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A liquid chromatography-tandem mass spectrometry method for the simultaneous determination of exemestane and its metabolite 17-dihydroexemestane in human plasma

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A simple and sensitive liquid chromatography-tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the quantitation of exemestane (Exe) and its main metabolite 17-dihydroexemestane (DhExe) in human plasma. The analytes were extracted by protein precipitation with acetonitrile, containing stable ^{13}C-labelled Exe ($^{13}C_3$-Exe) as internal standard, and measured by LC–MS/MS. The best chromatographic separationof the analytes from the interferences was achieved by using a Phenyl column operating under isocratic regime conditions. The total chromatographic runtime was 5.0 min and the elution of Exe and DhExe occurred at 2.5 min and 2.9 min, respectively. Quantitation was performed by employing the positive electrospray ionization (ESI) technique and multiple reaction monitoring mode (MRM). The monitored precursor to product-ion transitions for Exe, DhExe and $^{13}C_3$-Exe internal standard were *m/z* 297.0 → 120.8, *m/z* 299.1 → 134.9 and *m/z* 300.0 → 123.2, respectively. The lower limit of quantitation (LLOQ) was 0.1 ng/ml for DhExe and 0.2 ng/ml for Exe. The method was linear up to 36–51 ng/ml with $r^2 \geq 0.998$. The intra- and inter-assay precision were ≤7.7% and 5.1% for Exe and ≤8.1 and 4.9% for DhExe while deviations from nominal values were in the 1.5–13.2% and −9.0–5.8% ranges for Exe and DhExe, respectively. The analytical method resulted robust and suitable for pharmacokinetic monitoring of Exe and its main metabolite during adjuvant therapy in patients with breast cancer.

Keywords: exemestane; 17-dihydroexemestane; liquid chromatography; tandem mass spectroscopy; electrospray ionization; pharmacokinetics

Introduction

Exemestane (Exe, Aromasin®, 6-methylenandrosta-1,4-diene-3,17-dione) is an anti-estrogen drug used in the adjuvant treatment of advanced estrogen receptors (ER)-positive breast cancer in postmenopausal women.[1] Exe is a steroid analogue, structurally related to the endogenous androstenedione, which irreversibly binds to the active site of the aromatase enzyme involved in the conversion of androgens to estrogens.[2] The pharmacodynamic effect of aromatase inhibition by Exe consists in a significant drop (98%) of the circulating and the tissue level of estradiol and estrone.[3] This metabolic feature is considered important for the prevention from the recurrence of estrogen-responsive cancers and for improving the survival of patients treated with Exe as compared to those undergoing standard tamoxifen adjuvant therapy.[4] Moreover, Exe, over other anti-estrogen drugs, is characterized by lower effects on blood lipids, in particular on the accelerated bone loss associated to the long-term estrogen deprivation.[5] The *in vivo* metabolism of Exe likely boosts this feature by generating the DhExe metabolite which expresses an androgen agonistic activity which may contrast with the side-effect of estrogen deprivation.[6]

In spite of important clinical achievements, the pharmacological potential of Exe is still to be explored especially with regard to the emerging combined therapy of Exe with new target drugs.[7,8] The need for new clinical pharmacology studies that could lead to an improvement of the current knowledge on clinical pharmacology of Exe has increased the demand for reliable analytical methods. To date, few analytical approaches have been reported for the quantitation of Exe in biological fluids. An HPLC method with UV detection, that includes a liquid-liquid extraction (LLE) as pre-analytical step, has been described but its sensitivity was not suitable for measuring the low plasma concentration observed when a 25 mg standard dose of Exe was daily administered.[9] The poor sensitivity of the

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HPLC–UV methodologies was originally addressed with the use of specific radio immuno assay (RIA) preceded by a plasma sample preparation that consists in a solid phase extraction (SPE) and in a further HPLC drug fraction purification steps.[10] This RIA assay was characterized by the very low limit of quantification of 13.5 pg/ml, that enables the pharmacokinetics monitoring of low dose of Exe up to 96 h from administration. However, the extensive sample preparation, the potential cross reactivity of antibodies with the Exe metabolites, as well as their difficult commercial availability have strongly restricted the HPLC-RIA method to few clinical phase I investigations. In the last decade, liquid chromatography coupled to mass spectrometry (LC–MS and LC–MS/MS) has led to major breakthroughs in the field of quantitative bio-analysis due to its inherent specificity and sensitivity, thus becoming the preferred analytical tool for quantitating drugs and metabolites in biological matrices. In this context, Cenacchi *et al*. introduced a methodology that uses atmospheric pressure chemical ionization-tandem mass spectrometry (LC–APCI–MS/MS) to quantitate Exe down to 0.1 ng/ml by using 500 µl of starting volume of plasma sample and the use of SPE as pre-analytical step.[11,12] The method partially overcomes the typical limitations of the HPLC–RIA assay and so far this LC–APCI–MS/MS technique, approach is the only one currently used in clinical pharmacology investigations of Exe. Recently, Mareck *et al*. reported a LC–APCI–MS/MS method for the quantitation of Exe and its prominent metabolite in human urine within the anti-doping investigations.[13] All these reported LC–MS/MS methods employ SPE or LLE sample preparation procedure. These pre-analytical approaches have the advantage of concentrating and purifying analytes before the MS analysis, contributing to improve sensitivity and to reduce at the same time the potential deleterious effect of matrix components on the measurement. However, these sample preparation techniques present the general disadvantage to be complex and time consuming and that may limit the overall throughput normally required for a day-to-day routine drug monitoring in a clinical environment.

Here we report the development and validation of a straight forth LC–MS/MS protocol to quantitate Exe and its metabolite DhExe in a small plasma volume by using a fast and cost effective sample preparation approach consisting of a simple plasma protein precipitation (PPP) step. The loss in sensitivity associated to the sample dilution and the limitation associated to the possible matrix effect (ion suppression) due to the raw plasma purification were overcome by the stringent optimization of both chromatographic and mass spectrometry conditions. The method was properly validated and successfully applied in pharmacokinetics investigation of Exe and DhExe along oral adjuvant therapy administration of 25 mg/day of Exe in breast cancer patients.

