Journal of Chromatography B, 879 (2011) 2984–2990

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

A sensitive combined assay for the quantification of paclitaxel, docetaxel and ritonavir in human plasma using liquid chromatography coupled with tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 20 July 2011
Accepted 26 August 2011
Available online 31 August 2011

Keywords:
Paclitaxel
Docetaxel
Ritonavir
Liquid chromatography/tandem mass spectrometry
Acid/alkaline mobile phases

ABSTRACT

A combined assay for the determination of paclitaxel, docetaxel and ritonavir in human plasma is described. The drugs were extracted from 200 μL human plasma using liquid–liquid extraction with tertiar-butylmethylether, followed by high performance liquid chromatography analysis using 10 mM ammonium hydroxide pH 10:methanol (3:7, v/v) as mobile phase. Chromatographic separation was obtained using a Zorbax Extend C_{18} column. Labelled analogues of the analytes are used as internal standards. For detection, positive ionization electrospray tandem mass spectrometry was used. Method development including optimisation of the mass transitions and response, mobile phase optimisation and column selection are discussed. The method was validated according to FDA guidelines and the principles of Good Laboratory Practice (GLP). The validated range was 0.5–500 ng/mL for paclitaxel and docetaxel and 2–2000 ng/mL for ritonavir. For quantification, quadratic calibration curves were used ($r^2 > 0.99$). The total runtime of the method is 9 min and the assay combines analytes with differences in ionisation and desired concentration range. Inter-assay accuracy and precision were tested at four concentration levels and were within 10% and less than 10%, respectively, for all analytes. Carry-over was less than 6% and endogenous interferences or interferences between analytes and internal standards were less than 20% of the response at the lower limit of quantification level. The matrix factor and recovery were determined at low, mid and high concentration levels. The matrix factor was around 1 for all analytes and total recovery between 77.5 and 104%. Stability was investigated in stock solutions, human plasma, dry extracts, final extracts and during 3 freeze/thaw cycles. The described method was successfully applied in clinical studies with oral administration of docetaxel or paclitaxel in combination with ritonavir.

1. Introduction

Docetaxel (Taxotere®) and paclitaxel (Taxol®) are both taxanes and share the baccatin ring structure (see Fig. 1) [1]. Paclitaxel was isolated in the early 70 s from *Taxus brevifolia* and docetaxel, a semi-synthetic derivative of a taxane from *Taxus baccata* was found a decade later [2]. Both taxanes are widely used as intravenously administered anticancer agents but oral formulations with paclitaxel and docetaxel are currently under investigation in both *in vitro* and in vivo studies [3]. The taxanes are subject to a complex detoxification mechanism involving both ABC drug transporters and drug metabolizing enzymes, which results in a low bioavailability after oral administration [4]. In vivo, both P-glycoprotein (PgP) and multidrug resistance-associated protein (MRP) 2 are involved in paclitaxel and docetaxel pharmacokinetics by decreasing exposure to the taxanes [5,6]. Thereby, MRP7 reduces in vivo tissue sensitivity to paclitaxel [7]. Paclitaxel is metabolized by Cytochrome P450 (CYP) 2C8 and CYP3A4, while docetaxel is primarily metabolized by the CYP3A subfamily [1]. Due to the involvement of the transporters and CYP enzymes, the oral bioavailability of the taxanes is limited and several studies have assessed enhancers in combination with oral formulations to increase the bioavailability [3]. One of the currently applied boosting agents is the CYP3A inhibitor ritonavir (Norvir®) (see Fig. 1). Low doses of ritonavir are also widely used as booster to increase

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doi:10.1016/j.jchromb.2011.08.034

