Journal of Chromatography B, 878 (2010) 3134–3142

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of an HPLC–MS/MS method to determine clopidogrel in human plasma. Use of incurred samples to test back-conversion

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ARTICLE INFO

Article history:
Received 7 April 2010
Accepted 22 September 2010
Available online 1 October 2010

Keywords:
Clopidogrel
Platelet aggregation
Liquid chromatography
Tandem mass spectrometry
Pharmacokinetics
Back-conversion

ABSTRACT

Quantitative methods using LC–MS/MS allow achievement of adequate sensitivity for pharmacokinetic studies with clopidogrel; three such methods, with LLOQs as low as 5 pg/mL, were developed and fully validated according to the well established FDA 2001 guidelines. The chromatographic separations were performed on reversed phase columns Ascentis RP-Amide (15 cm × 2.1 mm, 5 μm), Ascentis Express C8 (10 cm × 2.1 mm, 2.7 μm) and Ascentis Express RP Amide (10 cm × 2.1 mm, 2.7 μm), respectively. Positive electrospray ionization in MRM mode was employed for the detection and a deuterated analogue (d3-clopidogrel) was used as internal standard. Linearity, precision, extraction recovery, matrix effects and stability tests on blank plasma spiked with clopidogrel and stored in different conditions met the acceptance criteria. During the analysis of the real samples from the first pharmacokinetic study, a significant increase (>100%) of the measured clopidogrel concentrations in the extracts kept in the autosampler at 10 °C was observed. Investigations led to the conclusion that most probably a back-conversion of one or more of the clopidogrel metabolites is occurring. The next methods were optimized in order to minimize this back-conversion. After a series of experiments, the adjustment of the sample preparation (e.g. processing at low temperature and introducing a clean-up step on Supelco HybridSPE-Precipitation cartridges) has proven to be the most effective in order to improve the stability of the extracts. Incurred samples of real subjects were successfully used in the validation of the last two analytical methods to evaluate the back-conversion, while tests using only the known metabolites could not detect this important problem.

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1. Introduction

Clopidogrel is a potent drug inhibiting platelet aggregation [1]; an oxidative metabolite is responsible for this activity while the most quantitatively relevant metabolite in plasma, a carboxyl derivative, is pharmacologically inactive [2,3].

During drug development the inactive carboxyl metabolite was studied in order to understand clopidogrel pharmacokinetics, since the clopidogrel levels were too low to be measured with the analytical instruments available at that time [3]. Recently, LC–MS/MS methods with adequate sensitivity to quantify clopidogrel in biological fluids have been developed [4–6]. The main application of such methods was the development of generic formulations of clopidogrel according to bioequivalence regulations (EMEA, FDA, etc.); these norms require the measurement of the parent compound, whenever possible from the analytical point of view, since this is the most sensitive to formulation problems [7,8]. Recently, European authorities (EMEA) have published an additional document focused on clopidogrel bioequivalence [9], confirming the need to evaluate clopidogrel levels only. Interestingly, in the same document back-conversion of clopidogrel metabolites was considered a significant problem and validation of this aspect was introduced as a mandatory step in the validation of an analytical method for clopidogrel quantification [9]. It is noteworthy that back-conversion is now seen as a major problem in pharmacokinetic studies and EMEA is proposing to validate back-conversion in all analytical methods to be applied in bioequivalence studies, as stated in the draft guidance on bioanalytical validation [10].

In 2007 our group started the development and validated an analytical method to quantify clopidogrel and carboxy-clopidogrel in plasma samples of subjects involved in bioequivalence studies; the classical FDA bioanalytical validation rules (2001) were followed [11]. A fully validated method (Method 1) was applied to real clinical samples; during this work it was observed that extracts of real samples, reanalyzed after staying a few hours in

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doi:10.1016/j.jchromb.2010.09.022

the autosampler, showed clopidogrel levels much higher than the fresh ones.

Prompted by these results a new method to determine only clopidogrel in plasma (Method 2) was validated, using incurred samples of real subjects in order to verify the plasma sample and extract stability. Use of an unusually low autosampler temperature (−5 °C) was needed to obtain stable results in real samples, in clear contrast to the results achievable in blank plasma spiked with clopidogrel and/or carboxy-clopidogrel, which was stable. Finally, a third method with a special plasma extraction procedure (Method 3) resulted in good clopidogrel stability in real samples without the need to maintain temperatures below 0 °C.

These three methods will be presented in this paper, together with discussion of validation procedures to check back-conversion in biological samples.

2. Materials and methods

2.1. Chemicals

Clopidogrel bisulfate standard (according to USP) was obtained from LGC Promochem GmbH (Germany). The internal standard (d3-clopidogrel hydrogensulfate) and clopidogrel carboxylic acid hydrochloride were purchased from SynFine Research (Richmond Hill, Canada). Methanol, acetonitrile (both gradient grade for LC), dimethylsulfoxide (analytical grade) and trichloroacetic acid (analytical grade) were purchased from Merck (Darmstadt, Germany); while formic acid 88.0% was from Sigma–Aldrich (Deisenhofen, Germany). Ultra pure water was obtained with a Milli-QPlus system (Millipore, Molsheim, France).

2.2. Stock and working standard solutions

The stock solutions of clopidogrel and clopidogrel carboxylic acid were prepared at a concentration of 1 mg/mL in dimethylsulfoxide; the internal standard (d3-clopidogrel) was dissolved at the same concentration in methanol. All of these solutions were stored at −20 °C and used up to 1 month from preparation. Working dilutions from stock solutions of the analytes or internal standard were freshly prepared when needed.