Materials and Methods

Chemicals and standards

The Exe reference standard (purity ≥98%) was supplied by Pifzer (Pfizer Inc, Groton, UK). The internal standard $^{13}C_3$-labeled-Exe (purity ≥98%) was provided by Nerviano Medical Science Laboratories (Nerviano, Milan, Italy). The DhExe metabolite (purity >95%) was a generous gift by Dr. Mareck (Institute of Biochemistry, German Sport University Cologne, Germany). Acetic acid, methanol and acetonitrile LC–MS grade were supplied by Baker (J.T. Baker, Deventer, NL). Ultra-pure water was obtained from an ELGA apparatus (ELGA, High Wycombe, UK) fed with pre-distilled water. The drug-free human plasma was obtained from healthy volunteers afferent to central transfusion unit of Centro di Riferimento Oncologico, Aviano, Italy. All the donators as well as the patients enrolled in the pharmacokinetics investigation signed a written consent to participate in the study, according to the Institutional ethical committee rules.

Instruments

The liquid chromatographic system was an Ultimate 3000 stack (Dionex, Sunnyvale, CA-USA) consisting of a degasser, two binary gradient pumps, a thermostated autosampler fitted with a 50-µl sample loop and a column oven. The analytical column was a 50 × 2 mm i.d., 3 µm Gemini C6-Phenyl preceded by a 4.0 × 2.0 mm SecurityGuard™ column packed with the same chromatographic material (Phenomenex, Torrance, CA USA). The mass spectrometer was the hybrid API 4000 Q-Trap (Applied Biosystems-MDS/Analytical Technologies, Foster City, CA-USA). Nitrogen, zero grade air and compressed air were supplied by an integrated compressor and gas generator unit (Peak Scientific Instruments, Renfrew, UK). Analyst software version 1.4.2, was used for data acquisition and processing.

Chromatographic and mass spectrometer condition

Exe and DhExe were separated under isocratic conditions using an aqueous solution of 0.1% acetic acid as mobile phase and containing 32% of acetonitrile flowing at a rate of 0.6 ml/min. The Exe and DhExe were measured through tandem mass spectrometry (MS/MS) using the electrospray ionization (ESI) technique. Parameter optimization was performed by the post-column infusion of reference standard of Exe and DhExe solution both at 1.0 µg/ml concentration in 0.1% acidic acid/acetonitrile 50/50. The infusion was performed at 5 µl/min through a tee connected to the column effluent by a Model 11 Plus syringe pump (Harvard Apparatus, Holliston, MA-USA). The optimized parameters for positive ion mode operation resulted in an ESI voltage of 4500 V, both nebulizer gas (air) and turbo gas (air) set at a pressure of 40 psi and with a nominal turbospray gun temperature of 650 °C. Curtain gas (nitrogen) was set at 20 psi and operating pressure was at 8×10^{-3} Torr with a CAD gas setting at 8 (arbitrary units). Declustering potentials were 50 V for Exe and $^{13}C_3$-Exe-IS, and 60 V for DhExe. The multiple reaction monitoring (MRM) experiment relied on the fragmentation of the pseudo-molecular ions $[Exe\text{-}H]^+$ at *m/z* 297.0, $[^{13}C_3\text{-}Exe\text{-}H]^+$ at *m/z* 300.0 and $[DhExe\text{-}H]^+$ at *m/z* 299.1 into the respective fragments at *m/z* 120.8, *m/z* 123.2 and *m/z* 134.9 with collision energies of 28 and 26 eV. Quadrupoles 1 and 3 were run at unit resolution. Dwell time was set at 200 ms for all the analytes.

The MS/MS spectra of the chromatographic interferences were recorded by exploiting the linear ion trap capability of the API 4000 QTrap through the enhanced product ion (EPI) scan mode on the precursor ion at *m/z* 297 and by using a collision energy (CE) range from 20 to 55 eV. With an ion trap filling time set at 100 ms combined with the Q0 trapping mode, the MS/MS spectra were recorded from *m/z* 50 to 700 at a scan rate of 4000 amu/sec.

Preparation of standards and quality control samples

Two independent stock solutions of Exe (1.28 and 1.60 mg/ml) and DhExe (0.90 and 1.35 mg/ml) and the $^{13}C_3$-Exe-IS solution

(1.01 mg/ml) were prepared in methanol. The stock solutions were further diluted to give two independent series of diluted working solutions of both Exe and DhExe analytes in acetonitrile : water (50 : 50**%**) solution. One solution series was used for preparing 'spiked' quality control samples (Qc) by adding each working solution to a control-blank human plasma in order to obtain final concentrations of 0.64 ng/ml (VERY LOW: VLQc), 3.84 ng/ml (LOW: LQc), 19.2 ng/ml (MED: MQc), 38.4 ng/ml (HIGH: HQc) for Exe and 0.5 ng/ml (VLQc), 2.7 ng/ml (LQc), 13.5 ng/ml (MQc), 27.0 ng/ml (HQc) for DhExe. The second working solution series was used for the daily preparation of Exe and DhExe plasma calibrators ($n \geq 8$) with concentrations from 0.19 to 51.2 ng/ml for Exe and from 0.09 to 36.0 ng/ml for DhExe.

The stock solutions were stored at −80 °C while the working solutions were stored at 4 °C in polypropylene tubes. All plasma samples including Qc were stored at −80 °C.

Sample preparation

After thawing at room temperature, plasma samples were vortex-mixed and centrifuged at 6000 × *g* for 5 min. A 100 µl-aliquot of clear plasma was transferred in a 1.5 ml polypropylene tube and added by 200 µl of the acetonitrile solution containing 5 ng/ml of $^{13}C_3$-Exe-IS. Tubes were quickly and vigorously vortex-mixed for 1 min using a Desyre-Mix (Zinsser Analytic, Frankfurt, Germany) and centrifuged at 6000 × *g* for 10 min at 4 °C. Then, 200 µl of supernatant were diluted with an equal volume of water and transferred into a vial of the autosampler kept at 4 °C for a 30 µl-injection.