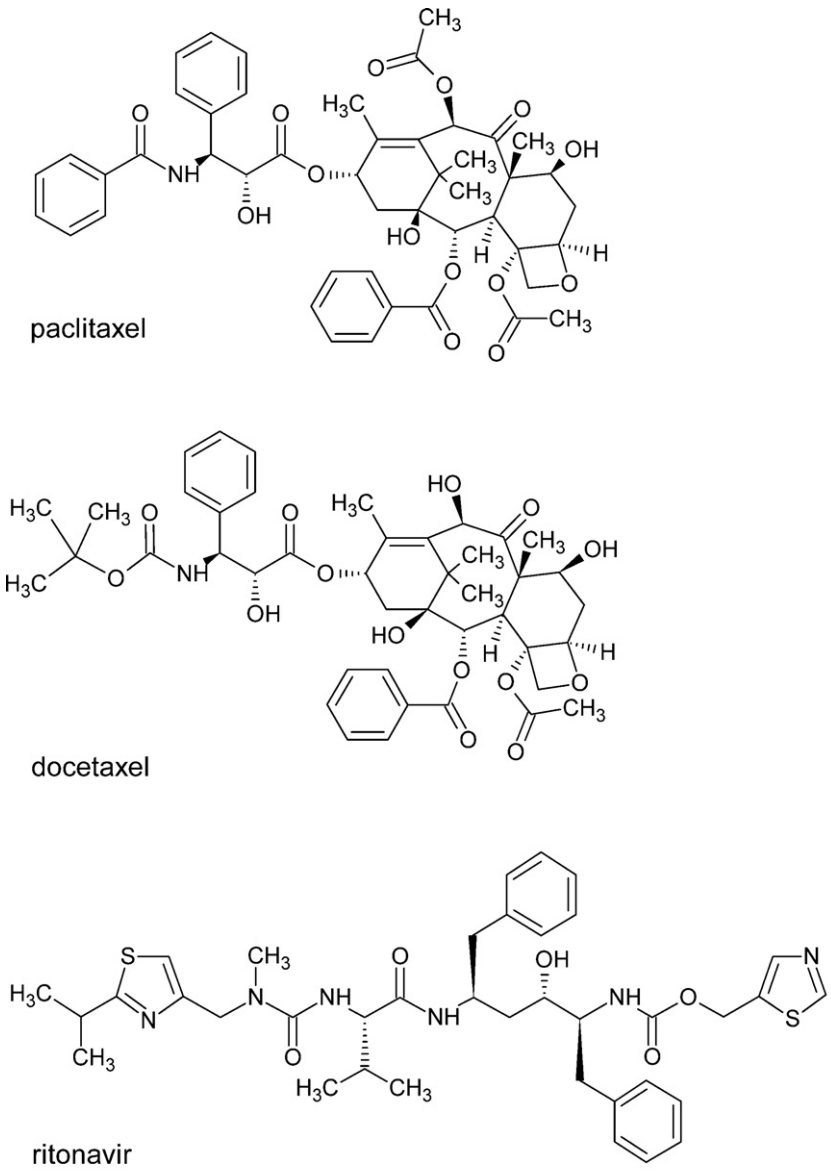

Fig. 1. Structures of paclitaxel, docetaxel and ritonavir.

the bioavailability of protease inhibitors in HIV therapy [8]. Previously, several methods for the (combined) quantification of taxanes in human plasma are described [9–20] and separate methods for the quantification of ritonavir mostly in combination with other antiretroviral drugs [21–25]. To support further studies with ritonavir-boosted oral taxanes, we developed and validated a sensitive and fast liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous detection of docetaxel, paclitaxel and ritonavir. We aimed for a lower limit of quantification of 0.5 and 2 ng/mL for the taxanes and ritonavir, respectively. According to previous pharmacokinetic profiling [14,26–28], these limits provide sufficient sensitivity to support clinical studies.

2. Material and methods

2.1. Chemicals

Paclitaxel and docetaxel were purchased from Sequoia Research Products (Oxford, UK). Ritonavir, $^{13}C_3$-labelled ritonavir and D_9-labelled docetaxel were purchased from Toronto Research Chemicals (North York, ON, Canada). $^{13}C_6$-labelled paclitaxel was kindly provided by Pharmacia Corporation (Nerviano, Italy). Methanol (HPLC grade) was obtained from Biosolve Ltd. (Amsterdam, the Netherlands), tertiar-butylmethylether (tert.-butylmethylether/TBME, Analytical grade) and water for chromatography (LiChrosolv) were obtained from Merck (Darmstadt, Germany). Drug free lithium-heparinized human plasma was obtained from Bioreclamation LLC (New York, NY, USA).

2.2. Mass spectrometric and chromatographic conditions

The chromatographic conditions were based on previously developed assays for quantification of docetaxel and paclitaxel [14,29]. An API 4000 triple quadrupole MS with electrospray ionisation (ESI) (AB Sciex, Foster City, CA, USA) was coupled to an Agilent 1100 liquid chromatographic system (Agilent Technologies, Palo Alto, CA, USA). The Agilent 1100 system consisted of a binary pump, an autosampler, a mobile phase degasser and a column oven. The mobile phase consisted of methanol:10 mM ammonium hydroxide in water (70:30, v/v) at a flow of 0.3 mL/min.

Table 1
MS/MS parameters and mass transitions of the analytes.

Parameter	Setting
Entrance potential	10.0 V
Ionspray voltage	5500 V
Collision gas	5.0 psi
Curtain gas	20.0 psi
Ionsource gas 1	60.0 psi
Ion source gas 2	50.0 psi
Temperature	400 °C

Parameter	PAC	DOC	RTV	IS PAC	IS DOC	IS RTV
Declustering potential (V)	60	56	81	60	56	81
Collision energy (V)	23	15	93	23	15	39
Collision cell exit potential (V)	16	14	18	16	14	18
Scan time (s)	0.2	0.2	0.2	0.2	0.2	0.2
Precursor ion (*m*/*z*)	854	808	721	860	817	726
Product ion (*m*/*z*)	509	527	196	515	527	296
Typical R.T. (min)	3.8	4.4	6.9	3.8	4.3	6.8

Abbreviations: PAC: paclitaxel; DOC: docetaxel; RTV: ritonavir; IS PAC: $^{13}C_6$-labelled paclitaxel; IS DOC: D_9-labelled docetaxel; IS RTV: $^{13}C_3$-labelled ritonavir.

