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Ultra-fast quantitation of saquinavir in human plasma by matrix-assisted laser desorption/ionization and selected reaction monitoring mode detection

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ABSTRACT

We present herein an ultra-fast quantitative assay for the quantitation of saquinavir in human plasma, without prior chromatographic separation, with matrix-assisted laser desorption/ionization using the selected reaction monitoring quantitation mode (MALDI-SRM/MS). The method was found to be linear from 5 to 10,000 ng/ml using pentadeuterated saquinavir (SQV-d5) as an internal standard, and from 5 to 1000 ng/ml using reserpine as internal standard (IS). Accuracy and precision were in the range of 101–108%, 3.9–11% with SQV-d5 and in the range 93–108%, 3.5–15% with reserpine. Plasma samples (250 μl) were extracted with a mixture of ethyl acetate/hexane. MALDI spotting of the extract was automated using electrodeposition and the dried droplet method using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. A 96 spots MALDI plate was prepared within 20 min in a fully unattended manner. Each sample was spotted four times and quantitation was based on the average of their analyte/IS area ratio. Samples were analyzed on a triple quadrupole linear ion trap (QqQ_{LIT}) equipped with a high repetition laser source (1000 Hz). The analysis time of one sample was approximately 6 s, therefore 96 samples could be analyzed in less than 10 min. With liquid–liquid extraction sample preparation no significant matrix effects were observed. Moreover, the assay showed sufficient selectivity for samples to be analyzed at the lower limit of quantification (LLOQ) in the presence of other antiretroviral drugs, without prior chromatographic steps. In parallel, to assess the selectivity of the assay with real samples, a liquid chromatography (LC)–SRM/MS method was developed and a cross validation with clinical samples was successfully performed.

1. Introduction

From early stage drug discovery to therapeutic drug monitoring (TDM), liquid chromatography coupled to atmospheric pressure ionization tandem mass spectrometry (LC–MS/MS) is nowadays a well-established technique to quantify low molecular weight compounds (LMWC) in biological matrices such as plasma or urine [1–3]. In contrast matrix-assisted laser desorption/ionization [4] usually combined with time-of-flight analyzers (MALDI–TOF), has been used for the qualitative analysis of large biomolecules (i.e. peptides, proteins, oligonucleotides and polymers) [5,6]. The interfering background due to MALDI matrix ions makes it at a first glance less attractive for the analysis of LMWC. As a result, MALDI has not given rise to applications comparable to LC–MS/MS for the quantitation of LMWC in biological matrices. Specific strategies have been proposed to deal with this issue. Porphyrin-based MALDI matrices have been shown to eliminate the chemical noise in the low-mass range. The analytes were quantitated based on their adducts with alkaline metal salts (cesium acetate or lithium iodide) by MALDI–TOF [7]. More recently van Kampen et al. [8] achieved quantitation of HIV protease inhibitors in peripheral blood mononuclear cells using Fourier transform mass spectrometry. Another example of quantitative MALDI is the determination of free fatty acids in rat plasma by Yu et al. [9]. Another approach to improve selectivity is to perform the analysis on a MALDI–MS/MS rather than on a MALDI–MS system. MALDI–TOF/TOF has been used by Notari et al. [10] for the quantitation of antiretroviral drugs (lamivudine, lopinavir and ritonavir) in human plasma with the standard addition method. Atmospheric pressure MALDI coupled to an ion trap has been used by Cui et al. [11] to quantify lysergic acid diethylamide in urine in the range 1–100 ng/ml.

Recently, high repetition rate laser MALDI combined with a triple quadrupole mass spectrometer has been introduced as a new alternative. Design and performance of such instrumentation have been discussed respectively by Corr et al. [12] and Gobey et al. [13]. The first great benefit of this combination results from the intrinsic selectivity of the selected reaction monitoring (SRM) experiment which reduces dramatically the interfering background. The second

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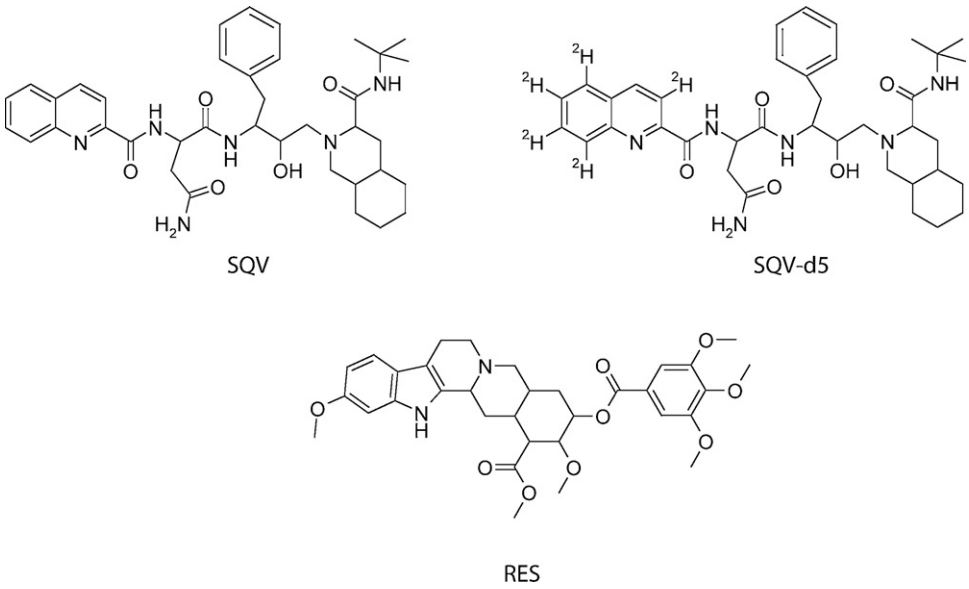