2.3. Calibration curves and quality control samples preparation

In Method 1 spiked calibration standards and quality control (QC) samples were prepared with clopidogrel and clopidogrel carboxylic acid together; the concentrations ranged from 50 to 30,000 pg/mL for clopidogrel, and they were 100 times higher for clopidogrel carboxylic acid. For Methods 2 and 3, spiked calibration curves were prepared with clopidogrel only, at the following concentrations in plasma: 5–10–30–90–250–600–1300–2000 pg/mL. Spiked quality control (QC) samples for these methods were prepared also with clopidogrel only, at the following concentrations in plasma: 9–100–900–1800 pg/mL. In order to prepare the highest concentration calibration standard or QC, aliquots of pooled plasma from healthy volunteers were spiked with working dilutions of clopidogrel. The other calibration points and QCs were obtained by sequential dilutions with blank plasma starting from the corresponding more concentrated points. Calibration standards and QC samples in plasma were stored below −20 °C for Method 1 and below −70 °C for Methods 2 and 3.

2.4. Clopidogrel isolation from plasma samples

Sample preparation was based in all three procedures on protein precipitation with different reagents; in Method 3 an SPE cleanup step was added.

In Method 1, spiked plasma samples (calibration curve, QC, validation samples) as well as samples of the PK study were processed as follows:

1. Aliquots of plasma (0.1 mL) were transferred in 1.5 mL polypropylene tubes containing 0.02 mL of internal standard solution (50 ng/mL d3-clopidogrel hydrogensulfate in methanol) and 0.1 mL of trichloroacetic acid 20% in water/methanol (1/1; v/v) were finally added.
2. Samples were vortexed for 5 min and than centrifuged 3 min (8200 × *g*).
3. Aliquots (0.08 mL) of the clear supernatant were aspirated and transferred in autosampler 96-well plates, and then 0.12 mL of distilled water was added in each well.

After vortexing for 3 min, the samples were transferred in the autosampler stack thermostatted at 10 °C (±1 °C) and analyzed by LC–MS/MS as described below.

In Method 2, all plasma samples (calibration standards, QCs, validation samples and clinical samples) were kept on crushed ice during sample preparation, and the precipitation reagent was changed to acetonitrile. Aliquots of plasma (0.1 mL) were transferred in 2 mL polypropylene tubes containing 0.04 mL of internal standard solution (d3-clopidogrel hydrogensulfate 1 ng/mL in methanol) and 0.2 mL of acetonitrile were added. Samples were mixed for 5 min, and then centrifuged for 5 min at 2500 × *g* (10 °C). The clear supernatants were transferred in autosampler polypropylene 0.5 mL vials, vortexed for 30 s and transferred in the autosampler equipped with sample racks refrigerated at −5 °C (±1 °C) by an external cryothermostat.

In Method 3, plasma aliquots (0.2 mL) were transferred in 2 mL polypropylene tubes (chilled with crushed ice) containing 0.02 mL of internal standard solution (d3-clopidogrel hydrogensulfate 2.5 ng/mL in methanol) and 0.4 mL of methanol were added in each tube. After mixing (3 min) and centrifugation (5 min at 8800 × *g* and 7 °C), the clear supernatants were loaded on precipitation plates (Supelco HybridSPE-Precipitation, containing 50 mg bed) and then transferred under vacuum in 2.2-mL deep well plates, using a VacMaster-96 system (Biotage, Uppsala, Sweden). Finally, the deep well plates were transferred in the autosampler cooled stack maintained at +5 °C (±1 °C) during LCMS/MS analysis.

2.5. Chromatographic separations

The LC systems were composed of quaternary pumps model 1100 Series (Method 1) or binary pumps 1200SL Series (Methods 2 and 3) from Agilent (Santa Clara, CA, USA) and CTC PAL HTS autosampler (Schlieren, Switzerland).

In Methods 2 and 3, in order to get a high sample processing speed, a high-throughput HPLC system model Aria LX-2 developed by Cohesive Technologies (Thermo Fisher Scientific, San Jose, CA, USA), running 2 columns in parallel, was employed.

Different chromatographic conditions were used in each validated method as detailed in the next paragraph.

In Method 1, separations were performed on a reversed phase column Ascentis RP-Amide 15 cm × 2.1 mm, 5 μm silica packing (Supelco, Bellefonte, PA, USA), eluted at 0.4 mL/min with mobile phase consisting of water with 0.1% formic acid (A)–methanol (B), in gradient conditions. The injection volume was 20 μL and the samples in the autosampler were thermostatted at 10 °C (±1 °C) in the CTC cooled stack.

In Method 2, samples were injected on a reversed phase Ascentis Express C8, 10 cm × 2.1 mm, 2.7 μm column (Supelco), maintained at 55 °C in a column thermostat Eppendorf (Hamburg, Germany) model CH 30, the flow rate was 0.35 mL/min and the mobile phase composed of methanol/water (55/45; v/v) with 0.1% formic acid

Table 1
Mass spectrometry parameters.

	Clopidogrel			Clopidogrel carboxylic acid			d3-Clopidogrel			Instrument	IS[c]	DP[d]	TP[e]
	MRM transition	CE[a]	CXP[b]	MRM transition	CE[a]	CXP[b]	MRM transition	CE[a]	CXP[b]				
Method 1	322.2/184.1	31	10	308.2/95.0	77	4	327.2/217.1	23	16	API 4000 QTrap	5000	46	450
Method 2	322.2/184.1	33	14	N/A	–	–	327.2/159.9	53	20	API 5000	5000	50	500
Method 3	322.2/184.1	33	14	N/A	–	–	327.2/159.9	53	20	API 5000	5000	70	450

[a] Collision energy (V).
[b] Collision cell extraction potential (V).
[c] Ionspray voltage (V).
[d] Declustering potential (V).
[e] Source temperature (°C).

(A) and methanol with 0.1% formic acid (B), also in gradient. The injection volume was 20 μL and samples in the autosampler were thermostatted at −5 °C (±1 °C) using a liquid cooled sample tray (circulator Lauda model RM 6 (Brinkmann, Germany) or HUBER (Offenburg, Germany) model CC1 were used for this purpose).

