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RESEARCH PAPER

Laser diode thermal desorption atmospheric pressure chemical ionization tandem mass spectrometry applied for the ultra-fast quantitative analysis of BKM120 in human plasma

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Abstract A sensitive and ultra-fast method utilizing the laser diode thermal desorption ion source using atmospheric pressure chemical ionization coupled to tandem mass spectrometry (LDTD-APCI-MS/MS) was developed for the quantitative analysis of BKM120, an investigational anticancer drug in human plasma. Samples originating from protein precipitation (PP) followed by salting-out assisted liquid-liquid extraction (SALLE) were spotted onto the LazWell™ plate prior to their thermal desorption and detection by tandem mass spectrometry in positive mode. The validated method described in this paper presents a high absolute extraction recovery (>90 %) for BKM120 and its internal standard (ISTD) [D_8]BKM120, with precision and accuracy meeting the acceptance criteria. Standard curves were linear over the range of 5.00 to 2000 ng mL^{-1} with a coefficient of determination (R^2) >0.995. The method specificity was demonstrated in six different batches of human plasma. Intra- and inter-run precision as well as accuracy within ±20 % at the lower limit of quantification (LLOQ) and ±15 % (other levels) were achieved during a three-run validation for quality control (QC) samples. The post-preparative stability on the LazWell™ plate at room temperature was 72 h and a 200-fold dilution of spiked samples was demonstrated. The method was applied successfully to three clinical studies (n= 847) and cross-checked with the validated LC-ESI-MS/MS reference method. The sample analysis run time was 10 s as compared to 4.5 min for the current validated LC-ESI-MS/MS method. The resultant data were in agreement with the results obtained using the validated reference LC-ESI-MS/MS assay and the same pharmacokinetic (PK) parameters were calculated for both analytical assays. This work demonstrates that LDTD-APCI-MS/MS is a reliable method for the ultra-fast quantitative analysis of BKM120 which can be used to speed-up and support its bioanalysis in the frame of the clinical trials.

Keywords Laser diode thermal desorption · Liquid chromatography tandem mass spectrometry · Salting-out assisted liquid-liquid extraction · Bioanalysis · Validation · Pharmacokinetic

Introduction

Bioanalysis has become a critical process in the development of new chemical entities (NCEs). During the long process of clinical trials, involving several steps and high numbers of human volunteers as well as patients, the quantification of NCEs in biological fluids is of paramount importance. The quantitative data are generally used to gain insights into the absorption, distribution, metabolism and excretion of a given compound. In addition, these data are essential to understand in depth the pharmacokinetic properties of a NCE. In this respect, there is a need to develop reliable and robust analytical methods for the quantitative determination of NCEs in human biological fluids. The number of samples to be analyzed has increased during the last decade. Thus, it is obvious that the development of ultra-fast analytical methods saves

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significant time concerning a large scale and multi-site clinical program.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) has been widely used for the quantitative analysis of compounds in biological fluids due to its selectivity, sensitivity and high precision [1–3]. During the past years, various approaches including sub-2 μm particle columns [4, 5], fused-core LC columns [6–8], high flow rates using shorter columns [9], ballistic gradients [10] and fast isocratic LC-MS runs [11] have been developed to accelerate or improve the chromatographic performances. Although the LC-separation time has been reduced below one minute, the injection time which takes the washing cycles and the software initialization into account is still relatively long accounting up to 50 % of the total LC-MS/MS runtime [8]. Since the overall time of a LC-MS/MS method is predominantly governed by the injector-performance, having a method without an auto-sampler would represent an attractive option for the development of ultra-fast assays.

The laser diode thermal desorption (LDTD) is a relatively new direct sample introduction technique in combination with conventional atmospheric pressure chemical ionization (APCI) [12]. The interface exists in different versions on the market which makes the installation on various models of mass spectrometers possible. The analyte is desorbed, ionized and introduced directly into the mass spectrometer without previous separation. Consequently, the analysis time can be decreased to a few seconds representing one of the benefits of this ion source. The principles and the thermal desorption mechanisms of the LDTD-APCI ion source have been previously discussed [13]. The LDTD-APCI ion source has been applied for qualitative and quantitative analyses of different compounds in environmental science [13–16] as well as in the food area [17–19]. More recently, the use of the LDTD-APCI ion source has gained importance in bioanalysis and several authors have demonstrated its benefits in the field of quantitative analysis of small molecules in various biological fluids [20–26].

The purpose of the present study was to develop a LDTD-APCI-MS/MS method for the quantitative analysis of BKM120 in human plasma, an active compound against breast, ovarian, brain, and colon cancer [27–30] which is currently under investigation in clinical trials.

A full method validation of the LDTD-APCI-MS/MS was performed according to guidelines set by the US Food and Drug Administration (FDA) [31] and the European Medicines Agency (EMA) [32] in terms of selectivity, sensitivity, linearity of response, accuracy, precision, extraction recovery, matrix effect, and on-plate BKM120 stability. In addition, the validated LDTD-APCI-MS/MS method was applied to the analysis of several hundreds of human plasma samples originating from three different clinical studies which were previously measured by the validated LC-ESI-MS/MS reference method.

Experimental

Chemicals, reagents, and stock solutions

BKM120 (Fig. 1a) and [D_8]BKM120 (Fig. 1b) were synthesized by Novartis Pharma AG (Basel, Switzerland) with a purity of 100.0 and 99.4 %, respectively. All solvents and reagents were of high analytical grade and were used without further purification. Acetonitrile (ACN) was obtained from Fluka (Buchs, Switzerland) whereas methanol (MeOH), formic acid and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was deionized and purified using a Milli-Q gradient system from Millipore (Bedford, MA, USA). The different drug-free batches of human plasma used for the preparation of calibration standards (Cs) and quality control (QC) samples were obtained from healthy donors (Etablissements Français du Sang (EFS) d'Alsace, Strasbourg).