Chromatographic separation was obtained using a Zorbax Extend C_{18} column (150 mm × 2.1 mm I.D., particle size 5 μm; Agilent Technologies) protected with an inline filter (0.5 μm). The column oven was set at 35 °C, while the autosampler was thermostatted at 4 °C. A sample volume of 25 μL was injected and the injection needle was washed for 3 s with methanol after each injection. Mass transitions were optimized for each compound in positive ion mode. Ion specific parameters were optimized for each analyte separately. An overview of the mass transitions and MS/MS settings is listed in Table 1. The total run time was 9 min. A switching valve was used to direct the eluent during the first 3 min of the run to waste. For quantification, the multiple reactions monitoring (MRM) chromatograms were acquired with Analyst software version 1.5 (AB Sciex).

2.3. Preparation of stock and working solutions

Two stock solutions of each analyte from independent weightings were prepared in methanol at a concentration of 1 mg/mL. The stock solutions of the three analytes were diluted to combined working solutions with methanol. One set of working solutions was used for the preparation of calibration standards, while the other set was used for the preparation of quality control (QC) samples. For the internal standards $^{13}C_3$-ritonavir, D_9-docetaxel and $^{13}C_6$-paclitaxel stock solutions of, respectively, 0.5, 1.0 and 0.1 mg/mL were prepared in methanol. The three internal standards were diluted to one combined internal standard working solution of 500, 40 and 200 ng/mL ($^{13}C_3$-ritonavir, D_9-docetaxel and $^{13}C_6$-paclitaxel, respectively). All solutions were stored at −20 °C.

2.4. Preparation of calibration standards and quality controls

Calibrations standards (CAL) were prepared by diluting a fixed amount of working solution containing each analyte in blank human plasma. The CALs contained the analytes in a range of 0.5–500 ng/mL (paclitaxel/docetaxel) and 2–2000 ng/mL (ritonavir). In a similar way, QC samples at three concentrations were prepared from another set of working solutions. The QCs contained the taxanes in concentrations of 1.5, 100 and 400 ng/mL and ritonavir in concentrations of 6, 400 and 1600 ng/mL. For validation purpose, additional QCs were made at lower limit of quantification (LLOQ) level (0.5/2 ng/mL; taxanes/ritonavir) and higher than the upper limit of quantification (ULOQ; 2000/8000 ng/mL; taxanes/ritonavir). Samples were transferred to 2.0 mL polypropylene tubes (Eppendorf, Merck) in aliquots of 200 μL and stored at −20 °C.

2.5. Sample preparation

To 200 μL sample, 20 μL internal standard working solution was added and the sample was vortex-mixed for 10 s. Blank samples were spiked with 20 μL of methanol instead of internal standard working solution. After mixing, 1.0 mL of tertiar-butylmethylether was added and again, the sample was mixed for 10 s. Samples were successively shaken automatically for 10 min at 1250 rpm (L46, Labinco, Breda, The Netherlands) and centrifuged for 5 min at 23,000 × *g* (5403 Eppendorf, Netheler Hinz GmbH, Hamburg, Germany). The aqueous layer was frozen in a bath of ethanol and dry ice and the organic layer was transferred into a clean 1.5 mL tube. The sample was dried under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in methanol: water (1:1, v/v), vortex-mixed for 10 s and centrifuged for 3 min at 23,000 × *g*. The supernatant was transferred to a glass autosampler vial with insert and 25 μL was injected onto the LC–MS/MS system.

2.6. Validation

For the assay, a full validation program was executed, including calibration model, accuracy, precision, carry-over, dilution test, specificity and selectivity, matrix effect, recovery and stability. Stability of each analyte separately during 3 freeze/thaw cycles [14,30,31] has been previously determined at our institute, so only long-term stability in human plasma and stability of the dried extract, the processed sample stability and re-injection reproducibility were executed. Long-term stability was tested for each analyte separately, while all other stability testing was executed with all analytes combined. The validation was executed according to the FDA guidelines [32] on bioanalytical method validation and to the guidelines of the 3rd AAPS/FDA bioanalytical workshop [33]. The validation was performed in compliance with the OECD principles of Good Laboratory Practice (GLP) [34].