In Method 3, chromatographic separations were carried out on a column Ascentis Express RP Amide, 10 cm × 2.1 mm, 2.7 μm (Supelco) maintained at 55 °C using the Eppendorf thermostat, and eluted with a mobile phase composed of water with 0.1% formic acid in water (A) and methanol (B), at 0.2 mL/min, in gradient conditions as follows: (1) short (10 s) isocratic step at 35% B; (2) linear gradient up to 70% B in 30 s; (3) isocratic step at 70% B for 4 min; (4) step gradient (6 s) to 85% B followed by 1 min isocratic under these conditions for better column clean-up; (5) rapid (0.1 min) restoring of the initial conditions (35% B), followed by 2.0 min re-equilibration with this last composition. The injection volume was 20 μL and samples in the autosampler were thermostatted at +5 °C (±1 °C) using a liquid cooled sample tray.

2.6. Mass spectrometry

Analytical procedures were carried-out on Applied Biosystems-Sciex (Toronto, Ontario, Canada) mass spectrometers; for the first method a quadrupole-linear ion trap model API 4000 QTrap instrument was used, while the other 2 methods were validated on a triple quadrupole model API 5000. Both instruments were equipped with an atmospheric pressure electrospray ionization source (Applied Biosystems model Turboionspray) and data system based on Dell computer with Windows XP as operating system and Applied Biosystems-Sciex software Analyst 1.4.2 for data acquisition and processing. Research grade nitrogen was used as curtain and CAD gas while the Turboionspray source was supplied with zero grade air.

To select the appropriate detection conditions and to optimize the mass spectrometer parameters for clopidogrel, clopidogrel carboxylic acid (when applicable), and d3-clopidogrel, these compounds, diluted at 1 μg/mL in water/methanol (50/50, v/v), were infused, at 10 μL/min employing a syringe pump Harvard model "11 PLUS" (Holliston, USA), directly in the ionization source. Quantitative data were acquired in multiple reaction monitoring (MRM) positive electrospray ionization mode.

The MS parameters for the three methods are summarized in Table 1.

In all three analytical methods the eluent from the HPLC column was introduced in the MS interface without splitting and, in order to minimize the ionization source contamination by salt and proteins the mobile phase was diverted to the waste for a short period after injection (1.7–2.7–3.5 min, in the three methods, respectively) using a computer controlled switching valve. The acquisition duration was 3.8–1.5–3.5 min, respectively.

In Method 1, the turbo gas and nebulizer gas were optimized both at 45 psi, and the curtain gas at 14 psi. In Method 2, the nebulizer and turbo gas were regulated both at 50 psi, and the curtain gas at 15 psi, while in Method 3 the turbo gas was set at 50 psi, the nebulizer at 40 psi and the curtain gas at 25 psi. The turbo gas temperature was 450 °C in Methods 1 and 3, while in Method 2 a temperature of 500 °C was used. The nebulizer and curtain gases were not warmed up.

2.7. Validation procedures

All procedures were carried out on plasma collected with EDTA as anticoagulant. The validation of the first HPLC–MS/MS method for the determination of clopidogrel and clopidogrel carboxylic acid in plasma samples was performed in accordance with FDA rules [11]. For the last two methods, besides FDA criteria, back-conversion experiments were introduced; these experiments were carried out before the release of the EMEA document requesting to validate the back-conversion [9,10].

The analytical range to be validated was chosen on the basis of the expected plasma concentrations [4]. Spiked plasma samples were prepared for calibration curves (8 points) and quality controls (4 different concentration levels) as previously detailed in Section 2.3. The following validation tests were carried out:

(1) *LLOQ evaluation*: The response (peak area) in each blank plasma sample (8 replicates from different subjects including hemolytic and lipemic plasma) compared with spiked LLOQ samples prepared with plasma from the same volunteers. The peak areas in blank samples cannot exceed 20% of the mean clopidogrel LLOQ peak areas, with a precision of ±20%. The precision and mean accuracy of back-calculated LLOQ replicate concentrations must be respectively < 20% and ±20%.
(2) *Selectivity*: Analyses were performed on 8 blank plasma samples (including a hemolytic and a lipemic plasma) collected from different healthy volunteers without addition of internal standard, then with addition of the internal standard or clopidogrel; no interfering peaks should appear at the same retention time with the compounds of interest.
(3) *Calibration curves, regression model, precision and accuracy*: A calibration curve was prepared in replicate ($n = 6$) and analyzed. The accepted correlation coefficient (r), obtained using the simplest regression model giving the best fitting in the whole range of tested concentrations, must be >0.99, with precision and accuracy, for the back-calculated concentrations of the calibration points, within ±15%, except for the lower quantification limit (±20%).
(4) *Within-run and between-run variability*: In the case of within-run evaluation, a minimum of 9 quality controls at each concentration level were prepared and analyzed in the same sequence. For the between-run, quality controls at each concentration levels were prepared and analyzed in at least 3 different sequences and in different days. In both conditions the pre-

cision and accuracy for replicated quality controls at various concentrations must be situated within ±15%.

(5) *Sample stability* (*plasma samples and extracts*): According to the clinical protocol the plasma samples were stored below −20 °C in the first bioequivalence study and below −70 °C in the following two studies. Spiked plasma samples stored in these conditions for different time frames were analyzed to exclude the possibility that clopidogrel undergoes a significant degradation. Spiked samples were also evaluated after 3 freeze-thawing cycles in order to verify if they can be re-analyzed, if needed, after repeated freezing without compromising the results. The stability was also tested on spiked plasma samples kept at room temperature or chilled with crushed ice, to evaluate the clopidogrel stability during the sample preparation. The stability of the extract, at different temperatures, was tested in each of the three methods. The stability of clopidogrel under transportation conditions (dry ice) was estimated keeping sets of spiked samples at −70 °C for up to one month. Finally, the stock solution stability of clopidogrel and the internal standard were tested for 6 h at room temperature and 1 month at −20 °C, comparing peak areas to those of freshly prepared solutions.

