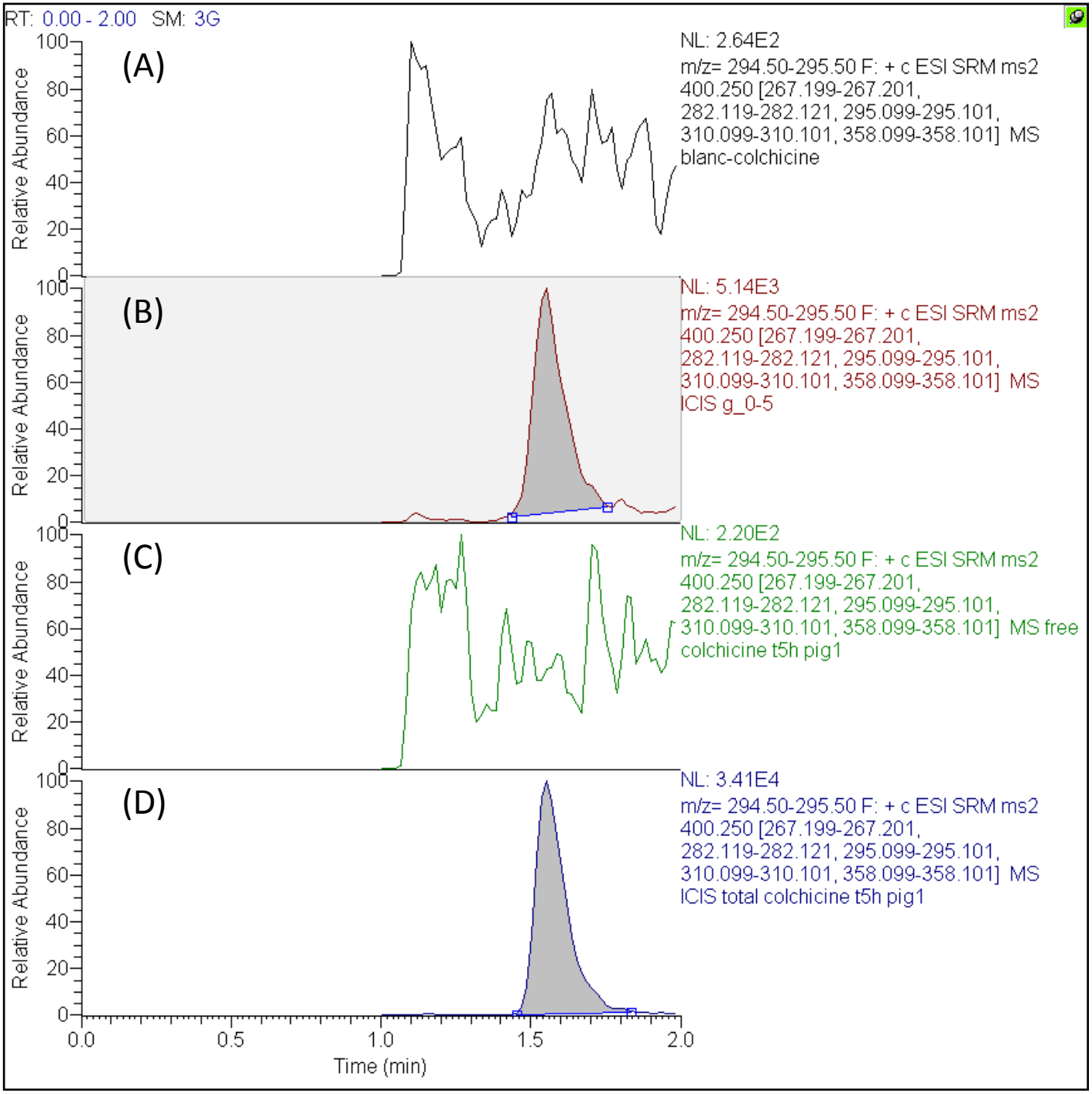
Table 1. Mean slope, intercept, determination coefficient and calibration standard (CS) range (minimum and maximum), CV and bias for the different methods.

Table 2. Mean matrix effect ± coefficient of variation (%) and extraction recovery in plasma and liver.

Table 3. Intra-day and inter-day coefficient of variation and mean bias observed for the limit of quantification (n=6) and quality controls (n=6).

Table 4. Total and free colchicine levels (ng/mL) in plasma and urine measured during the experiment.

Table 5. Post-mortem colchicine levels found in organs.