3. Results and discussion

3.1. Mass spectrometric and chromatographic conditions

3.1.1. Mass transition

The molecular ions ($[M+H]^+$) of paclitaxel, docetaxel and ritonavir observed at *m*/*z* 854, 808 and 721, respectively, were used as precursor ions to generate product ion spectra. The most abundant product ions of paclitaxel and docetaxel were optimized for multiple reactions monitoring (MRM) (Table 1). For ritonavir the product ion at *m*/*z* 196, and not the most abundant product ion at *m*/*z* 296, was optimized for MRM since back-calculated calibration concentrations at the transition 721–196 provided the lowest total bias across the range. For the internal standard $^{13}C_3$-ritonavir, the isotope ion at *m*/*z* 726 was selected as precursor ion, instead of the protonated molecular ion at *m*/*z* 724, as the molecular isotopic ions of ritonavir monitored at *m*/*z* 724 and 725 interfered with the molecular isotopic ions of $^{13}C_3$-ritonavir at these mass transitions. At mass transition *m*/*z* 726, ritonavir showed no isotope ions, while $^{13}C_3$-ritonavir showed isotope ions at an intensity which was 2-fold lower compared to the intensity at *m*/*z* 724. To increase the sensitivity for the detection of $^{13}C_3$-ritonavir, the most abundant product ion at *m*/*z* 296 was selected instead of the same product ion as used for ritonavir to obtain a maximum response. For D_9-docetaxel and $^{13}C_6$-paclitaxel the molecular ions observed at *m*/*z* 860 and 817, respectively, were used as precursor ions and the most abundant product ions were used for MRM (Table 1).

3.1.2. Acid versus alkaline mobile phase

During development of the method, 0.1% formic acid (pH 2.7) and 10 mM ammonium hydroxide (pH 8–10) were tested as

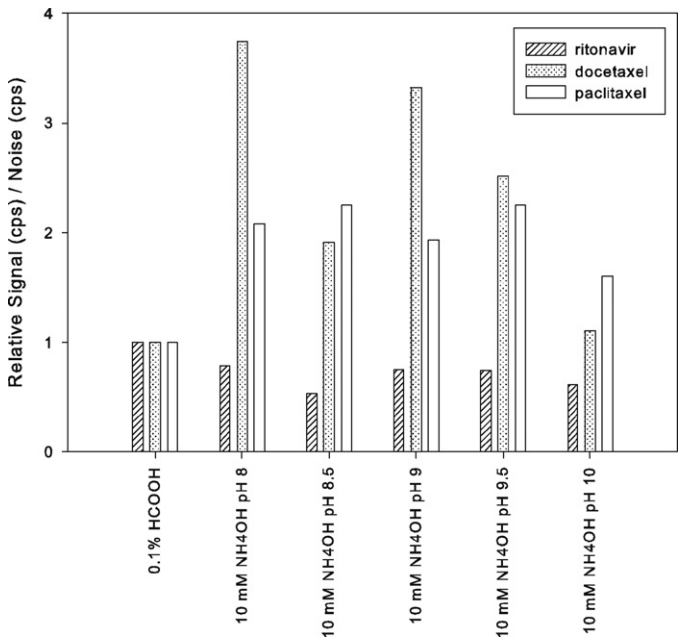

Fig. 2. Relative signal-to-noise ratios of ritonavir, docetaxel and paclitaxel after flow injection analysis. During the experiments no column was used and the mobile phase contained 70% (v/v) methanol.

aqueous phases of the eluent. Docetaxel and paclitaxel responses increased 1.1–3.7-fold when alkaline mobile phases were applied, while the response of ritonavir decreased (see Fig. 2). For these tests, a small volume (2 μL) was injected from a solution containing all analytes at a concentration of 5000 ng/mL (taxanes) or 20,000 ng/mL (ritonavir). Compared to 10 mM ammonium hydroxide pH 9.5, a mobile phase of pH 10 showed a slightly higher response of docetaxel, but also a higher background signal. Both observations resulted in a lower signal-to-noise ratio for 10 mM ammonium hydroxide pH 10. It was observed that the background signal of alkaline mobile phases was reduced when a column was used. Due to the reduction in noise by using a column, a mobile phase of pH 10 resulted in the highest signal-to-noise ratio and the best performance at LLOQ concentration level. Therefore 10 mM ammonium hydroxide pH 10 was selected as aqueous phase. It is known that the disposition of the analytes in the formed droplets has an effect on the ESI response as described in detail by Chech and Enke [35]. It is hypothesized that by changing the mobile phase from acid to alkaline, the disposition of the analytes in the droplets formed by ESI is changed resulting in an increase of the docetaxel and paclitaxel responses. The change of disposition could be the result of changed chemical characteristics of the mobile phase or the analytes due to pH changes (e.g. protonation or changed surface-activity) or adduct forming of the analyte with ammonium. The results of the experiments with different pH types suggest that the increase in response is due to a change in pH and not due to the presence of ammonium ions. Although the mechanism is not clear yet, the increase in the response of docetaxel by changing the pH of the mobile phase with ammonium hydroxide is supported by observations from other research groups [12–14,17,19].