(6) *Extraction recovery*: The extraction recoveries of clopidogrel and d3-clopidogrel were measured comparing the peak areas of spiked quality control sets in plasma at different concentrations, prepared according to the above described protocol, to those of samples containing clopidogrel and d3-clopidogrel diluted from standards in mobile phase, at the concentration expected if the recovery would be 100%, and added over extracted blank samples. A recovery of 50% in the whole range of expected plasma concentrations was considered enough to obtain an adequate sensitivity.

(7) *Matrix effect*: In compliance with FDA/AAPS Crystal City 2007 White Paper recommendations [12], a deuterated internal standard (d3-clopidogrel) was used to compensate matrix effects. These effects have been evaluated by comparing the signal of a solution in mobile phase against the signal of the same mixture added over a blank extract [13].

(8) *Back-conversion tests on known metabolites*: Tests of back-conversion were performed on plasma samples separately spiked with the known clopidogrel metabolites, clopidogrel carboxylic acid, oxo-clopidogrel and clopidogrel active metabolite, using the analytical Method 2.

First, plasma samples were spiked with clopidogrel carboxylic acid at 5000.0 ng/mL (starting from the stock solution of 1 mg/mL), and they were then kept for 24 h at room temperature. Following this period the samples were processed as the other validation samples and the clopidogrel content was measured using normal calibration curves. Similar experiments were also carried-out on freshly spiked plasma samples keeping the sample extract, after precipitation, for 24 h at room temperature. A test of precipitation with methanol, using the same proportion, was also performed and sample extracts were equally analyzed after 24 h.

Back-conversion of oxo-clopidogrel was then evaluated using the same procedures (final concentration and storage conditions); the oxo-clopidogrel standard was obtained from SynFine (Richmond Hill, Canada) and a stock solution at 5 mg/mL was prepared in methanol.

In the case of clopidogrel active metabolite, due to the fact that this compound was not commercially available, an *in vitro* oxidation of oxo-clopidogrel with human liver microsomes was performed as already described [2]. Briefly, a solution (10 μL) of 5 mg/mL oxoclopidogrel in methanol was added over 0.5 mL buffer containing sodium phosphate 0.2 M, sodium fluoride 1 M and reduced glutathione 0.1 M, then 50 μL human liver microsomes (producer Celsis, Chicago, USA) and 100 μL solution of reduced NADPH 10 mM were added, and the mixture was incubated for 1 h at 37 °C. The obtained active metabolite was purified (semipreparative HPLC on Supelco RP-Amide column), quantified (UV peak areas) and biologically characterized (*in vitro* anti aggregant effect).

Diluted solutions with purity >90% and concentrations >5 μg/mL were typically obtained. Back-conversion experiments were carried-out as for the other two metabolites, but keeping in consideration the quite low levels present in plasma the concentration was reduced to 50 ng/mL.

(9) *Back-conversion tests in incurred samples*: These tests were carried-out, both with Method 2 and 3 using samples of previous clinical studies (see Section 2.8). A group of randomly selected samples ($n = 30$) of each study, already analyzed once and adequately kept frozen, were prepared again and the measured concentrations were compared to the original-ones, to evaluate the risk of back-conversion during deep freeze storage. In order to detect back-conversion at room temperature or on ice aliquots of the same plasma were also kept for 3 h at room temperature or on crushed-ice and the measured value were compared to those obtained just after thawing. Finally, extracts of these last samples (preparation immediately after thawing) were re-analyzed after storage in different conditions in autosampler vials (18 h at room temperature, at 4 °C, or at −5 °C), and the results compared to the fresh-ones to establish the presence of back-conversion in the extracts.

2.8. Plasma samples from pharmacokinetic study

Separate bioavailability studies, duly approved by regulatory authorities and Ethical Committees, were performed in accordance with good clinical practice (GCP) norms on 3 groups of volunteers (12 subjects in the first study, 40 in the second and 62 in the last one); clopidogrel was orally administered (fasting conditions) as a single dose of 75 mg of the same pharmaceutical product. Blood samples (5 mL each) were collected, using potassium EDTA as anticoagulant (sampling tubes S-Monovette Hematology 7.5 mL from Sartstedt, Nümbrecht, Germany), before dosing (0.0) then 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 (only performed in the second study), 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0 (not sampled in the second study) 24.0, 36.0 and 48.0 (only performed in the second study) hours postdose. In the case of the second and third studies blood samples, immediately after sampling, were kept in crushed iced while in the first one no special measure was taken. Plasma was separated, within 15 min from sampling, by centrifugation at 4 °C and aliquots (1–2 mL) were kept frozen at −20 °C (first study) or −70 °C (studies 2 and 3) until analyzed.

3. Results and discussion

Product ion spectra of clopidogrel and d3-clopidogrel were similar to those reported [4]. The molecular ion corresponding to ^{35}Cl (322.2) was chosen as precursor ion for clopidogrel in the MRM method used for quantification, while for d3-clopidogrel the isotope ^{37}Cl was preferred, despite being less intense, in order to obtain optimal analytical selectivity. Preliminary tests showed a better chromatographic background for this ion in plasma samples; in addition, at high clopidogrel concentrations, due to the isotopic distribution, a significant cross-talk was observed on the transition of d3-clopidogrel when using as precursor ion the isotope with ^{35}Cl (325.2).

Typical MRM chromatograms for clopidogrel obtained with analytical methods 3 in blank, LLOQ and CAL 8 samples are presented in Fig. 1; as it can be seen an adequate sensitivity as well as background free from interferences were obtained.