Preparation of stock and working solutions

Stock solutions containing either BKM120 or [D_8]BKM120 were prepared in a mixture of MeOH/water (1/1, *v*/*v*) to give a final concentration of 200 μg mL^{-1} which were stored at 2-8 °C and were stable for 11 months. Subsequent working solutions of BKM120 for the use in Cs and QC samples were prepared by serial dilution of the stock solution in ACN. A protein precipitation solvent was prepared by diluting the [D_8]BKM120 stock solution with ACN resulting in a final concentration of 100 ng mL^{-1}. The working solutions and protein precipitation solvent were prepared freshly on each day of analysis and were discarded after use.

Preparation of Cs and QC samples

The Cs and QC samples were prepared freshly on each day of analysis by spiking human plasma with each individual BKM120 working solution yielding in Cs concentrations of 5.00 (LLOQ), 10.0, 50.0, 100, 500, 1,000 and 2,000 ng mL^{-1}. The QC concentrations prepared were 5.00, 15.0, 500, and 1,500 ng mL^{-1}. The samples were discarded after use.

Sample preparation and analysis

LDTD-APCI-MS/MS

In a 1.5-mL Eppendorf tube, a volume of 100 μL human plasma either from study samples, Cs, QC, blank or zero samples (blank plasma sample spiked with ISTD) was mixed with 200 μL ACN solution containing [D_8]BKM120 at a concentration of 100 ng mL^{-1}. Afterwards, 100 μL of saturated sodium chloride solution were added for salting-out assisted liquid-liquid extraction (SALLE). The samples were

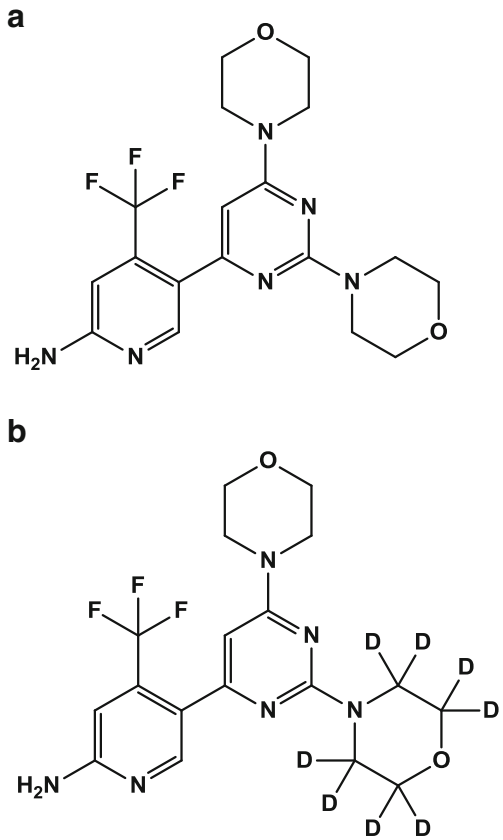

Fig. 1 Chemical structure of **a** BKM120 and **b** [D_8]BKM120

mixed using a vortex device and centrifuged at 4 °C for 10 min at 20,000×*g*. A volume of 3 μL of the supernatant was spotted onto a 96-well LazWell™ plate. The droplets were allowed to dry for approximately 10 min at room temperature prior to the LDTD-APCI-MS/MS analysis.

Ionization of BKM120 and that of its ISTD [D_8]BKM120 were achieved with the S-960 LDTD-APCI ion source, developed and manufactured by Phytronix Technologies (Quebec, QC, Canada), coupled to an API 4000 triple quadrupole mass spectrometer from Applied Biosystems (Foster City, CA, USA).

The LDTD-APCI sample optimization for MS/MS conditions in positive ionization mode was performed using the manual-tune function in the Analyst 1.4.2 software (Foster City, CA, USA) with BKM120 dissolved at a concentration of 1 μg mL^{-1} in pure ACN solution. An aliquot of this solution (2 μL) was spotted onto the LazWell™ plate and was evaporated to dryness at room temperature prior to the sample analysis by LDTD-APCI-MS/MS.

The mass spectrometer operated in a full scan mode ranging from *m/z* 100–500. The LDTD-APCI ion source parameters were set to 3 L min^{-1} for the carrier gas flow rate and 3 μA for the corona discharge current in positive mode. The laser pattern was set after 1 s to a 3 s ramp from 0 to 45 % of maximum energy, held for the next 2 s before shutting off. The MS parameters were set to the following values: curtain gas pressure 10 psi, entrance potential (EP) 6 V, collision cell exit potential (CXP) 12 V, declustering potential (DP) 80 V and collision energy (CE) 50 V for the MS/MS experiments.

Physical parameters of the LDTD-APCI ion source were optimized with spiked aliquots of human plasma in order to account for matrix on the deposition volume into the plate wells and on the laser power (laser pattern). Thus, BKM120 was spiked into human plasma resulting in a final concentration of 2 μg mL^{-1}. Each sample was analyzed in MRM mode using the optimized MS parameters corresponding to the selected precursor and product ions.