3.1.3. Column selection

During development of the method, several columns were tested. Chromatography of a Zorbax Extend C_{18} column (150 mm × 2.1 mm I.D., particle size 5 μm; Agilent Technologies), a Kinetex C_{18} column (150 mm × 2.1 mm I.D., particle size 2.6 μm; Phenomenex), an Xbridge C_{18} column (50 mm × 2.1 mm I.D., particle size 5 μm; Waters) and a Gemini C_{18} 110A column (150 mm × 2.0 mm I.D., particle size 5.0 μm; Phenomenex) were compared at a flow rate of 0.2 mL/min. All other mass spectrometric and chromatographic conditions were as described above, except for the Kinetex column. For the Kinetex column the mobile phase consisted of 80% methanol instead of 70% and the column oven was set at 60 °C. This was necessary to reduce the pressure in the system. The Zorbax Extend column was considered superior to the other columns in terms of accuracy, precision and carry-over for all analytes and mainly for docetaxel. Injection of spiked docetaxel samples on the Kinetex column resulted in an increase of background signal at *m/z* 808 at the retention time of docetaxel. The signal remained constant over three subsequent injections of water: methanol (1:1, v/v) and this is probably due to a memory effect of the Kinetex column. This effect was observed during multiple runs and not seen with any of the other tested columns. The chromatographic conditions of the Zorbax Extend column were further optimized resulting in a flow rate of 0.3 mL/min. To reduce the run time, a mobile phase containing 75% (v/v) methanol was tested but under these conditions an endogenous interference shifted towards the docetaxel peak. Finally, a Zorbax Extend Column was selected and a mobile phase containing 70% (v/v) methanol was used. The flow rate was set at 0.3 mL/min and the column oven was set at 35 °C.

3.1.4. Optimization of ritonavir response

The combination of the taxanes paclitaxel and docetaxel in one assay with ritonavir was challenging as not only the desired concentration range of ritonavir was 4-fold higher, but also because ritonavir is a better responder compared to the taxanes. Consequently obtaining sensitivity of docetaxel and paclitaxel at LLOQ level resulted in saturation of the response of ritonavir at the ULOQ concentration level. For paclitaxel and docetaxel the most abundant product ions were selected for quantification. To prevent saturation of the detector, not the most abundant product ion at *m/z* 296 was selected for ritonavir but an apparently suboptimal product ion at *m/z* 196. The response of this transition was almost 30 times lower compared to the transition 721–296, however, saturation of the signal response was still observed. To reduce the amount of product ions, the collision energy was changed from 86 to 93 V. The combination of the selected mass transition and the apparently non-optimal collision energy resulted in the most accurate and precise quantification of ritonavir, despite the differences in ionisation and target concentration ranges between ritonavir and the taxanes.

3.2. Sample pre-treatment

Sample pre-treatment as described previously by our group for the quantification of docetaxel [14] and paclitaxel [31] was followed. Liquid–liquid extraction (LLE) was compared with protein precipitation. Relative recovery of both pre-treatment procedures was comparable, however using LLE, cleaner plasma extracts were obtained. Furthermore sample concentration was favourable to decrease the LLOQ. During development the following solvents for reconstitution were tested: methanol–water (1:1, v/v), methanol–water (7:3, v/v), acetonitrile–water (1:1, v/v) or 10 mM ammonium hydroxide pH 5–acetonitrile. Methanol–water (1:1, v/v) was selected as reconstitution solvent since peak shapes improved and the lowest noise levels in the MRM chromatograms were observed. A reduction in noise levels was not expected since analytes are eluting far from time zero. Probably less matrix ions are dissolved during the reconstitution in methanol–water (1:1, v/v) resulting in improved noise levels compared to the other tested solvents.