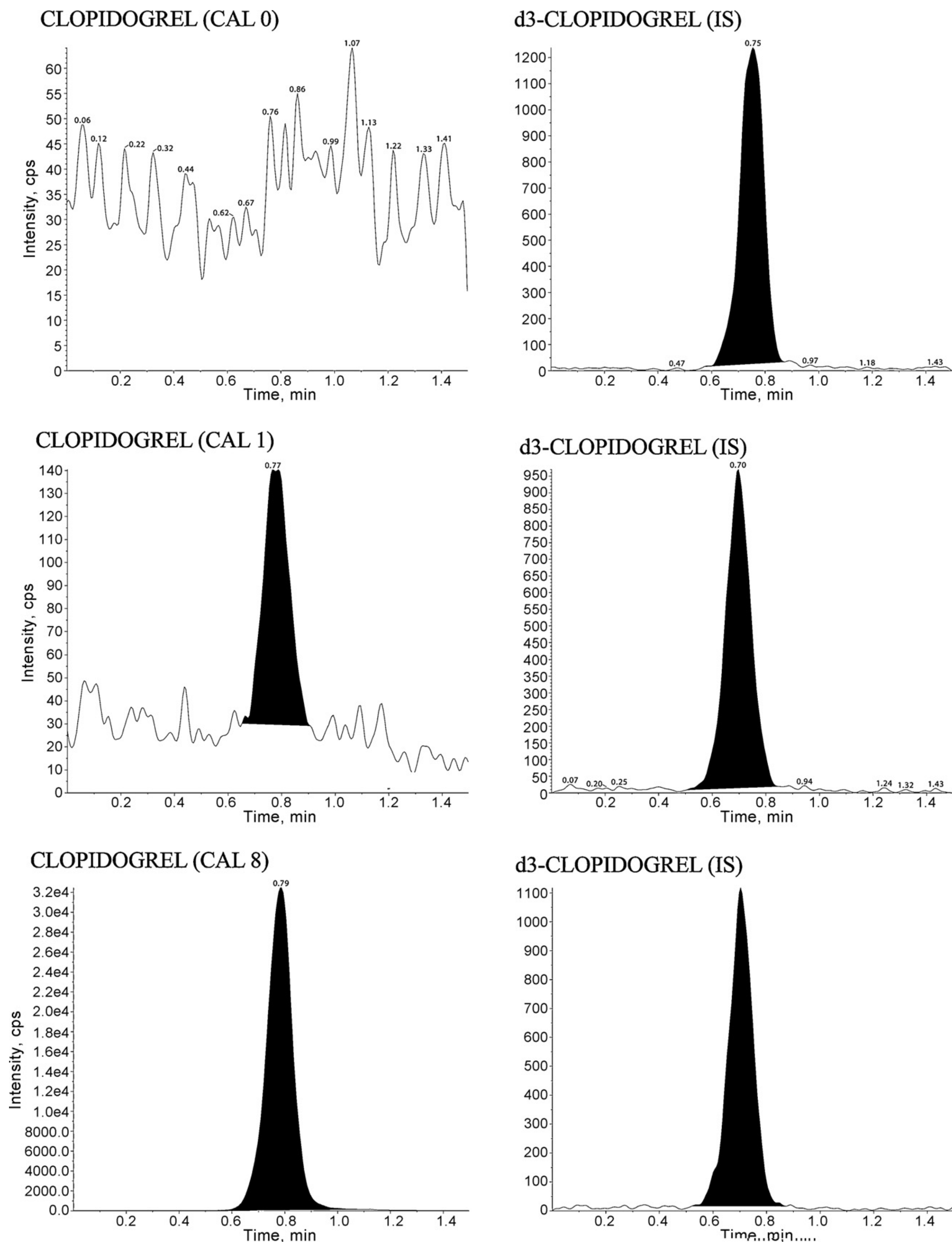

Fig. 1. Chromatograms recorded in multiple reaction monitoring (MRM) on the transitions of clopidogrel and d3-clopidogrel after the injection of blank plasma, Calibrator 1 (concentration 5 pg/mL in plasma) and Calibrator 8 (concentration 2000 pg/mL in plasma) processed with internal standard (concentration 80 pg/mL in plasma), according to the protocol. Data obtained with Method 3. Column: Ascentis Express RP Amide (10 cm × 2.1 mm, 2.7 μm), mobile phase – gradient of formic acid 0.1% in water and methanol.

Table 2
Validation data for LLOQ mean accuracy and precision; within-run mean accuracy and precision ($n = 9$ QC samples per concentration in the same analytical run) and between-run mean accuracy and precision ($n = 27$ QC samples per concentration in three days, four different analytical runs) for the analytical method 3.

LLOQ (5 pg/mL)	Mean accuracy (%)		Precision	
	100.3		10.3	
	Within-run		Between-run	
	Mean accuracy (%)	Precision	Mean accuracy (%)	Precision
QC1 (9 pg/mL)	93.8	13.9	96.5	12.2
QC2 (100 pg/mL)	96.9	3.5	97.0	4.0
QC3 (900 pg/mL)	102.7	2.2	101.8	2.7
QC4 (1800 pg/mL)	102.1	1.7	102.4	3.2

Table 3
Validation stability tests on spiked plasma results.