Assay validation

Method validation was performed according to the guidelines set by the United States Food and Drug Administration (FDA) for bio-analytical method validation.[14] The method was validated in terms of linearity, specificity, LLOQ, recovery, intra- and inter-day accuracy and precision, and stability of analyte during the sample storage and processing procedures. Each analytical run included a double blank sample (without internal standard), a blank sample (with internal standard), eight to nine standard concentrations for calibration and replicate sets ($n \geq 5$) of Qc samples for both Exe and DhExe.

Carry-over

The carry-over was measured by injecting a blank plasma sample following the injection of the highest calibration standard run in triplicate. Carry-over was expressed as the mean ratio percentage of Exe, $^{13}C_3$-Exe-IS and DhExe peak area of the blank sample *versus* peak area in the high calibration point run.

Recovery and matrix effect

Overall recovery of Exe, DhExe and $^{13}C_3$-Exe-IS was estimated by comparing in triplicate the peak area of the plasma samples at VLQc and HQc levels with the same concentrations of analytes in neat solutions of acetonitrile–water. The matrix effect was measured by comparing the peak response of the post-extracted spiked sample with those of the neat solution of Exe and DhExe in mobile phase. Post-column infusion experiments were performed with the same setting as described for the optimization parameters by injecting precipitated plasma matrix in order to assess the minimal interference along the elution window of Exe and DhExe peaks.

Linearity and LLOQ

Linearity of the method was assessed through the linear least squares regression calculation with a weighting factor of $1/\times$. The LLOQ was defined as the lowest point of the calibration curve fulfilling the requirement of a signal-to-noise ratio >5 and with both measurement accuracy and precision within ≤20%. Daily linear calibration curves were prepared with each sample batch to be analyzed.

Accuracy and precision

Assay accuracy and precision were established by analyzing $n \geq 5$ samples of each Qc at four concentration levels in 3 day runs. Intra-assay precision was calculated as the relative standard deviation (RSD = standard deviation × 100/mean value) resulting from each analytical run. Inter-assay precision was assessed by the RSD of the mean concentration per analytical run over three separate runs.

Intra- and inter-assay accuracy were expressed as the relative error (RE) which express the percentage deviation from the nominal concentration [RE = (observed concentration − nominal concentration)/nominal concentration × 100].

A dilution integrity experiment was performed to validate the dilution procedure used to quantitate real subject plasma sample with concentrations out of calibration range. Validation was carried out at about five times the upper limit of quantification (ULOQ) concentration for both Exe and DhExe by preparing three replicate samples each at 1/10 of 5 × ULOQ concentration by adding blank plasma. Concentrations were calculated by applying the dilution factor of 10 against the freshly prepared calibration curve for Exe and DhExe. The acceptance criteria for dilution protocol were met when precision and deviation from nominal value of back calculated concentration for both Exe and DhExe, were within 15%.

Specificity

The absence of cross-interferences from the endogenous plasma components that can potentially interfere with detection and quantitation of Exe, DhExe and $^{13}C_3$-Exe-IS was assessed in six drug-free plasma samples from different sources. Samples were analyzed as double blank (no internal standard spiked), blank (with internal standard only) and at VLQc level concentration in triplicate.

Stability

The stability of Exe and DhExe in the extracted solution, in plasma and in whole blood was checked by analyzing at VLQc and HQc concentration levels. Aliquots of the diluted plasma extracts, maintained at 5 °C in the autosampler rack, were injected at time intervals of 3 up to 72 h. Stability was assessed through linear trend analysis by plotting, for each concentration level, absolute peak areas of Exe, $^{13}C_3$-Exe-IS and DhExe *versus* time after first injection. Ideally analytes are considered stable over the storage period when the slope of the linear regression should equal zero. Instability of analytes is evidenced when the analyte concentration decreases over time with a negative slope statistically different from zero ($p < 0.05$) as evaluated by the t-test for linearity regression. The Exe and DhExe short-term stability was measured after storage of both plasma and whole blood samples at 4 °C for 48 h. The freeze/thaw stability was determined after three freeze/thaw cycles. For each cycle, samples were frozen at −20 °C for 21 h, thawed and kept

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at room temperature for 1 h. The concentrations obtained from all stability studies were compared with the freshly prepared Qc samples, and the percentage concentration deviation from nominal value was calculated. The analytes were considered to be stable when the concentrations were within an acceptance interval of 15% with respect to the fresh Qc concentration.

Pharmacokinetic investigations

In order to test the applicability of the hereby described method, the steady state plasma pharmacokinetics profile of Exe and DhExe was measured in a patient receiving 25 mg/day of Exe as adjuvant breast cancer treatment. Whole-blood sample were withdrawn from a permanently placed central venous line and collected in potassium EDTA-tubes before the daily dose and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0 and 24.0 h from Exe administration. In a group of six patients, blood samples were drawn at 1 h (C^{peak}) and 24 h ($C^{through}$) post-dose. After drawing, the blood samples were immediately processed to obtain the plasma fraction, and were then stored at −80 °C until analysis. A 'non-compartmental' model was used to estimate the main pharmacokinetics parameters such as the area under curve (AUC) from 0 to 24 h, and the terminal half-life from the concentration-time curve.

Results and Discussion

The main aim of the present study was the development of a LC–MS/MS method for rapid quantitation of Exe and DhExe in human plasma that does not require time consuming sample preparation procedures like SPE and LLE. Chromatographic process and ESI ionization were maximized to achieve adequate sensitivity and selectivity when just a PPP is used as pre-analytical step. The method was optimized and validated to perform the measurement on a 100-µl sample volume, and resulted suitable to perform pharmacokinetic monitoring of Exe and its main metabolite in breast cancer patients receiving anti-estrogen adjuvant therapy.