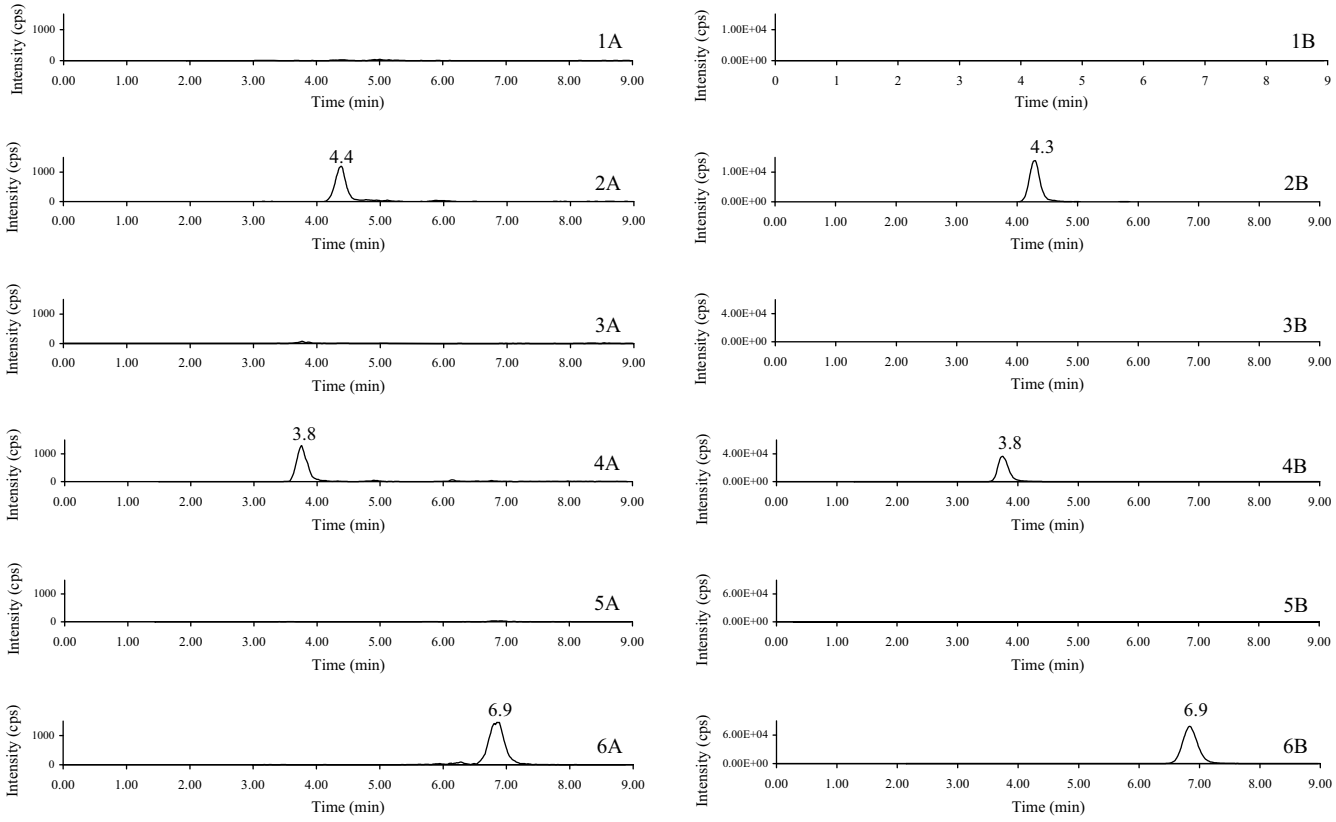

Fig. 3. Typical MRM chromatograms of the analytes in a blank plasma sample and at LLOQ level (0.5 ng/mL and 2 ng/mL for the taxanes and ritonavir, respectively). Panels show the response in a blank plasma sample and in a calibration sample at LLOQ level at the transition of docetaxel (1A and 2A, respectively), D_9-labelled docetaxel (1B and 2B), paclitaxel (3A and 4A), $^{13}C_6$-labelled paclitaxel (3B and 4B), ritonavir (5A and 6A) and $^{13}C_3$-labelled ritonavir (5B and 6B).

3.3. Validation of the method

3.3.1. Calibration model

CALs (8) with duplicate points at each concentration in the range 0.5–500 ng/mL (paclitaxel and docetaxel) and 2–2000 ng/mL (ritonavir) were prepared in control lithium heparinized human plasma and analyzed in three independent analytical runs. Calibration curves were fitted by quadratic regression of the peak area ratio with the internal standard versus the concentration with $1/x^2$ (the reciprocal of the squared concentration) as the weighting factor. At high concentration levels of the analytes, the calibration curves were not linear, resulting in a higher total bias in the upper ranges of the calibration curves when linear regression was applied instead of quadratic regression. Although both quadratic and linear regression met the criteria [32], quadratic regression was used to minimize the bias across the range of 0.5–500 ng/mL for the taxanes and 2–2000 ng/mL for ritonavir. Reduction of the calibration ranges was not desirable because this would result in an excessive number of re-analysis of study samples after dilution in control matrix. When calibration data was fitted by quadratic regression, correlation coefficients (r^2) of 0.9989 or better were obtained for all analytes. For every calibration curve the calibration concentrations were back-calculated from the response ratios. The deviations of the nominal concentrations should be within ±15%. At the LLOQ level a deviation of ±20% was permitted. For paclitaxel at all calibration standard concentration levels, the deviations of measured concentrations from nominal concentration were between −1.9 and 3.9% with coefficient of variation (CV) values of less than 12.5%. For the calibration standards of docetaxel, the deviations of measured concentrations from nominal concentration were between −1.9 and 1.7% with CV values of less than 4.3% and for ritonavir the deviations of measured concentrations were between −1.8 and 2.4% with CV values of less than 4.1%. Typical MRM chromatograms of blank plasma and the analytes at LLOQ-level are presented in Fig. 3.

3.3.2. Accuracy and precision

QC samples were prepared in control lithium heparinized human plasma and five replicates of each level were analyzed in three independent analytical runs. The accuracy was determined in percentage difference between the mean concentration and the nominal concentration. The CV was used to report the precisions. The intra- and inter-assay accuracies and precisions should be within ±20% and less than 20%, respectively, for the LLOQ concentration and within ±15% and less than 15%, respectively, for other concentrations. Assay performance data of paclitaxel, docetaxel and ritonavir are in Table 2. For all the analytes, all intra- and inter-assay accuracies and precisions fulfilled the required criteria [32,33].