Sample	Method 1		Method 2		Method 3	
	Storage conditions	Stability % (comparison vs. control)	Storage conditions	Stability % (comparison vs. control)	Storage conditions	Stability % (comparison vs. control)
QC1	Plasma 2 h room	105.8	Plasma 1 h ice	104.3	N/A	–
QC4	temperature	102.7		100.0		–
QC1	Plasma 6 h room	99.5	Plasma 3 h ice	97.6	N/A	–
QC4	temperature	99.7		99.4		–
QC1	Plasma 1 month	107.4	Plasma 1 month	97.0	N/A	–
QC4	−20 °C	101.5	−70 °C	96.2		–
QC1	Plasma 3 month	103.9	Plasma 6 month	112.1	N/A	–
QC4	−20 °C	100.7	−70 °C	102.3		–
QC1	Plasma freeze-thaw	106.4	Plasma freeze-thaw	100.2	Extract 24h	102.1
QC4	3 cycles	101.4	3 cycles	99.9	−5 °C	101.7
QC1	Samples extract	100.9	Samples extract	93.1	Extract 4 h	100.9
QC4	12 h 10 °C	100.6	4 h – 5 °C	101.2	+4 °C	99.7
QC1	Samples extract	101.0	Samples extract	111.1	Extract 24h	104.2
QC4	36 h 10 °C	100.0	18 h – 5 °C	101.4	+4 °C	102.9

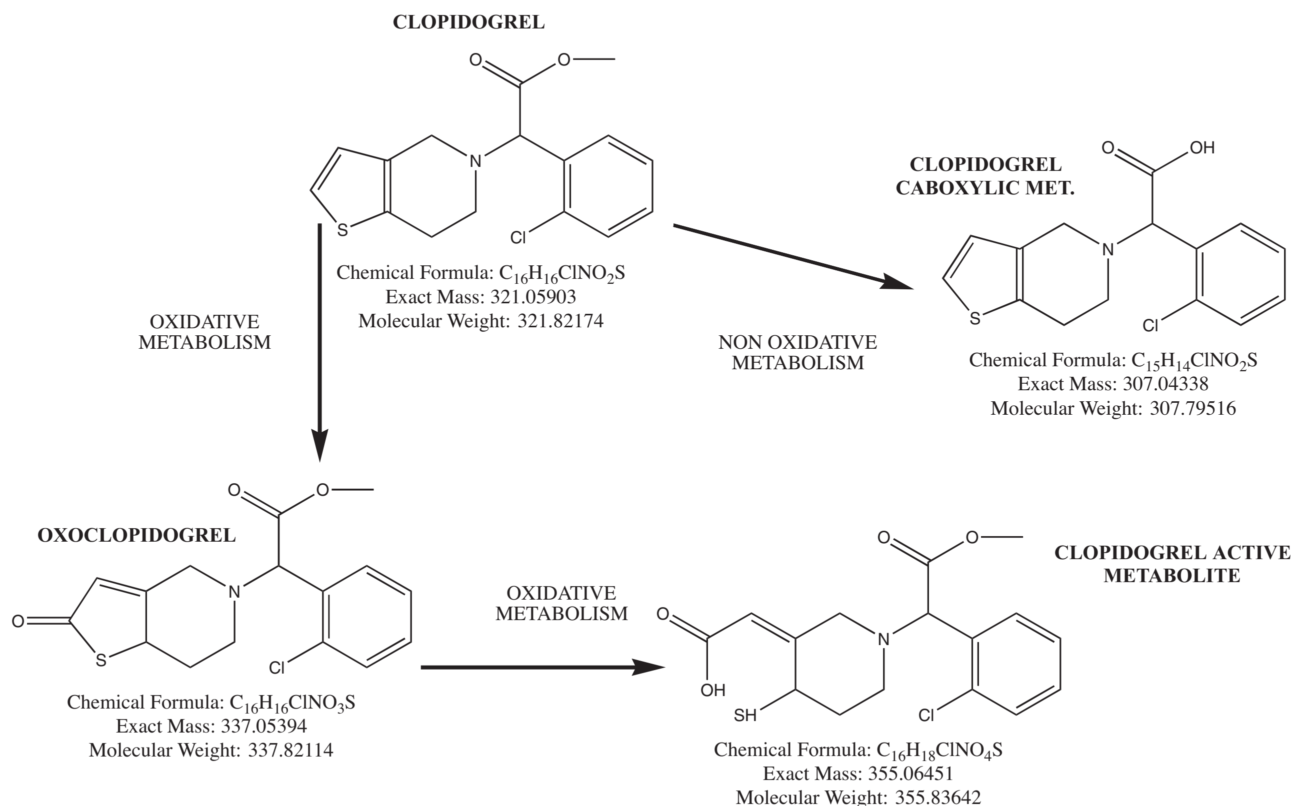

Fig. 2. Clopidogrel known metabolic pathway and related chemical structures (clopidogrel, clopidogrel carboxylic acid, oxo-clopidogrel and active metabolite).

Table 4
Back-conversion test Methods 2 and 3 on 15 incurred samples. Mean accuracy of the ratios concentration after storage vs. first measurement.

Storage conditions	Mean ratio	Mean accuracy (%)	CV (%)	Range
Method 2				
Plasma at −70 °C 8M	1.153	+15.3	10.1	0.9–1.3
Plasma on ice 3 h	1.020	+2	8.1	0.9–1.2
Plasma room temperature 3 h	1.739	+73.9	12.6	1–3.6
Sample extract at −5 °C 18 h	1.007	+0.7	7.7	0.9–1.2
Sample extract at 4 °C 18 h	3.713	+271.3	134.1	1.5–14.7
Sample extract room temperature 18 h	5.826	+482.6	61.1	2.1–30.5
Method 3				
Plasma at −70 °C 8M	0.953	−4.7	19.3	0.8–1.4
Plasma on ice 3 h	1.094	+0.94	8.2	0.9–1.3
Plasma room temperature 3 h	1.511	+51.1	16.6	1.2–4.1
Sample extract at −5 °C 18 h	0.980	−2	5.3	0.9–1.1
Sample extract at 4 °C 18 h	1.196	+19.6	16.9	0.9–1.6
Sample extract room temperature 18 h	1.861	+86.1	17.8	1.2–2.6

Calibration curves were linear (1/*x* weighted regression model) for all 3 methods, with correlation coefficients >0.99 and LLOQ results met the validation criteria with all procedures. The accuracy and precision obtained on spiked QC samples at all concentrations levels were within acceptance range (±15%) with all the three methods developed. The results obtained during the validation of Method 3 are summarized in Table 2. Looking more in detail to Method 3, extraction recoveries were 80.5% for QC1 level, 73.6% for QC 2, 75.2% for QC 3% and 76.8% for QC 4, with coefficients of variation (CV) of 6.6, 5.7, 2 and 1%, respectively. For the internal standard, a recovery of 72.4% with 5.4% CV was obtained. The matrix factor for clopidogrel, calculated as ratio between the areas in presence of matrix and the areas in absence of matrix, had a mean of 0.85 (14.9% CV), indicating mild ion suppression, while for the internal standard, d3-clopidogrel, the matrix factor was 0.81, with a CV of 12.8%; the results were in agreement with FDA/AAPS Crystal City 2007 White Paper recommendations [12].