Following optimization, the deposition volume was 3 μL while the laser energy was increased after 1 s from 0 to 35 % in a 3 s ramp, held for 2 s at 35 %, decreased to 0 % within 0.1 s and held for 4 s prior to the next sample analysis. The corresponding laser pattern nomenclature was 3-35-2. The remaining LDTD-APCI ion source settings were as follows: corona discharge needle voltage (2 μA), vaporizer temperature (ambient) and carrier gas flow rate (3 L min^{-1}). The LDTD-APCI ion source was controlled by the LazSoft 4.2 software (Phytronix Technologies, Quebec, QC, Canada). The optimum MS parameters used for the LDTD-APCI-MS/MS analyses are summarized in Table 1.

LC-ESI-MS/MS (reference method)

A volume of 100 μL human plasma was mixed with 50 μL aqueous solution of [D_8]BKM120 at 200 ng mL^{-1}. The mixture was vortexed and centrifuged at 4 °C for 10 min at 2,250×*g*. Off-line solid phase extraction was performed by passing 140 μL of supernatant fluids through an Oasis HLB 10 mg, 30 μm cartridge from Waters (Milford, MA, USA) which was pre-conditioned with 500 μL MeOH and 500 μL distilled water. Then, the cartridge was washed with 2× 500 μL 2 % MeOH in water. Finally, the cartridge was eluted with 2×250 μL MeOH containing 0.2 % formic acid. The eluent was evaporated to dryness under a nitrogen stream at 50 °C. A volume of 200 μL of MeOH/water (30/70, *v*/*v*) containing 0.2 % formic acid was added for reconstitution. The plate was shaken for 10 min and an aliquot of 20 μL was injected into the LC-ESI-MS/MS system. Sample analysis was performed on a LC-ESI-MS/MS system consisting of an API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray™ interface from Applied Biosystems (Foster City, CA, USA). The MS system was connected to a HTS PAL auto-sampler from CTC Analytics AG (Zwingen, Switzerland) and to an Agilent 1200 pump system (Wilmington, DE, USA). Chromatographic separation was performed at a flow rate of 400 μL min^{-1} on an Ascentis Express C_{18} 50 mm×2.1 mm, 2.7 μm column from Supelco (Bellefonte, PA, USA) with a binary gradient where the mobile phases consisted of 0.2 % formic acid in water (A) and 0.1 % formic

Table 1 Summary of obtained MRM transitions and optimized MS parameters for BKM120 and [D_8]BKM120 during LDTD-APCI-MS/MS method development

	BKM120					[D_8]BKM120				
Precursor ion	*m/z* 411.3	DP	EP	CE	CXP	*m/z* 419.5	DP	EP	CE	CXP
Product ion 1	*m/z* 367.3	80 V	6 V	45 V	10 V	*m/z* 371.2	70 V	6 V	45 V	6 V
Product ion 2	*m/z* 307.3	80 V	6 V	55 V	26 V	*m/z* 309.6	80 V	6 V	55 V	10 V

acid in MeOH (B). The elution gradient program (T(min), %(B)) was as follows: (0, 25), (0.8, 25), (2.0, 95), (3.0, 95), (3.1, 25) and (4.5, 25). The column temperature was maintained at 40 °C using a column heater. The system was operated in electrospray positive ionization using MRM mode. The different monitored MRM transitions used were as follows: BKM120 *m/z* 411.2→367.2 and for [D_8]BKM120 *m/z* 419.1→309.1. The other MS conditions were as follows: turbo ion spray 5,000 V, source temperature 550 °C, curtain gas 20 psi, Gas 1 80 psi, Gas 2 60 psi, dwell time 100 ms, DP 106 V, EP 10 V, CE 49 V and CXP 10 V. The LC-ESI-MS/MS method validation parameters are summarized in Table S1 in the Electronic Supplementary Material (ESM).

Desorption efficiency with LDTD-APCI

The desorption efficiency using the final assay conditions was assessed. Three QC concentration levels were spotted in triplicate onto the LazWell™ plate. Each well was impacted three times by the selected laser pattern. The percentage from each single shot was calculated to determine the desorption efficiency of the analyte according to the following equation

$$\text{Desorption efficiency}\,(\%) = 100 \times \frac{R_{\text{individual}}}{R_{\text{total}}}$$

where $R_{\text{individual}}$ represents the response at a single shot for a certain QC sample whereas R_{total} represents the total response of the three laser shots from a certain QC sample.

Statistical analysis and PK parameters calculations

Statistical analysis and graphical illustration was conducted with R (version 2.15.1) from R Foundation for Statistical Computing (Vienna, Austria) and with MedCalc® (version 12.7.8.0) from MedCalc Software bvba (Ostend, Belgium). PK parameters were calculated using stand-alone PK within Watson LIMS (version 7.4.1) from Thermo Fisher Scientific (Waltham, MA, USA).

Method comparison

Analytical method comparison between LDTD-APCI-MS/MS and LC-ESI-MS/MS was performed by visualizing the obtained concentrations in a scatterplot and by linear regression modelling with 95 % confidence intervals of the linear regression parameters (slope and intercept) using a correlation coefficient based on least squares.

Deviation from linearity was determined by the Cusum test with a significance level set to a p value <0.05.

The degree of agreement among the two analytical assays was evaluated by the Bland-Altman plot [33]. The mean concentration of a sample measured by both analytical methods was assigned as the x-axis and the difference between both measurements (% bias) on the y-axis. The 95 % limits of agreement were estimated by the mean bias between both analytical methods±1.96 times the standard deviation (SD) to identify possible outliers.

Results and discussion

LDTD-APCI parameters optimization

Four main LDTD-APCI physical parameters were optimized to result in a better signal intensity to gain maximum sensitivity: the laser power, the spotting volume, the carrier gas flow rate and the corona discharge current.