Method Development and Optimization

Mass spectrometry

The preliminary development step involved the selection of the appropriate mass spectrometry conditions for the detection and quantitation of the analytes. Positive mode ionization was selected after evaluating both polarities by infusion of Exe and DhExe standards in 0.1% acetic acid : acetonitrile (50 : 50) solution. Both APCI and ESI techniques have been considered for quantitation of steroids though ESI is not frequently proposed by literature without derivatization, since steroid molecules such as Exe and DhExe do not contain readily ionizable moiety that can predict a good sensitivity by ESI. As APCI involves a more energetic gas-phase ionization process, it is usually expected to provide better sensitivity. In this study we directly compared the optimized MS signals for Exe and DhExe of the APCI with the Turbospray™ (ESI) source. We found that for all the considered analytes the MS signal obtained with ESI was about five-fold higher in intensity than the one obtained by the APCI source. Such a difference can be related with the observation that newer generation ESI sources show amount-flow dependant response rather than concentration-flow dependant response, making ESI source unexpectedly more sensitive than APCI like in this specific case. Another rationale can be related to the presence of a extendedly conjugated α,β-unsaturated 3 carbonyl moiety between C6 and C3 that makes the molecule more prone than common steroids to be ionized by ESI in a liquid phase.

The product ion mass spectra of the protonated molecular ions $[M + H]^+$ of Exe, $^{13}C_3$-Exe-IS and the DhExe metabolite, were obtained from chemical standards and by using a CE of 30 eV (Fig. 1). The fragmentation pattern shows abundant fragments at *m/z* 120.8, 149.0 for Exe, at *m/z* 121.0, 123.2, 149.1 and 163.9 for $^{13}C_3$-Exe-IS, while a fragment at *m/z* 134.9 is the predominant one for the DhExe. The complexity of the MS/MS spectra makes it difficult to draw the entire pathways of fragmentation. Thevis *et al*. described some possible mechanism of fragmentation for steroid with an androstadiene-17β-ol-3-one nucleus.[15] According to such study the observed most abundant *m/z* 121.0 fragment ion may likely originate from the protoned cross-conjugated 3-carbonyl moiety through a fragmentation route that involves the cleavage of both C9–C10 and C6–C7, the loss of a CH_3 and a rearrangement leading to a stable tropylium cation-ion with *m/z* 121.0 (Fig. 2a). The proposed mechanism is in agreement with the presence in the $^{13}C_3$-Exe-IS MS/MS spectrum of an abundant *m/z* 123.2 ion that generates from the *m/z* 121.0 fragment plus 2 amu without the involvement of the C6-methylene group. However, the persistence of the *m/z* 121.0 ion, in the CID spectra of $^{13}C_3$-Exe-IS, seems to suggest that the *m/z* 121.0 fragment ion could also originate from the unlabelled 17-ketogroup moiety of Exe. The characteristic fragment ion at *m/z* 134.9 in the MS/MS spectrum of DhExe seems to be related to the same fragmentation mechanism that involves the cleavage of the C9–C10 and C7–C8 bonds (Fig. 2b).

The reaction monitoring transition (MRM) used for the quantitation was done by exploiting the most abundant product ions for each analyte: *m/z* 297.0 → 120.8 and *m/z* 300.0 → 123.2 for Exe and $^{13}C_3$-Exe-IS, respectively. The *m/z* 299.1 → 134.9 transition was chosen for the quantitation of the DhExe metabolite.

Liquid chromatography

The high selectivity offered by the tandem MS technique has often allowed to use a generic and very fast LC gradient in order to achieve a high analytical throughput. In this context, LC conditions are adjusted for obtaining the best peak shape within the shortest retention time for each analyte. However, a highly fast and, sometimes, very simple LC run is frequently a cause of selectivity problems when analyzing real clinical biological samples. A non-optimal chromatographic separation of matrix-related components from the targeted analytes can affect the ionization process of the latter ones if co-eluting and affects the final quantitative result. Stringent chromatographic conditions are also needed to eliminate other potential interferences associated to the presence in the sample of drug metabolites derived by both phase I and II enzyme reactions. Since these metabolites are chemically related to the parent drug they can generate, by themselves or by in-source reactions, a signal at the same MRM transition of parent drug. If such isobaric interference are not well resolved they may limit the quantitation of the targeted drug as recently reviewed by Yan *et al*.[16] In order to address this potential problem the LC optimization has been carried out by running extracts of real plasma samples as obtained from a patient under Exe adjuvant treatment (25 mg/day). Figure 3a report the MRM chromatograms of the extracted sample in comparison with a pre-dose sample by using a 3 min-gradient from 10 to 60%