The stability data of spiked plasma and extracts obtained with Methods 1 and 2 are displayed in Table 3. As it can be observed, all these experiments, performed with spiked plasma QCs extracted according to different protocols as aforementioned in this paper showed an adequate stability in the selected conditions.

While carrying-out the analytical part of a first pharmacokinetic study using Method 1, it was observed that extracted samples reanalyzed, after an overnight stay in the autosampler, in the conditions validated with spiked plasma QCs, provided concentrations definitively higher (more than double) than those measured in the first run. This fact prompted us to perform, first, back-conversion tests of the metabolites described in the literature (clopidogrel carboxylic acid, oxo-clopidogrel, clopidogrel active metabolite); the known metabolic pathway of clopidogrel is presented in Fig. 2.

In all of these experiments (data not reported here) no clopidogrel levels were measured, therefore it was necessary to further evaluate the back-conversion with incurred samples of pharmacokinetic studies in patients. Different storage conditions were used and the results are reviewed in Table 4. The incurred samples were randomly selected from the whole set due to the fact that if an unknown metabolite is responsible of back-conversion, not having PK information, it may be expected at any time in the sampling interval.

It can be easily observed that plasma samples of treated subjects maintained in the deep freezer (−70 °C) up to 8 months did not evidence relevant variation of concentration compared to the first measurement. Relevant increases of concentration were observed in plasma samples after storage at room temperature for 3 h. Also, extracted samples show remarkable increase of clopidogrel concentrations when kept at 4 °C for 18 h, suggesting the existence of an important back-conversion. The fact that such back-conversion take place also in deproteinated sample extracts suggests that a chemical reaction and not an enzyme based biotransformation is responsible for the phenomenon. Based on these findings different kind of anticoagulants, plasma stabilizers and restrictive storage conditions were evaluated on incurred samples in order to get stable results. Beside EDTA, heparin, sodium bisulfite, ascorbic acid, potassium oxalate and sodium fluoride were completely ineffective to improve sample stability (data not shown here). In contrast, the cold storage of the plasma samples during processing (on crushed ice) and the storage of the extracts at low temperature (below 0 °C), used in Method 2 were quite effective to increase incurred samples stability. This approach finally allowed obtaining adequate results, in terms of stability, in a reasonable time frame (12 h) to may run an analytical sequence (Table 4).

The third method was first developed in order to automate the precipitation procedure and to minimize the matrix effect as described in the technical information by the producer of the HybridSPE-Precipitation plates [14], but an unexpected improvement of stability was then observed when compared to Method 2. In fact a higher temperature (4 °C) was enough to maintain stable the sample extracts prepared with this method, while similar results could be achieved only at −5 °C (or below) with Method 2. Looking more carefully to these results it can be however appreciated that the storage of sample extracts at −5 °C was beneficial also with Method 3 (Table 4); the coefficient of variation of the ratios between concentrations of freshly analyzed samples and the extracts kept

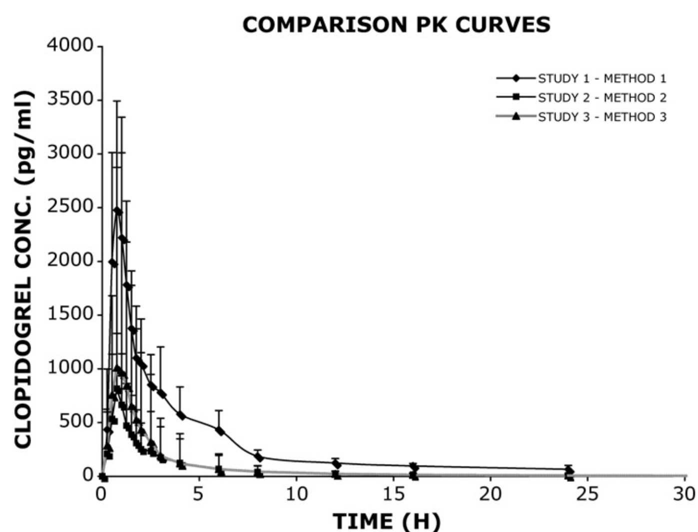

Fig. 3. Mean clopidogrel pharmacokinetic curves obtained on patients orally treated with clopidogrel 75 mg in 3 pharmacokinetic studies analyzed with Methods 1, 2 and 3.

Table 5
Comparison of clopidogrel pharmacokinetic data obtained with different analytical methods.

Author	Dose (mg) administered	CMAX	AUC0-INF	Extraction procedure	Precipitation agent/extraction solvent	Reconstitution solvent
Lainesse et al. [4]	150	7921.5	29944.6	Liquid/liquid extraction	No data presented	No data presented
Robinson et al. [5]	150	2128.5	4625.7	Liquid/liquid extraction	Ammonium acetate pH 6.8/diethyl ether	Acetonitrile with 0.1% formic acid/water with 0.1% formic acid 1/1 (v/v)
Nirogi et al. [6]	75	1019.0	1880.0	Liquid/Liquid extraction	Diethyl ether/n-hexane (8/2, v/v)	20% ammonium formate/80% methanol
Method 1	75	3610.0	13998.0	Protein precipitation 4 °C	20% TCA in water/methanol 1/1 (v/v)	N/A[a]
Method 2	75	803.3	1919.4	Protein precipitation −5 °C	Acetonitrile	N/A[a]
Method 3	75	1216.4	2244.5	Protein precipitation Hybride SPE filtration Plate 4 °C	Methanol	N/A[a]

[a] Not applicable.