Different volumes in the range of 2 to 8 μL were spotted onto the LazWell™ plate and analyzed by varying the maximum laser energy from 25 to 65 % whereas the ramping and holding time of the laser were kept constant to a value of 3 and 2 s, respectively. Each combination of both parameters was analyzed in replicates of five. The optimal settings for both parameters were determined by the comparison of the mean peak area, its standard deviation and the resulting peak shape. Among the tested laser patterns, the maximum MS peak area of BKM120 was observed with the laser pattern 3-35-2 regardless to the deposition volume (Fig. 2a). Increasing the laser did not produce a higher MS response for BKM120. As a matter of fact, thermal fragmentation of the analyte and matrix components, caused by elevated desorption energy, generated fragments which were in proton affinity competition with the analyte, leading to ion suppression. Hence, the 3-35-2 pattern was selected as the optimal one for the quantitative analysis of BKM120. The MS signal of BKM120 obtained with the selected laser pattern was only significantly enhanced when increasing the spotting volume from 2 to 3 μL which was

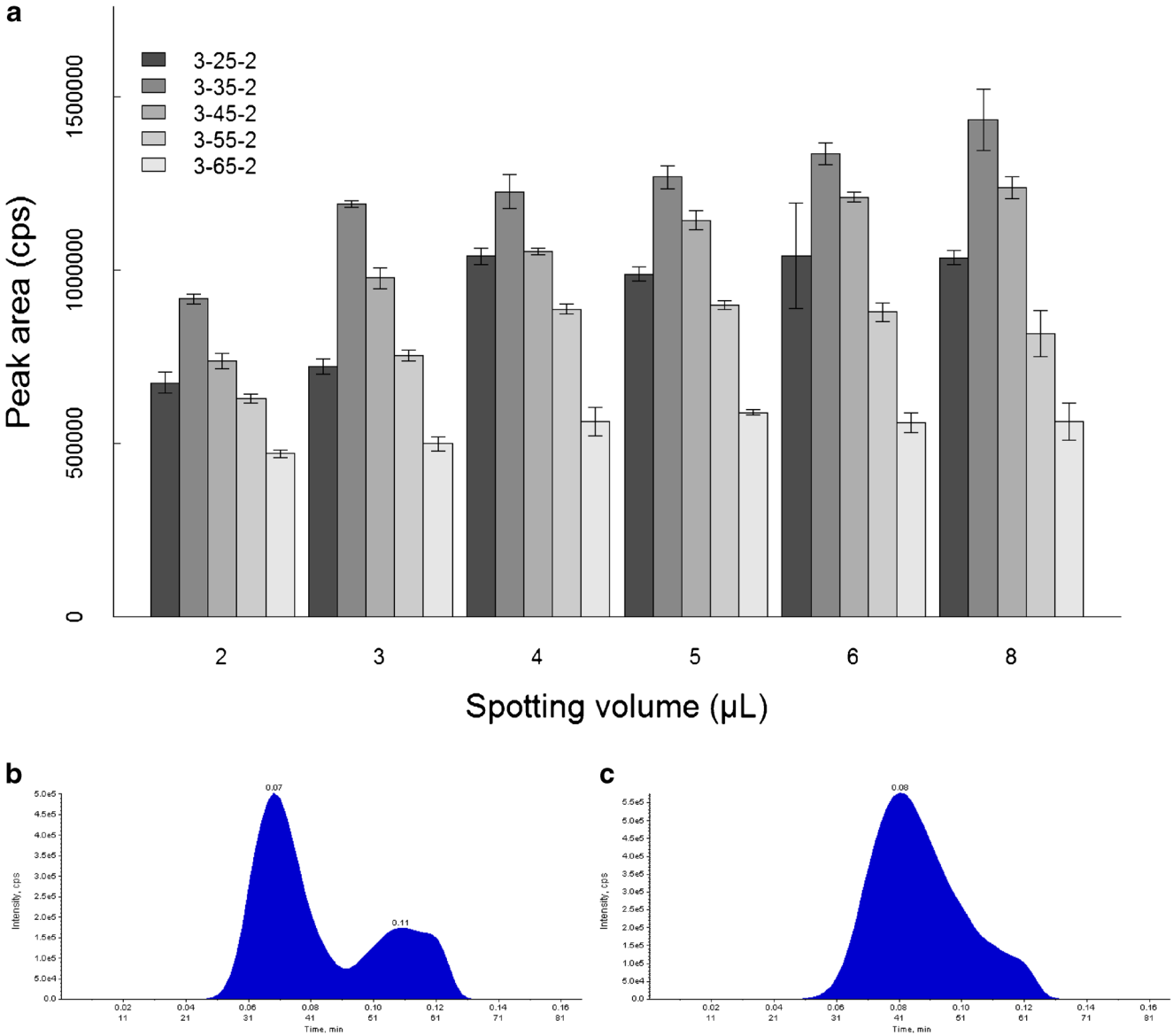

Fig. 2 **a** Results from peak area comparison of various laser patterns and spotting volumes for BKM120 (n=5). Peak shape obtained with 3-35-2 laser pattern and spotting volume of **b** 8 µL indicating plate overloading and **c** 3 µL using a human plasma sample spiked with BKM120 at 2 µg mL^{-1}

evaluated by a stepwise comparison using the analysis of variance (ANOVA) with a significance level set to a p value <0.05. There was only a marginal MS signal enhancement beyond a spotting volume of 3 µL (Fig. 2a). Increasing the deposit volume might lead to an overload in the well, resulting in poor analyte peak shape as depicted in Fig. 2b. Hence, the optimized spotting volume for BKM120 was set to 3 µL which gave a proper Gaussian peak shape (Fig. 2c). Furthermore, the lowest standard deviation in peak area was also observed with a spotting volume of 3 µL considering only the samples with the 3-35-2 laser pattern (Fig. 2a).