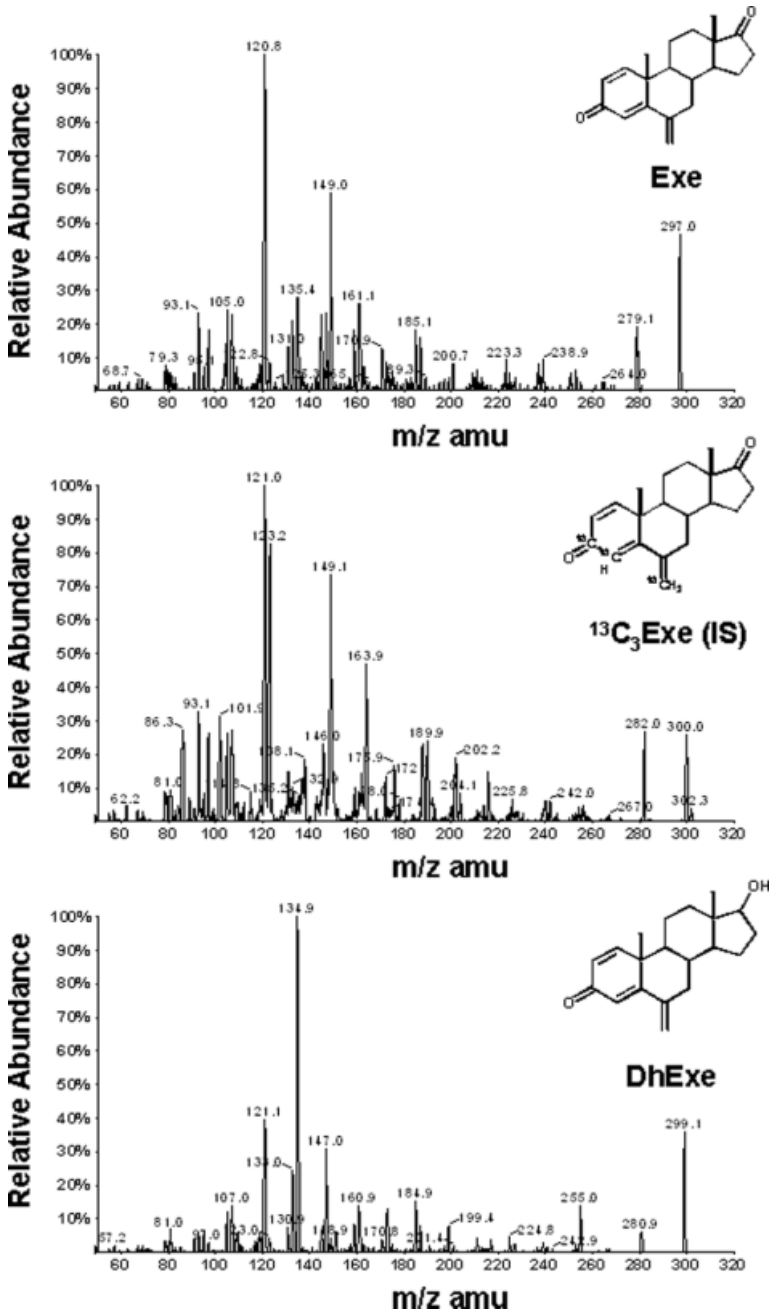

Figure 1. ESI product ion MS/MS spectra of Exe, $^{13}C_3$-Exe-IS and DhExe protonated molecular ions.

of acetonitrile in 0.1% acetic acid water flowing at 0.5 ml/min into a 50 × 2.0 mm Onyx C18 Lith column kept at 30 °C. The extracted ion chromatogram (XIC) revealed a shoulder on peak shape of the Exe that can be attributed to an interference likely originated by the drug administration since it is not present in the pre-dose sample. There is as well a strong signal emerging at 0.6 min on the same Exe MRM channel but it does not affect the targeted Exe quantitation. The chromatographic optimization through the column type selection, the mobile phase composition choice and the gradient profile programming were driven in order to achieve the best resolution between Exe and the interfering peak within the shortest running time. This led to use a 50 × 2.0 mm, 3 µm Gemini C6-Phenyl column (Phenomenex, Torrance, CA USA) maintained at 60 °C and running in isocratic mode with a mixture of 0.1% acetic acid in 32% acetonitrile as mobile phase flowing at 0.6 ml/min. Under the above conditions the column back pressure was 120 bar which arose to 150 bar after the run of more than 200 samples. The MRM chromatograms of the pre-dose and $C^{through}$ extracted plasmas from patients are shown in Fig. 3b. The chosen chromatographic condition assured an adequate separation ($Rs \geq 2$) between Exe and the isobaric interference. For maintaining the column performances during long running batches, a 5 min-washing step with 100% acetonitrile was introduced after every 20 samples in order to reduce any possible accumulation of plasma contaminants into the column.

Figure 4 shows the product ion MS spectra of the two interferences appearing at 0.6 and 3.5 min, obtained by the

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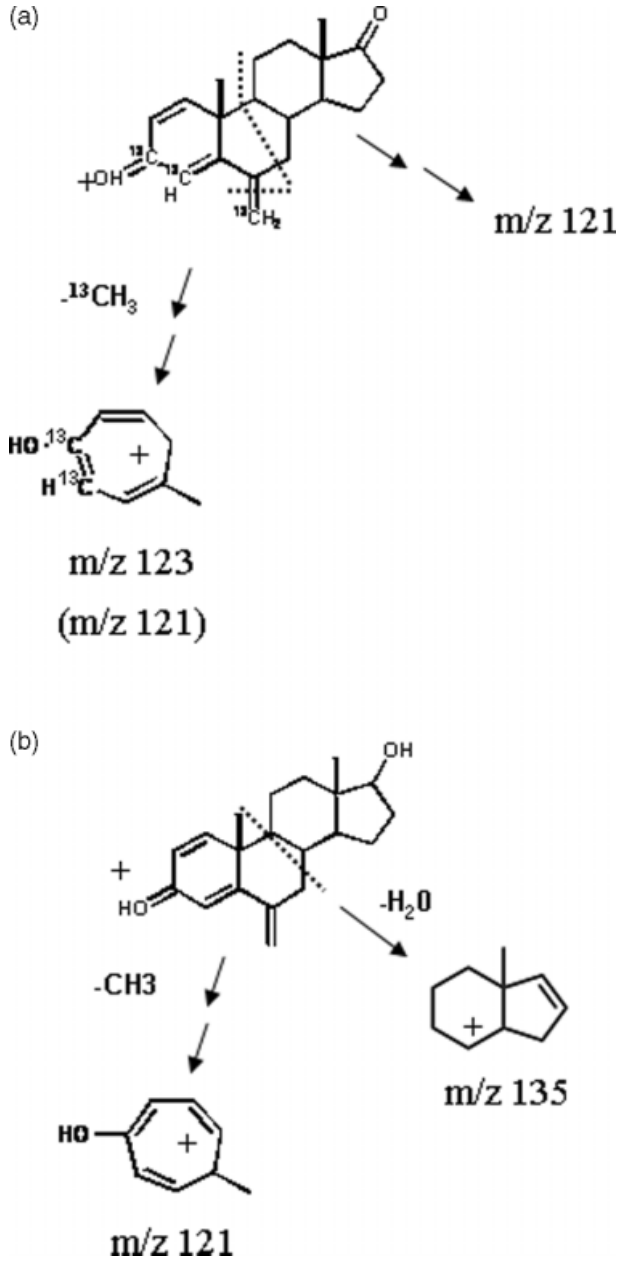

Figure 2. Proposed fragmentation schemes for Exe and DhExe generating the product ion at *m/z* 121 and *m/z* 135, respectively.