18 h was 16.9% at 4 °C and only 5.3% at −5 °C. Also, the mean accuracy (expressed as percent difference of the respective ratios from 1) was improved at −5 °C, being −2%, compared to +19.6% at 4 °C. However, with both storage conditions >67% of the results were within ±20% of the first reading (14 out of 15 at −5 °C and 12 out of 15 at 4 °C – individual data not reported here), as required for incurred samples reanalysis in the EMEA draft guideline on bioanalytical methods validation [10]. Due to the enhancement of stability, several experiments were performed in order to clarify if the removal of unstable clopidogrel metabolite(s) by the HybridSPE plate can be the basis of these results. Preliminary tests (data not presented) showed that the active clopidogrel metabolite, and a putative glucuronide conjugate of clopidogrel acid metabolite were strongly retained on the SPE-precipitation plate, while clopidogrel and oxoclopidogrel were not. The identification of the conjugation metabolite has been completed only recently with the availability of a commercial standard of this glucuronide (the results of these experiments will be the object of a publication under preparation). It is important to note that the precipitation conditions of Method 3 (pure methanol and no acidification) selected after our experiments were quite different from those recommended by the producer [14] in order to eliminate the plasma phospholipids often involved in problems of matrix effect. All these findings are also in agreement with the data recently reported by Havard et al. in a poster presentation [15]; these authors observed the back-conversion of a clopidogrel metabolite, other than clopidogrel carboxylic acid, in methanolic conditions during sample extraction. These results however may be surprising and intriguing due to the fact that methanol was used as precipitation agent in Method 3. Just recently, studying the stability of clopidogrel carboxylic acid acyl glucuronide, it has been observed that its back-conversion is critical in methanolic extracts, but it proceeds slower in plasma kept cold (data will be presented in a future paper), explaining how in Method 3 back-conversion was adequately controlled by the removal of metabolite(s) on HybridSPE and plasma samples cooling.

Mean pharmacokinetic curves calculated from plasma samples of the three studies with volunteers orally treated with the same dose (75 mg) and formulation of clopidogrel, but analyzed each with a different method are displayed in Fig. 3. While results obtained using Methods 2 and 3 are quite similar, concentrations determined with Method 1 are definitively higher; as a mean an increase of up to four times was observed.

In Table 5 some of the pharmacokinetic data of clopidogrel, which is available in literature, were summarized and compared with the results of the studies performed by our group; two of the studies, the first ones in the table, were performed with a twofold higher dose (150 mg).

In order to compare these studies, it is important to take into account that clopidogrel has a linear pharmacokinetic behavior within this dose range, as reported in the literature [16]. Keeping this in mind, and dividing by two the pharmacokinetic data from the studies with a twofold higher dose, the results of the first study in the table [4] are very similar to those obtained with the first method here presented, which was inadequate to control back conversion. The results of the other published studies [5,6] are very close to the results gathered with Methods 2 and 3 in conditions controlling back conversion.

Looking to these different methods, all were satisfactory according to the classic FDA 2001 guidelines for Bioanalytical method validation [11]. More recently, EMEA has published a draft of new bioanalytical validation rules [10] requesting to test back-conversion on plasma spiked only with standards of known metabolites.

The results obtained here tend to show that, because for too many compounds the metabolism is still poorly understood, an approach based only on known metabolites could be misleading. It is interesting to compare the complex metabolic pathway of ticlodipine [17], compound closely related to clopidogrel, and the relatively simple scheme described for clopidogrel metabolism.

On the other hand the use of incurred samples proved to be quite effective for investigating back-conversion and its application seems simple and very promising. It is evident that in case of new method development, this part of validation has to be carried out *a posteriori* after completion of a first clinical study (except when samples from another trial are available).

Larsson and Han [18] used incurred samples for quality assessment in the quantification of rifalazil, having however in view to test the risk of interference by unknown metabolites. Tan et al. [19] have definitively chosen this approach in order to investigate potential metabolite (e.g. glucuronide) back-conversion during the analysis of ramipril and ramiprilat in human EDTA plasma. The importance of incurred samples analysis during bioanalyti-

cal method development was underlined also by Jemal et al. in an interesting paper published in 2010 [20]. The authors emphasize de difference between incurred samples reanalysis as used in the current practice only to assess method reproducibility, and incurred samples usage in the method development to test potential interferences, stability issues or back-conversion due to metabolites.

4. Conclusions

With all three methods developed we could obtain an adequate sensitivity to determine clopidogrel at levels expected in pharmacokinetic studies and similar to the performance achieved by other authors [4–6].

An unexpected and scarcely described back conversion problem was observed after developing the first analytical method. Sample preparation and extract handling at low temperatures were mandatory to minimize this back-conversion; tests on incurred samples were the key to develop and validate this procedure. The presence of an unknown metabolite (or more) is the only possible explanation for this back-conversion as indirectly confirmed by the last method developed. Introducing a purification step on a column probably removed this metabolite(s), improving significantly the real samples stability.

It seems therefore important to conclude that back conversion need special attention during validation. The idea to perform these tests only with synthetic standards, based on the fact that metabolites are generally known as proposed in the new draft by EMEA [10], can prove inadequate. Under certain conditions, like unclear metabolic pathway in combination with very high metabolization rate, giving low parent compound levels and very relevant concentrations of metabolite(s), most probably only incurred samples can help to solve the problem. Regarding this last issue of the validation with incurred samples it is however clear that these tests must be carried-out in different storage conditions, while the random repetition of a fraction of a study samples, using only the validated conditions may fail to put in evidence the existence of back conversion.

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