RT: 0.00 - 2.00 SM: 3G
(A)
NL: 2.64E2
m/z= 294.50-295.50 F: + c ESI SRM ms2 400.250 [267.199-267.201, 282.119-282.121, 295.099-295.101, 310.099-310.101, 358.099-358.101] MS blanc-colchicine
(B)
NL: 5.14E3
m/z= 294.50-295.50 F: + c ESI SRM ms2 400.250 [267.199-267.201, 282.119-282.121, 295.099-295.101, 310.099-310.101, 358.099-358.101] MS ICIS g_0-5
(C)
NL: 2.20E2
m/z= 294.50-295.50 F: + c ESI SRM ms2 400.250 [267.199-267.201, 282.119-282.121, 295.099-295.101, 310.099-310.101, 358.099-358.101] MS free colchicine t5h pig1
(D)
NL: 3.41E4
m/z= 294.50-295.50 F: + c ESI SRM ms2 400.250 [267.199-267.201, 282.119-282.121, 295.099-295.101, 310.099-310.101, 358.099-358.101] MS ICIS total colchicine t5h pig1
Relative Abundance
0
20
40
60
80
100
0.0
0.5
1.0
1.5
2.0
Time (min)

	Slope ± SD	Intercept ± SD	R^2 ± SD	CS CV (min;max,%)	CS bias (min;max,%)
Plasma free colchicine (n=4)	0.167 ± 0.101	0.080 ± 0.123	0.9992 ± 0.0004	1.53;8.47	-0.37;-10.2
Plasma total colchicine (n=4)	0.276 ± 0.022	0.009 ± 0.031	0.9983 ± 0.0011	2.24;11.67	-1.15;3.33
Urine free colchicine (n=4)	0.180 ± 0.023	0.014 ± 0.029	0.9975 ± 0.0007	1.15;4.72	-3.39;-11.67
Urine total colchicine (n=4)	0.365 ± 0.064	-0.001 ± 0.053	0.9986 ± 0.0006	3.14;10.5	0.15;-6.00
Liver (n=5)	0.031 ± 0.001	0.100 ± 0.054	0.9993 ± 0.0002	0.62;7.15	0.48;-7.19

Table 1.

		1.5 ng/mL	75 ng/mL
Plasma free colchicine	Matrix effect	85±10%	73±3%
	Extraction yield	94±14%	91±3%
Plasma total colchicine	Matrix effect	84±13%	72±9%
	Extraction yield	33±6%	31±9%
Urine free colchicine	Matrix effect	86±7%	98±10%
	Extraction yield	90±6%	78±4%
Urine total colchicine	Matrix effect	67±4%	97±10%
	Extraction yield	65±8%	69±12%
		15 pg/mg	**260 pg/mg**
Liver	Matrix effect	90±4%	99±9%
	Extraction yield	58±4 %	73±5%

Table 2.

QC		0.5 ng/mL		1.5 ng/mL		15 ng/mL		75 ng/mL	
		CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Plasma free colchicine	**Intra-day**	5.4	-10.1	4.6	6.4	3.7	9.7	2.5	1.8
	Inter-day	14.1	-4.8	8.8	3.0	5.5	6.3	5.7	5.2
Plasma total colchicine	**Intra-day**	4.2	3.5	3.7	-10.2	5.1	-6.3	4.2	-7.6
	Inter-day	6.4	-5.5	4.7	-6.1	4.3	-6.7	3.2	-2.2
Urine free colchicine	**Intra-day**	8.6	-0.6	8.5	3.1	4.5	6.1	3.3	4.1
	Inter-day	1.4	-5.6	9.4	-2.6	4.4	3.9	4.4	4.2
Urine total colchicine	**Intra-day**	8.1	1.3	2.0	-3.0	3.9	4.6	3.7	-4.6
	Inter-day	10.0	5.0	6.2	-2.9	3.5	6.0	7.0	3.9
QC		**5 pg/mg**		**15 pg/mg**		**90 pg/mg**		**260 pg/mg**	
Liver	**Intra-day**	5.2	-4.1	4.8	1.2	1.4	1.5	0.6	0.8
	Inter-day	2.4	-3.5	5.0	1.6	2.3	0.4	3.0	-0.6

Table 3.

Time post colchicine-infusion		**T0h**	**T1h**	**T5h**	**T48h**
Plasma (ng/mL)	**Free colchicine**	<0.5	190	<0.5	1.6
	Total colchicine	<0.5	181	110	19
Time post colchicine-infusion		T0h	T3h	T6h	T48h
Urine (ng/mL)	**Free colchicine**	<0.5	6255	<0.5	130
	Total colchicine	<0.5	6835	1965	208

Table 4.

	Colchicine (pg/mg)
Heart	78
Liver	73
Lung	75
Muscle	90
Small intestine	114

Table 5.

	Slope ± SD	Intercept ± SD	R^2 ± SD	CS CV (min;max,%)	CS bias (min;max,%)
Plasma free colchicine (n=4)	0.167 ± 0.101	0.080 ± 0.123	0.9992 ± 0.0004	1.53;8.47	-0.37;-10.2
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Urine total colchicine (n=4)	0.365 ± 0.064	-0.001 ± 0.053	0.9986 ± 0.0006	3.14;10.5	0.15;-6.00
Liver (n=5)	0.031 ± 0.001	0.100 ± 0.054	0.9993 ± 0.0002	0.62;7.15	0.48;-7.19

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	Inter-day	1.4	-5.6	9.4	-2.6	4.4	3.9	4.4	4.2
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	Inter-day	10.0	5.0	6.2	-2.9	3.5	6.0	7.0	3.9
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