The corona discharge current and carrier gas flow rate on BKM120 signal were optimized by using the 3-35-2 laser pattern and a spotting volume of 3 µL with a fixed corona discharge current ranging from 1 to 5 µA while changing the carrier gas flow rate (1–5 L min^{-1}). The highest MS peak areas were observed with a carrier gas flow rate of 3 L min^{-1} irrespectively of the corona discharge current (ESM Fig. S1A and Fig. S1B). Badjagbo et al. [16] observed the same effect during the analysis of BTEX in air. They discovered that the residence time in the APCI region which is controlled by the carrier gas flow rate had an influence on the signal intensity. Furthermore, maximal signal intensity at a carrier gas flow rate of 3 L min^{-1} was also observed from other groups optimizing the LDTD-APCI parameters indicating that the carrier gas flow rate is not compound dependent but rather a combination of physical factors which influence the ionization efficiency [13, 14, 18]. The optimal corona discharge current

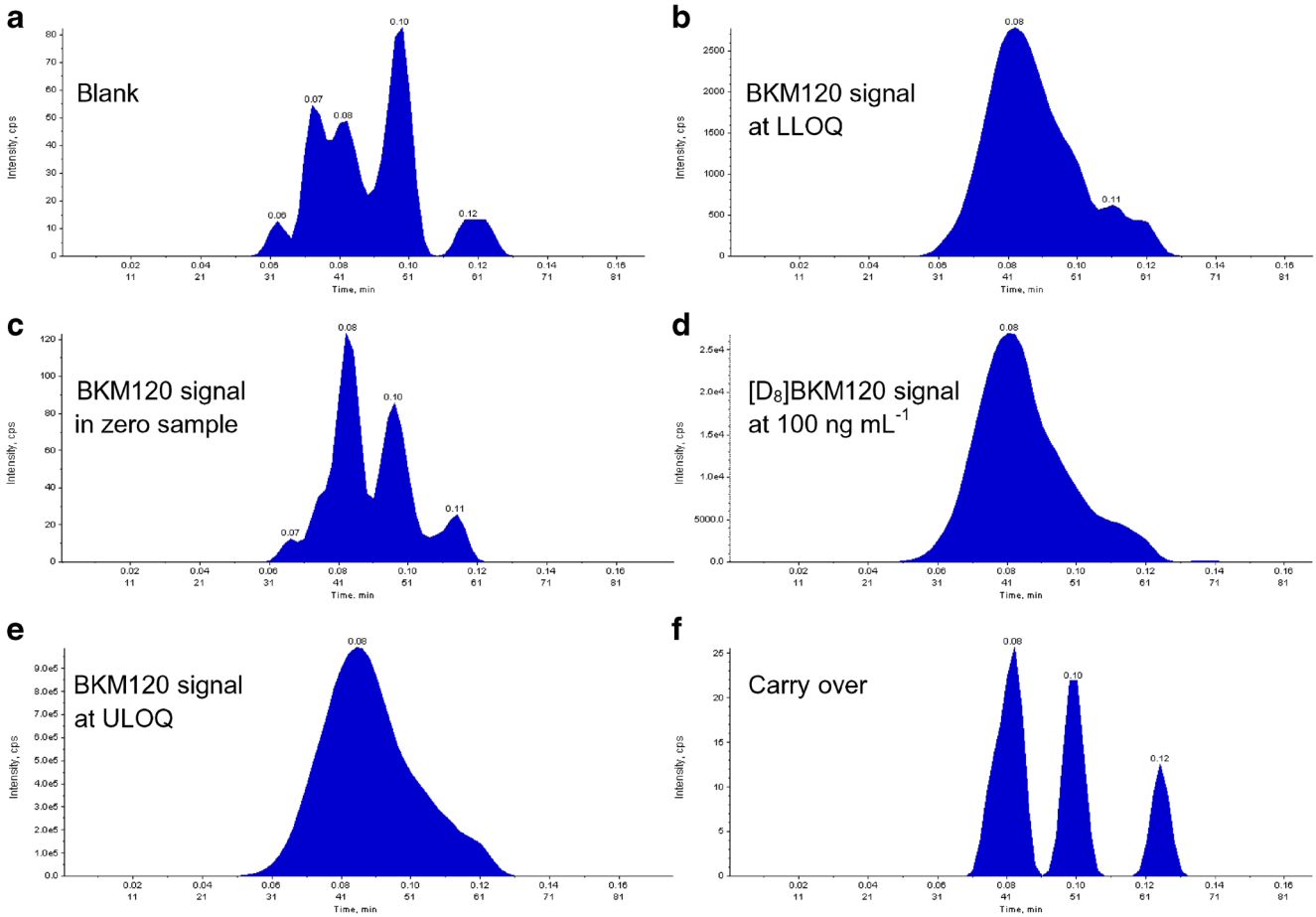

Fig. 3 LDTD-APCI-MS/MS desorption peaks showing MRM transition signal of BKM120 for **a** extracted blank plasma sample; **b** extracted plasma sample spiked with BKM120 at a concentration of 5.00 ng mL^{-1} (LLOQ); **c** BKM120 in the zero extracted sample; **d** extracted blank plasma sample spiked with $[D_8]$BKM120 at 100 ng mL^{-1}; **e** extracted plasma sample spiked with BKM120 at a concentration of 2,000 ng mL^{-1} (ULOQ) and **f** blank extracted sample injected right after the ULOQ

was set to a value of 2 μA since the peak area was slightly increased for $[D_8]$BKM120 (ESM Fig. S1B) compared to BKM120 (ESM Fig. S1A). Under the selected experimental conditions, the overall analysis time was 10 s per sample.