Fig. 1. Chemical structures of saquinavir (SQV), pentadeuterated saquinavir (SQV-d5) and reserpine (RES).

benefit is the high-speed capability of the system. Several thousands of samples can be analyzed on a daily basis. Nevertheless, due to sample matrix effects, sample preparation remains mandatory and is the time limiting step. Kovarik et al. discussed these method development aspects for the quantitation of talinolol in human plasma using MALDI-SRM/MS, showing the importance of the chosen sample preparation strategy [14].

We illustrate in this paper the potential of MALDI-SRM/MS for the high-throughput quantification of saquinavir in human plasma, without any prior chromatographic step. Saquinavir, a HIV-protease inhibitor drug largely prescribed for the treatment of AIDS [15], has been chosen as a model analyte using pentadeuterated SQV and reserpine as internal standards (Fig. 1). Various LC–MS/MS methods have been published for its quantification in biological fluids [16,17].

2. Experimental

2.1. Chemicals and materials

Saquinavir (SQV) was obtained from the Geneva University Hospital (Geneva, Switzerland). Pentadeuterated saquinavir (SQV-d5) was obtained from F. Hoffman La Roche AG (Basel, Switzerland). Reserpine (RES), acetonitrile and *n*-hexane were obtained from Sigma–Aldrich (Buchs, Switzerland). The matrix α-cyano-4-hydroxycinaminic acid (CHCA), ethyl acetate, ethanol and triethylamine were obtained from Fluka (Buchs, Switzerland). Formic acid was obtained from Merck (Darmstadt, Germany). The 384 spot MALDI plates were from Applied Biosystems (Foster City, CA, USA). The 0.2 ml skirted 96-well PCR plates were from ABgene (Epsom, UK). For electrodeposition the conductive pipette tips from Advion BioScience (Ithaca, NY, USA) were used. Citrate plasma was obtained from the Geneva University Hospital (Geneva, Switzerland).

2.2. Preparation of spiked human plasma samples

Stock solutions were prepared in a mixture H_2O/methanol (50/50, v/v). Saquinavir spiked sample sets were prepared by spiking 20 μl of appropriate stock solutions in 980 μl plasma to obtain a final concentration ranging from 5 to 10,000 ng/ml. Each set was divided into ten calibration standards (5, 10, 12.5, 25, 100, 250, 500, 1000, 5000 and 10,000 ng/ml) and ten quality control samples (same concentrations).

2.3. Liquid–liquid extraction of SQV in human plasma

Liquid–liquid extraction (LLE) of human plasma samples was performed as follows. To 250 μl of spiked plasma were added 25 μl of internal standard (SQV-d5 or RES, 1 μg/ml in H_2O/MeOH 1/1, v/v), 10 μl of triethylamine 10% in H_2O and 1 ml of hexane/ethyl acetate (1/1, v/v). After agitation (15 min) and centrifugation (5 min at 16,435 × *g*), samples were placed in an isopropanol/dry ice bath. Once the aqueous layer was frozen, the organic layer was removed, evaporate to dryness and reconstituted in 25 μl of CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH for MALDI-SRM/MS analysis and in 200 μl of the same solution for LC–SRM/MS analysis.

2.4. Protein precipitation (PP) of SQV in human plasma

Protein precipitation of human plasma samples was performed as follows. To 100 μl of spiked plasma were added 10 μl of internal standard (SQV-d5 or RES, 1 μg/ml in H_2O/MeOH 1/1) and 200 μl of CH_3CN/EtOH (1/1, v/v). After centrifugation (15 min at 16,435 × *g*), the supernatant was evaporated to dryness and reconstituted in 25 μl of CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH.

2.5. Automated MALDI spotting

MALDI spotting was automated and was performed according to the dried droplet technique. Human plasma samples resulting from LLE or PP were transferred into a 0.2 ml 96-well plate, which was then sealed with an adhesive aluminium foil to prevent evaporation. CHCA (5 mg/ml in CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH) was used as MALDI matrix and conditioned in a second, sealed 0.2 ml 96-well plate. Pipetting, mixing and spotting steps have been automated using an Xcise processing station (Shimadzu Biotech, UK) adapted to perform electrodeposition with conductive pipette tips (Fig. 2).