3.3.3. Carry-over

Carry-over was tested by injecting two processed blank matrix samples subsequently after injection of an ULOQ sample in three independent runs. The response in the first blank matrix at the retention time of the analytes or internal standards should be less than 20% of the mean response of a LLOQ sample for the analytes and less than 5% of the mean response for the IS. The response in the first blank matrix at the retention time of the analytes was less than 6% of the mean response at the LLOQ for all analytes. At the retention times of the internal standards no response was observed. Therefore, the carry-over test was found to be acceptable.

Table 2
Assay performance for paclitaxel, docetaxel and ritonavir.

Compound	Nominal conc. (ng/mL)	Mean measured conc. (ng/mL)	Inter-assay bias (%)	Inter-assay precision (%)	No. of replicates
Paclitaxel	0.504	0.459	−8.8	3.8	15
	1.51	1.45	−4.1	2.7	15
	101	92.6	−8.3	1.9	15
	403	383	−4.9	1.4	15
Docetaxel	0.5	0.459	−8.2	7	15
	1.5	1.45	−3.2	6.8	15
	100	95.9	−4.1	5.2	15
	400	398	−0.5	5.3	15
Ritonavir	1.98	1.91	−3.5	2.9	15
	5.94	5.96	0.4	1.7	15
	396	387	−2.2	1.3	15
	1580	1570	−0.8	0.9	15

Abbreviation: conc.: concentration.

3.3.4. Dilution test

To assess the reliability of the method at concentration levels above the ULOQ (500/2000 ng/mL; taxanes/ritonavir), an intra-assay accuracy and precision test was executed. A sample at a concentration level above the ULOQ was diluted 2 (800–400 ng/mL; taxanes), 5 (800–160 ng/mL; taxanes), 10 (2000–200 ng/mL; taxanes) and 100 times (2000–200 and subsequently 200–20 ng/mL; taxanes) with human blank plasma. All samples also contained ritonavir in 4 times higher concentrations compared to paclitaxel and docetaxel. Diluted samples were processed in 5-fold and analyzed. Accuracy and precision were determined as described for QC samples and should be within ±15% and less than 15%, respectively. For all dilution factors, accuracies and precisions fulfilled these acceptance criteria [32,33].

3.3.5. Specificity and selectivity

From 6 different batches of control human heparinized plasma, blank (without the internal standards) and spiked samples (with paclitaxel, docetaxel, ritonavir and the internal standards) at the LLOQ concentration level were prepared. The samples were prepared to determine whether endogenous compounds interfere at the mass transitions chosen for the analytes and internal standards. Samples were processed according to the described procedures and analyzed. Interferences co-eluting with the analytes or internal standards should not exceed 20% of the peak area of the analytes at LLOQ or 5% of the internal standard areas. Deviations of the nominal concentrations should be within ±20%. MRM chromatograms of the double blanks did not show peaks that co-eluted with one of the analytes with areas exceeding 11% of the area at LLOQ level or peaks that co-eluted with the internal standards with areas that exceeded 0.3% of the internal standard area. Deviations from the nominal concentrations at the LLOQ level of the analytes in all batches were between −12.6 and 5.8%.

To assess cross-analyte/internal standard interferences, samples containing only one of the analytes at ULOQ level or one of the internal standards in control human heparinized plasma were processed and analyzed. Interferences were less than 20% of the peak area of the analytes at the LLOQ or 5% of the internal standard areas. During cross-analyte interference tests, the interferences at the retention times of paclitaxel, docetaxel and ritonavir were less than 5.1, 18.5 and 7.1% of the area of their LLOQ standards, respectively. Interference at the retention times of $^{13}C_6$-paclitaxel, D_9-docetaxel and $^{13}C_3$-ritonavir were less than 0.3, 1.4 and 5% of their area, respectively. Therefore the cross analyte/internal standard interferences were considered acceptable for all analytes and internal standards.

3.3.6. Matrix factor and recovery

The matrix factor was determined by comparing the signals of the analytes in processed QC samples at low, mid and high concentration levels to the signals of the same concentration levels in methanol: water (1:1, v/v). Responses were corrected for the internal standard area. The variability in matrix factor, as measured by the coefficient of variation should be less than 15% [33]. The matrix factor for paclitaxel, determined as the area ratio with and without matrix ions present, was between 0.992 and 1.02 for all concentration levels, with CV values less than 1.8%. For docetaxel, the matrix factor was between 0.933 and 0.99, with CV values less than 4.7%, when one outlier at low concentration was rejected. For ritonavir, the matrix factor was between 1.00 and 1.02, with CV values less than 1.9%. Overall, the results (matrix factor around 1) indicate that the stable-isotopically labelled internal standards of all analytes are most effective minimizing the influence of matrix effects.

The overall recovery was calculated by comparing the absolute areas in a processed QC sample to the areas measured in an unprocessed sample. The overall recovery was determined for all analytes at 3 concentration levels. The total recoveries for paclitaxel, docetaxel and ritonavir were between 77.5 and 104% with CV values of less than 15%. It was observed, that recovery increased with the concentration level. An underestimation of the total recovery at low concentration was caused by disproportional high areas of the analytes in the samples in absence of matrix ions. However, the total recovery was constant, precise and reproducible.