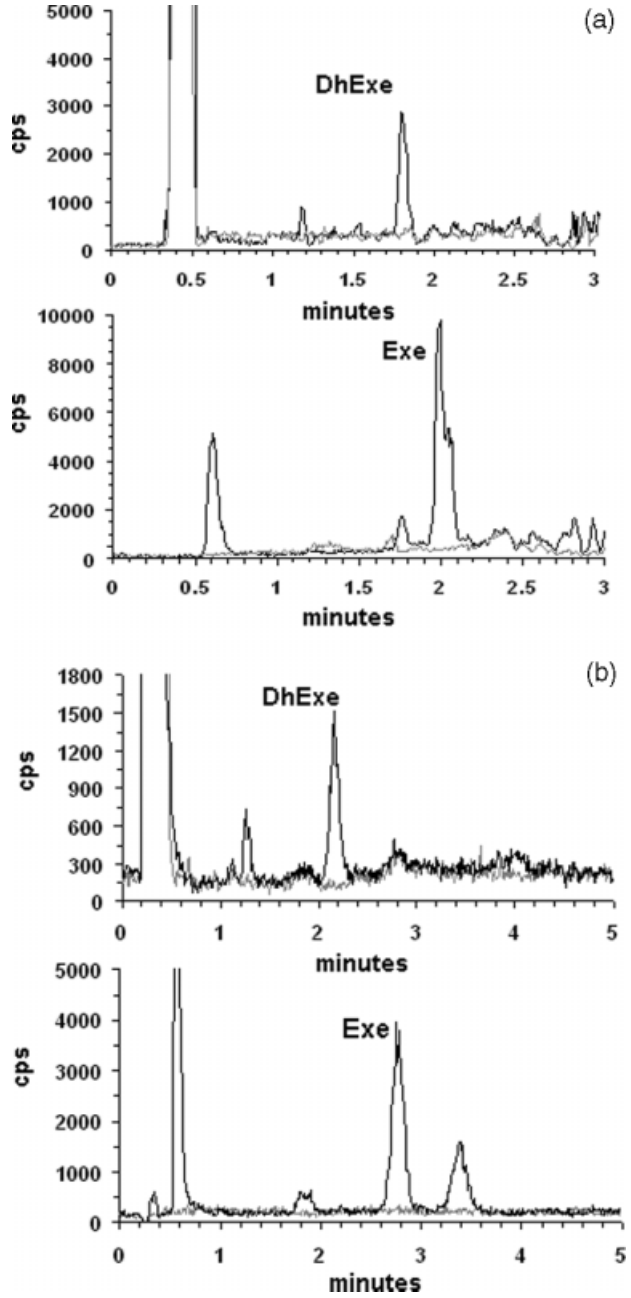

Figure 3. Extracted ion chromatogram (XIC) of Exe and DhExe present in a pre-dose clinical plasma sample (gray line) and after 24 h (black Line) from the administration of 25 mg/day of Exe. (a) The use of a fast LC gradient on 50 × 2.0 mm C18-Onyx Lith-column while (b) reports the run of the same sample using isocratic condition on 50 × 2 mm, 3 µm C6-Phenyl Gemini column.

enhanced product ion (EPI) scan experiment. The EPI spectrum of interference at 0.6 min with a predominant ion at *m/z* 121.1 (Fig. 4a) was superposable to the one of Exe, while for the interference at 3.5 min it shows a prominent fragment-ion at *m/z* 147.1 (Fig. 4b). The shorter retention time of the interference at 0.6 min could suggest the presence of an hydrophilic Exe conjugate (either glucoronide or sulfate) that, in source, dissociates back to the parent drug.[16]

However, after glucoronidase/sulfatase treatment of the $C^{through}$ plasma sample no reduction of isobaric interference was observed, neither a variation of concentration of Exe and DhExe was revealed. This should suggest that no glucoronide of sulfated metabolites are likely present in the investigated plasma sample.

For the interference at 3.5 min it can be hypothesized the presence of a Exe stereoisomer, however, further specific studies are needed to draw definitive conclusion about the effective structure of these observed isobaric interferences.

Carry over, recovery and matrix effect

The mean carry-over for Exe and DhExe was less than 0.003% large lower than 20% of LLOQ signal. A high mean overall recovery higher that 97% was reported for both the analytes when evaluated at VLQc and HQc levels, and evidences a high efficiency and reproducibility of the extraction protocol consisting in a PPP step. The high overall recovery also denotes a low ion suppression

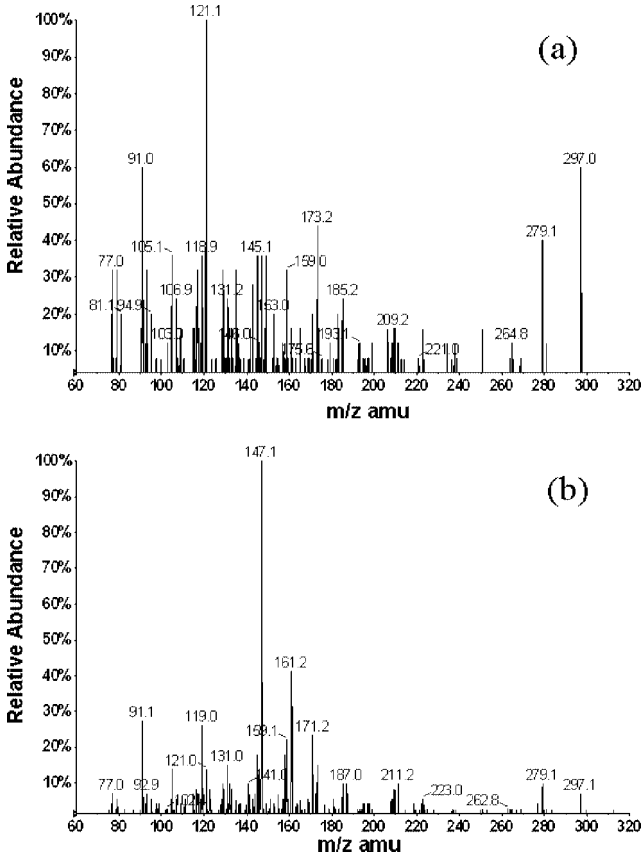

Figure 4. Enhanced product ion scan (EPI) of isobaric interferences eluting (a) at 0.6 min and (b) at 3.5 min.