The desorption efficiency of BKM120 was evaluated under the selected LDTD-APCI-MS/MS parameters. The BKM120 desorption was consistent over the three investigated QC concentrations (ESM Table S2). In average, 81.3±2.0 % of BKM120 was obtained after the first desorption process whereas less than 14 % was desorbed with the subsequent one. This demonstrated that the applied optimized conditions allow the majority of the spotted BKM120 to desorb after one shot, independently of the concentration.

Table 2 Daily variation of calibration parameters obtained in human plasma. Calibration parameters *a* and *b* of the calibration function $y=ax+b$ with a weighting factor of $1/x^2$ and coefficient of determination (R^2) on three days of the validation

	a	*b*	Coefficient of determination (R^2)	LLOQ (ng mL^{-1})	ULOQ (ng mL^{-1})
Day 1	0.01850	−0.004946	0.9965	5.00	2000
Day 2	0.01879	−0.005602	0.9959	5.00	2000
Day 3	0.02208	−0.004286	0.9986	5.00	2000
Mean	0.01979	−0.004945	0.9970		
SD	0.001988	0.0006580	0.001418		
CV (%)	10.05	−13.31	0.1422		
n	3	3	3		

Table 3 Statistical summary of intra- and inter-day accuracy and precision of QC samples on three different days

		Nominal concentration (ng mL^{-1}) in human plasma			
		1,500	500	15.0	5.00
		Intra-day accuracy and precision (n=6)			
Day 1	Mean (ng mL^{-1})	1,580	538	16.0	5.56
	Intra-day bias (%)	5.3	7.6	6.7	11.2
	Intra-day precision (% CV)	2.0	2.1	5.3	6.6
Day 2	Mean (ng mL^{-1})	1,620	517	15.5	5.42
	Intra-day bias (%)	8.0	3.4	3.3	8.4
	Intra-day precision (% CV)	1.0	5.2	1.8	4.1
Day 3	Mean (ng mL^{-1})	1,520	496	14.4	5.34
	Intra-day bias (%)	1.3	−0.8	−4.0	6.8
	Intra-day precision (% CV)	2.7	2.9	2.2	4.6
		Inter-day accuracy and precision (n=18)			
Overall	Mean (ng mL^{-1})	1,570	517	15.3	5.44
	Inter-day bias (%)	4.7	3.4	2.0	8.8
	Inter-day precision (% CV)	3.4	4.8	5.7	5.2

Validation

Selectivity

Since no chromatography separation was associated to our method, it was essential to assess its selectivity. This was established by assaying six lots of human blank plasma and comparing the response of each blank relative to the lowest calibration standard. The signal found on the BKM120 MRM channel of the blank extracted human plasma sample was less than 20 % of the one obtained on human plasma spiked at the LLOQ (Fig. 3a, b). The signal found on the BKM120 MRM channel of the zero sample was similar to the one obtained in the blank extracted sample (Fig. 3c, a), indicating that the [D_8]BKM120 does not contribute to BKM120 signal. We also observed that BKM120 did not contribute to the signal of the ISTD (data not shown). This indicated that our method was highly selective for both BKM120 and [D_8]BKM120.

Linearity and sensitivity

The concentrations of BKM120 were calculated by a seven point calibration curve using the parameter a and b of the linear calibration function in the form $y=ax+b$ with a weighting factor of $1/x^2$ where y was the peak area ratio of the response for BKM120 to the response of [D_8]BKM120 and x represented the concentration of BKM120 in the Cs samples. The resultant coefficients of determination obtained on three days were $R^2>0.9950$ (Table 2). Furthermore, the daily variation of 10.05 % represented by the coefficient of variation (CV) showed that the assay was suitable for the routine analysis of BKM120 over the range of 5.00–2,000 ng mL^{-1}. The lowest concentration meeting the acceptance criteria of ±20 % and ≤20 % regarding accuracy and precision was 5.00 ng mL^{-1}.

Carry over

The carry over (injection of blank samples directly after a sample at the ULOQ) observed for BKM120 and [D_8]BKM120 in four different batches of human plasma was less than 1.5 % (ESM Table S3) fulfilling the acceptance criteria of ≤20 % of the response observed for BKM120 at the LLOQ and ≤5 % of the response observed for [D_8]BKM120. In addition, the MS response observed in a blank human plasma sample (Fig. 3f) injected right after a sample spiked at ULOQ (Fig. 3e) was similar to the one found in the blank sample (Fig. 3a). These data demonstrated the absence of carry over with our current LDTD-APCI-MS/MS method.

Precision and accuracy

The precision and accuracy for the four QC levels at 5.00, 15.0, 500, and 1,500 ng mL^{-1} were assayed as the relative standard deviation in % CV and as the percent error (% bias), respectively. Six different replicates of each QC level were measured at each day of the validation to evaluate the intra-day precision and accuracy. In total, 18 samples at each QC concentration were used to determine the inter-day accuracy and precision (Table 3). The criteria for the inter-day values of less than 15 % were fulfilled since the accuracy ranged from 2.0 to 8.8 % and the precision was determined between 3.4 and 5.7 %. The results showed that the developed LDTD-APCI-MS/MS method was accurate and precise.