3.3.7. Stability

Stock solutions in methanol are stable for at least 12, 21 and 36 months for paclitaxel, docetaxel and ritonavir, respectively, when stored at nominally −20 °C. All analytes are stable in human plasma at ambient temperature for at least 24 h and at least 19 months (paclitaxel), 31 months (docetaxel) or 36 months (ritonavir) at nominally −20 °C and after three freeze/thaw cycles. The stability in dry extract and final extract samples containing all analytes was evaluated at nominally 2–8 °C at low and high level. The analytes were considered stable in the matrix when 85–115% of the initial measured concentration was found. Stability was demonstrated for at least 8 days at 2–8 °C under both conditions. The re-injection reproducibility was evaluated in processed samples of human plasma containing all analytes after storage at nominally 2–8 °C for 9 days. The bias was within ± 15% of the nominal concentration for all the analytes. Therefore it is concluded that the samples can be re-injected within 9 days when kept at nominally 2–8 °C.

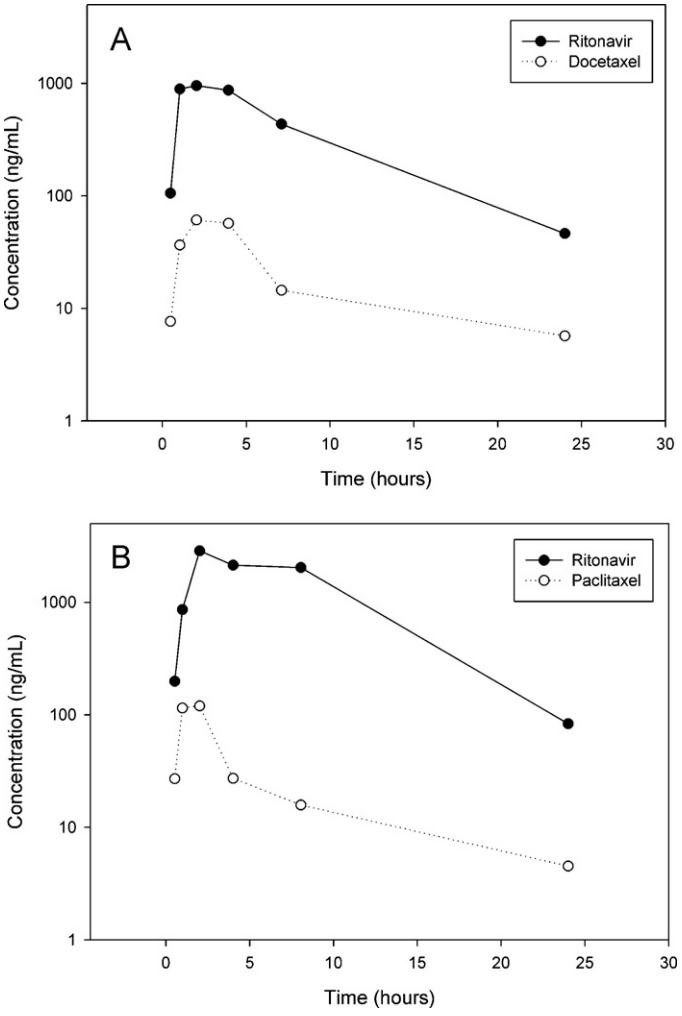

Fig. 4. Concentration–time curves after oral co-administration of 200 mg ritonavir with 60 mg docetaxel (A) or 200 mg ritonavir with 100 mg paclitaxel (B).

4. Application of the method

The validated assay is currently in use to support clinical studies. In these studies patients receive either paclitaxel or docetaxel in combination with ritonavir. In Fig. 4 concentration–time curves are presented of two patients receiving taxanes in combination with ritonavir, both orally administered. One patient received paclitaxel and ritonavir in doses of 100 mg and 200 mg, respectively. The other patient also received 200 mg ritonavir, co-administered with 60 mg docetaxel. Maximal plasma concentrations of oral paclitaxel and docetaxel were 120 and 61 ng/mL, respectively. The maximal plasma concentrations of ritonavir were 956 and 2879 ng/mL. The variation in plasma concentrations of ritonavir is observed in modelling of population pharmacokinetics after administration of 100 mg ritonavir bi-daily [36]. Phase I and II studies are currently ongoing to further investigate the oral co-administration of taxanes and ritonavir.

5. Conclusion

The development and validation of a combined assay for the quantification of paclitaxel, docetaxel and ritonavir in human plasma is described. The validated range for the taxanes was 0.5–500 ng/mL and for ritonavir was 2–2000 ng/mL using 200 μL plasma aliquots. The assay was validated according to FDA guidelines and has been successfully applied in clinical studies. During development we showed that using an alkaline mobile phase instead of an acid mobile phase the sensitivity for docetaxel and paclitaxel can be increased. Moreover, we demonstrated that each of the applied drugs in this single assay could be quantified successfully, although the individual chemical properties, concentration ranges and ionisation responses are diverse.

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