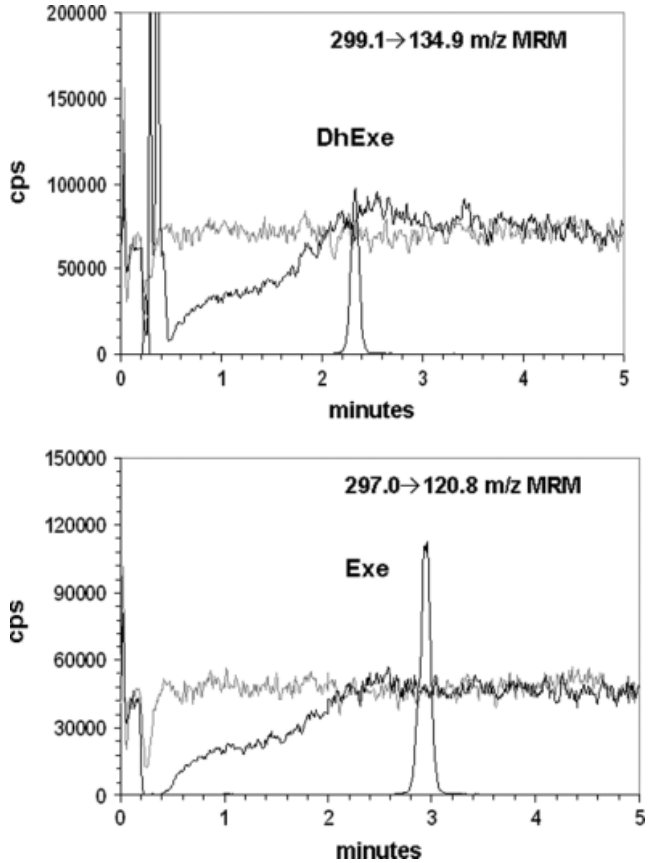

Figure 5. Regions for ionization suppression from the injection of mobile-phase (gray) and extracted plasma blank (black) with a post-column infusion of (a) DhExe and (b) Exe.

effect as associated to the potential co-eluting of plasma matrix co-extractants. Figure 5 shows the marginal ion suppression effect within the elution time region of the Exe and DhExe when a blank extracted plasma sample was injected in combination with the post-column infusion of standard analytes. The result confirms that ion suppression did not significantly influence the quantitative analysis of both analytes.

Linearity and LLOQ

Calibration standards prepared in human control plasma were analyzed through different analytical runs over a concentration range spanning from 0.09 to 36.0 ng/ml and from 0.19 to 51.2 ng/m for DhExe and Exe, respectively. When a $1/\times$ weighting linear fit was used, the correlation coefficients (r^2) measured over six batches were $\geq$0.9945 for Exe and $\geq$0.9973 for DhExe. The deviation from nominal value of the back-calculated concentrations were $\leq$9.8% and $\leq$12.4% for Exe and DhExe, respectively, while the RSD values were $\leq$12.0.% for Exe and $\leq$13.2% for DhExe.

The LLOQ resulted in 0.09 and 0.19 ng/ml for DhExe and Exe, respectively with a calculated signal to noise ratio (S/N) of about 8:1 and 11:1. The mean inter-day RE and RSD for the two analytes were less than 9.7 and 10.1% and are therefore within the acceptable criteria.

The LLOQ for Exe resulted two-fold higher as compared to the method reported by Cenacchi *et al.*[11] However, it is worth noting that the high level of sensitivity of the method was obtained by using a labour intensive off-line SPE extraction procedure and by starting from 500 µl of plasma. The hereby-described method presents the advantage of an increased selectivity accompanied by an off-line sample preparation, limited to a quick and cost effective batch protein precipitation step, and by using just 100 µl of plasma sample.

Precision and accuracy

The overall results on assay precision and accuracy are reported in Table 1. Intra and inter-day precision expressed as RSD for the VLQc, LQc, MQc, and HQc ranged from 0.87 and 7.71% for the Exe, while for the DhExe it was between 2.09 and 8.13%. The Intra and inter-day accuracy expressed as RE (relative error) ranged from 1.51 to 13.18% for Exe and from −8.96 to 5.78% for DhExe. The analysis of spiked samples with Exe and DhExe concentration up to 5 × ULOQ reported a RSD and RE less than 10% for both analytes meaning that plasma sample with concentration outside the ULOQ can be diluted and precisely measured.

Specificity

A selectivity test was performed to ensure the absence of any potential adverse effect of the endogenous compounds present in plasma samples. Five different EDTA plasma samples obtained from different breast cancer patients not treated with Exe were investigated. The MRM chromatogram of double blank, blank and sample spiked at VLQc concentrations did not show any detectable co-eluting peaks for Exe, DhExe and $^{13}C_3$-Exe-IS in all

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Table 1. Intra and inter-day precision and accuracy for Exe and DhExe in human plasma