Table 4 Extraction recovery (BKM120 and [D_8]BKM120) and normalized matrix factor (BKM120) in human plasma for three different QC concentrations (n=3)

	QC concentration (ng mL^{-1})		
	1500	500	15.0
BKM120 extraction recovery			
Mean extraction recovery (%)	88.5	106	79.0
CV (%)	1.3	15.7	14.7
Overall mean recovery (%)	91.0		
Inter-concentration CV (%)	14.8		
[D_8]BKM120 extraction recovery			
Mean extraction recovery (%)	80.3	111	80.4
CV (%)	11.2	9.8	1.9
Overall mean recovery (%)	90.5		
Inter-concentration CV (%)	19.4		
BKM120 normalized MF			
Overall mean normalized MF	0.86	0.87	0.86
CV (%)	5.8	7.8	9.2
Mean normalized MF inter-concentration level	0.86		
CV (%) inter-concentration level	7.4		

Extraction recovery and matrix effect

The extraction recovery was evaluated by the comparison of the analytical response of a blank matrix spiked with analyte and extracted (pre-spike) compared to the response of a blank matrix first extracted and spiked afterwards with the analyte (post-spike). The resultant mean extraction recoveries for BKM120 and [D_8]BKM120 over three QC levels were 91.0 % (14.8 % CV) and 90.5 % (19.4 % CV), respectively (Table 4). The variation of the [D_8]BKM120 extraction recovery was slightly higher compared to the one of the analyte, but the acceptance criterion of ≤20 % was fulfilled.

The matrix effect was determined by the matrix factor (MF) which was the ratio of the analyte response in the presence of matrix ions (blank extract spiked with analyte and ISTD) compared to the analyte response in absence of matrix ions (neat/unextracted solution). The normalized MF was determined by the ratio of the MF for the analyte divided by the MF of the ISTD. The mean normalized MF obtained was 0.86 (7.4 % CV) (Table 4). The latter results indicated that there was a slight influence from the matrix within the developed method using LDTD-APCI-MS/MS leading to 14 % ion suppression and that [D_8]BKM120 was required for the quantification of BKM120 in human plasma.

Post-preparative stability on LazWell™ plate

Stability investigation during the validation of the LDTD-APCI-MS/MS method was restricted to the post-preparative stability, as other stability tests were performed during the LC-ESI-MS/MS method validation. The only post-preparative stability was investigated at room temperature on two QC levels at 1,500 ng mL^{-1} and 15.0 ng mL^{-1}. The samples were analyzed by LDTD-APCI-MS/MS 2, 4, and 72 h after spotting. The resultant concentrations were compared with those observed at t_0 and the normalized difference in percent was determined. The observed mean bias was less than 15 % indicating that BKM120 was stable on the LazWell™ plate for the investigated duration at room temperature (ESM Table S4).

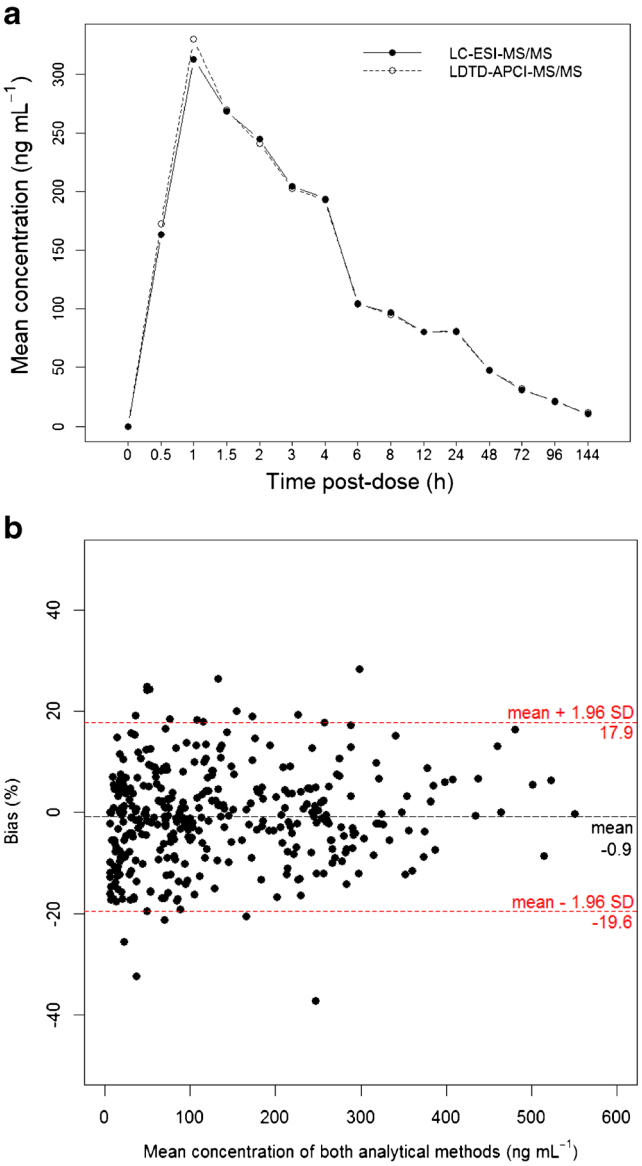

Fig. 4 **a** Mean plasma concentration-time profiles of 27 subjects administrated a single dose of 50 mg BKM120 once daily in clinical study 1 measured either with LC-ESI-MS/MS or LDTD-APCI-MS/MS (n=405) and **b** Bland-Altman plot with 95 % limits of agreement (*dashed lines*) for assessment of agreement between both analytical assays (n=368)

Table 5 Summary of cross-check and agreement between LDTD-APCI-MS/MS and LC-ESI-MS/MS for all three investigated BKM120 clinical studies

Clinical study	*n* samples	Criterion of ±20 % met during cross-check (%)	Linear regression of both assays during cross-check	Correlation of obtained concentrations between both methods	Cusum test *p* value	Within 95 % limits of agreement
1	405	97	$y=0.959x+0.577$	$R^2=0.9855$	0.25	95
2	378	94	$y=1.031x+0.282$	$R^2=0.9793$	0.53	94
3	64	97	$y=0.971x+1.522$	$R^2=0.9829$	0.41	94

Dilution factor

The QC sample at 10 μg mL^{-1} was diluted 200-fold and analyzed in replicates of five resulting in a nominal concentration of 50 ng mL^{-1}. The dilution was performed with blank human plasma. The back calculated mean concentration was 10.1 μg mL^{-1} with a bias of 1.0 % and a variation of 2.9 % (ESM Table S5).