	Intra-day			Inter-day
EXE	Day 1 ($n = 10$)	Day 2 ($n = 5$)	Day 3 ($n = 5$)	Day 1–3 ($n = 20$)
0.64 ng/ml				
Mean (ng/ml)	0.68	0.68	0.69	0.69
RE (%)	6.55	6.72	8.56	7.28
SD (ng/ml)	0.04	0.02	0.03	0.03
RSD (%)	5.37	2.61	3.86	4.33
3.84 ng/ml				
Mean (ng/ml)	4.26	4.35	4.34	4.31
RE (%)	10.96	13.18	12.89	12.34
SD (ng/ml)	0.14	0.05	0.04	0.11
RSD (%)	3.17	1.16	0.87	2.50
19.2 ng/ml				
Mean (ng/ml)	21.13	20.86	20.95	20.98
RE (%)	10.05	8.65	9.11	9.27
SD (ng/ml)	0.51	1.61	0.89	0.92
RSD (%)	2.40	7.71	4.24	4.40
38.4 ng/ml				
Mean (ng/ml)	42.12	38.98	40.96	41.05
RE (%)	9.69	1.51	6.67	6.89
SD (ng/ml)	1.12	2.92	1.21	2.10
RSD (%)	2.67	7.48	2.95	5.13
DhEXE				
0.5 ng/ml				
Mean (ng/ml)	0.46	0.46	0.48	0.46
RE (%)	−8.96	−8.40	−3.40	−7.43
SD (ng/ml)	0.02	0.01	0.01	0.02
RSD (%)	3.76	2.24	2.77	4.00
2.7 ng/ml				
Mean (ng/ml)	2.84	2.86	2.79	2.83
RE (%)	5.04	5.78	3.19	4.76
SD (ng/ml)	0.15	0.15	0.09	0.13
RSD (%)	5.25	5.40	3.07	4.71
13.5 ng/ml				
Mean (ng/ml)	13.65	14.22	14.06	13.90
RE (%)	1.11	5.33	4.15	2.93
SD (ng/ml)	0.35	1.16	0.54	0.68
RSD (%)	2.57	8.13	3.85	4.92
27.0 ng/ml				
Mean (ng/ml)	27.03	26.04	26.98	26.77
RE (%)	0.11	−3.56	−0.07	−0.85
SD (ng/ml)	0.78	1.90	0.56	1.14
RSD (%)	2.87	7.28	2.09	4.25

SD: standard deviation, **RE –** relative error: mean percentage deviation from nominal value, **RSD:** relative standard deviation.

the plasma samples tested. The measurement deviation from the nominal value was ≤8.68% with a precision ≤12.78% indicating that the assay performance is independent from the sample matrix. However it is worth noting that compounds which can interfere with quantitation of Exe are present only in plasma of patients under Exe treatment (Fig. 4) indicating that probably the interference is originated by the *in vivo* metabolism of Exe.

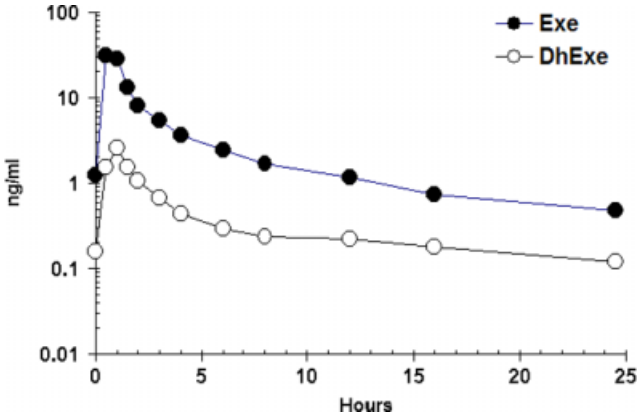

Figure 6. Steady state concentration–time profile of Exe (filled circle) and DhExe (open circle) evaluated after 8 week of adjuvant therapy with 25 mg/day of Exe in a patient with breast cancer.

Stability

The stability results indicate that both Exe, DhExe and $^{13}C_3$-Exe-IS did not show significant degradation in the diluted PPP extract stored up to 96 h at 5 °C. The same stability was found when plasma sample were stored at both 4 °C and at room temperature for over 48 h.

Conversely, the stability for Exe appears to be critical when monitored in whole blood. In EDTA-whole blood samples stored at 4 °C, the Exe concentration resulted significantly reduced of about 20% after the first 3 hand more than 50% after 24 h. Conversely, the DhExe metabolite resulted stable up to 24 h in whole blood stored at both 4 °C and room temperature. This stability suggests that blood samples should be quickly processed after the drawing in order to obtain trustable quantitative Exe results. In the freeze/thaw and long-term stability experiments, the observed maximum degradation was within 9.1% confirming previous findings, and fulfilling the acceptance criteria.

Pharmacokinetic application

The proposed method was applied to determine the steady state of the pharmacokinetic profile of Exe and DhExe in patients after 8 week of treatment with 25 mg/day of oral Exe. The Exe and DhExe plasma concentrations *versus* time curve, reported in Fig. 6, shows the peak plasma concentration of parent drug and metabolites occurring at about 1.0 h from administration. The disposal phase shows a characteristic biphasic decay pattern with a fast initial elimination rate followed by a slower elimination process. The terminal half-lives were 10.1 and 14.3 h for of Exe and DhExe. The AUC calculated from time 0 up 24 h for Exe was 74.5 $\mu g \times l^{-1} \times h$ while that of DhExe was 8.8 $\mu g \times L^{-1} \times h$, about nine-fold lower than the parent drug. In a group of six breast cancer patients the C^{peak} and $C^{through}$, levels were 24.4 ± 5.6 ng/ml, 1.0 ± 0.6 ng/ml and 2.4 ± 0.8 ng/ml, 0.3 ± 0.2 ng/ml, respectively for Exe and DhExe within the inter patient variability already observed in previously clinical pharmacology studies.[12,17] In all the investigated patients the DhExe and Exe $C^{through}$ were higher than the respective LLOQ indicating that the performances of the present LC–MS/MS method resulted suitable to well depict the plasma pharmacokinetics of Exe with adequate sensitivity.

Conclusions

A sensitive, specific, accurate and reproducible HPLC–ESI–MS/MS method has been developed and validated for the simultaneous pharmacokinetic monitoring of the Exe and its major metabolite DhExe. Compared to the previously reported methods the present assay have a similar sensitivity with the advantage to use a smaller amount of plasma and a simple plasma preparation approach consisting on a rapid and cost effective PPP technique. The method has an LLOQ of 0.1 and 0.2 ng/ml for both DhExe and Exe which was found suitable for the pharmacokinetics monitoring up to 24 h from administration. The present LC–ESI–MS/MS represents an effective analytical tool for supporting large clinical pharmacology investigations.

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