Method reproducibility

The method reproducibility was assessed on incurred plasma samples. In total, 50 samples obtained from a clinical study were extracted and analyzed on two different days. The normalized difference between first and repeat assay was within ±20 % for 88 % of investigated samples. The acceptance criterion was met as at least 2/3 of all investigated samples had to be within ±20 % normalized difference indicating that our method was reproducible and reliable for the analysis of BKM120 in clinical samples (ESM Table S6).

Application to clinical studies

The method was applied to samples from three clinical studies with subsequent PK parameter determination. In total, 847 human plasma samples were re-analyzed with LDTD-APCI-MS/MS. For the clinical studies, doses of 10, 30, and 50 mg BKM120 were administrated to patients once daily. Blood samples were taken at distinct time points ranging from 0–144 h or from 0–24 h post-dose at two different days were assayed. Blood sampling was performed either via direct venipuncture or an indwelling cannula inserted in a forearm vein. At each sampling time-point, between 3 and 4 mL blood were collected into tubes containing K_3-EDTA as anticoagulant. Immediately after each tube of blood was drawn, it was inverted gently several times to ensure the mixing of tube contents. Within 30 min after collection, the samples were centrifuged for 10 min at 1,500×*g* and at 4 °C, then the top layer (at least 0.7 mL of plasma) was transferred to polypropylene tubes. The tubes were kept frozen below −70 °C for pending analysis.

The mean concentration-time profiles from 27 subjects administrated a single dose of 50 mg once daily (clinical study 1) obtained either with the LC-ESI-MS/MS reference method or our new validated method were similar (Fig. 4a). Although 405 samples were selected from clinical study 1 for the re-analysis, only 395 samples had a concentration above 5.00 ng mL^{-1}. Hence, the comparison of data between our method and the LC-ESI-MS/MS reference method only included those samples. An excellent correlation during the cross-check between both analytical systems was obtained with a coefficient of determination of almost 0.99 indicated by the corresponding linear regression of $y=0.959x+0.577$ (Table 5). No significant deviation from linearity was revealed by the Cusum test, resulting in a *p* value of 0.25. The majority of investigated samples from clinical study 1 (97 %) met the bias acceptance criterion of ±20 % between the results from the LDTD-APCI-MS/MS analysis and those generated by the LC-ESI-MS/MS reference method (Table 5).

The mean bias between both analytical methods displayed in the Bland-Altman plot (Fig. 4b) was −0.9 %, whereas the samples where the concentrations were measured zero were

Table 6 Summary of BKM120 calculated PK parameters for all three investigated BKM120 clinical studies containing median T_{max}, mean C_{max} and AUC_{last} with SD (n=number of subjects) obtained with the LDTD-APCI-MS/MS method compared to the reference LC-ESI-MS/MS method

	Clinical study 1 (*n*=27)		Clinical study 2 (*n*=27)		Clinical study 3 (*n*=3)	
	LDTD-APCI-MS/MS	LC-ESI-MS/MS	LDTD-APCI-MS/MS	LC-ESI-MS/MS	LDTD-APCI-MS/MS	LC-ESI-MS/MS
T_{max} (h)	1.0±0.51	1.0±0.51	1.0±0.49	1.0±0.73	1.5±0.98	1.8±1.0
C_{max} (ng mL^{-1})	259±89.4	267±102	347±124	346±118	915±534	935±578
AUC_{last} (ng h mL^{-1})	4,542±1401	4,761±1410	6,434±2345	6,566±2121	10,065±7397	10,040±7330

excluded. Despite the fact that a few outliers were present and were located outside the 95 % limits of agreement (ranging from −19.6 to 17.9 %, represented by the dashed lines), 95 % of all samples were within the limits of agreement (Table 5). The obtained data for the other two remaining clinical studies during the cross-check are summarized in Table 5. The resultant PK parameters obtained for all three clinical studies were consistent between both analytical methods (Table 6) demonstrating that the two methods gave equivalent results.

Conclusion

A high-throughput and ultra-fast method for the quantitative analysis of a pharmaceutical compound (BKM120) in human plasma using LDTD-APCI-MS/MS has been successfully developed and validated. The overall performances (extraction recovery, precision, accuracy and calibration) of the method were comparable to those of the previously validated LC-ESI-MS/MS reference method but offer the advantage of ultra-fast analysis resulting in a 27-fold reduction in analysis time (10 s per sample). The comparative data between our method and the LC-ESI-MS/MS reference assay demonstrated that LDTD-APCI-MS/MS is an efficient approach for the quantitative analysis of BKM120 in biological matrices. The high-throughput nature of LDTD-APCI-MS/MS method would alleviate the clinical sample analysis. This work and those previously published demonstrate the applicability of LDTD-APCI-MS/MS for the quantitative analysis of small molecules in the bioanalysis field. The analysis was achieved without using chromatography. Hence, the cost and environmental impact can be reduced significantly regarding solvent and energy consumptions as well as material disposal. This is well aligned with the green chemistry principle.

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