The Xcise consists of a robot arm with a holder for eight-pipette tips for either conductive or non-conductive pipette tips and a 96-position rack. Eight samples can be processed in one cycle, by using

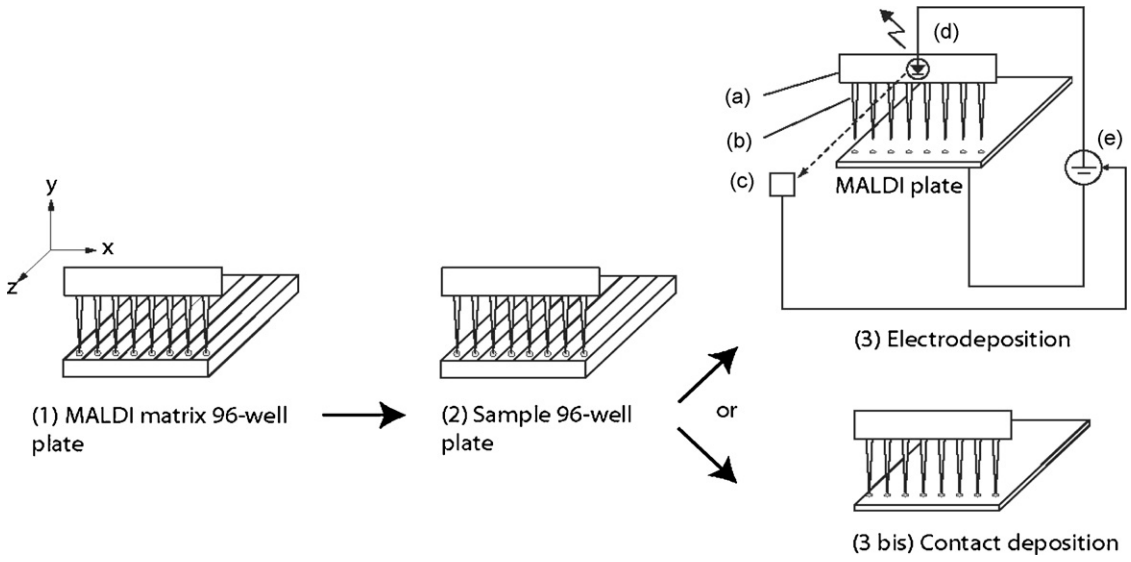

Fig. 2. Schematic overview of the electrodeposition apparatus: (a) robot arm (programmable motion in *x*, *y*, *z* directions), (b) conductive pipette tips, and (c) reflective part of (d) the optical sensor triggering the (e) power supply.

a user-defined macro as follows. One microliter of matrix was first aspirated into each of the eight pipette tips, followed by an air gap of 0.5 μl. Then, 1 μl of sample was withdrawn and mixed into the tip by 2 cycles of aspirate/dispense processes. Next, the solution was dispensed outside the tip, forming at its end a droplet, which was electrodeposited onto the MALDI plate by means of a 1000 V difference between the conductive tips and the MALDI plate. For each cycle, one new pipette tip series was picked up in the rack and ejected at the end of the cycle. Each sample was spotted four times (four distinct spots obtained with four different tips).

2.6. Selectivity of the MALDI-SRM/MS assay

To assess potential selectivity issues, a methanolic mixture of eight antiretroviral drugs (amprenavir, atanazavir, indinavir, lopinavir, nelfinavir, ritonavir, efavirenz and nevirapine) was prepared. The concentrations were 100 μg/ml for each compound except for amprenavir: 150 μg/ml.

This mixture (25 μl) was added to 250 μl of 5 ng/ml SQV in human plasma sample. LLE was then performed as described above.

2.7. Extraction recovery and matrix effects

Matrix effects and LLE recovery have been estimated at SQV concentration levels of 12.5 and 1000 ng/ml, using three sets of samples. Each set consisted of three samples prepared independently.

The first set consisted of standard solutions. Two hundred and fifty microliter solutions of SQV (12.5 and 1000 ng/ml in ACN) were evaporated under vacuum centrifugation and reconstituted in 25 μl of CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH.

The second set of samples consisted of extracted blank plasma which were further spiked with SQV. To 250 μl of blank human plasma were added 10 μl of triethylamine (10% in H_2O) and 1 ml of hexane/ethyl acetate (1/1, v/v). To the evaporated extract were added 250 μl of a solution of SQV in CH_3CN (12.5 and 1000 ng/ml, same stock solution as set 1). The mixture was evaporated to dryness and reconstituted in 25 μl of CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH. The third set of samples consisted in SQV-spiked plasma samples was further extracted by LLE as described previously. Each sample was then mixed with 25 μl of CHCA 5 mg/ml in CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH and spotted manually (1 μl) four times onto the MALDI plate.

2.8. MALDI-SRM/MS analysis

MALDI-SRM/MS experiments were performed on a MALDI–4000 Q TRAP equipped with a prototype MALDI source (AB/MDS Sciex, Concord, ON) (Fig. 3). The laser was a high repetition rate (1000 Hz), frequency-tripled (355 nm) Nd:YAG laser. Mass spectrometric detection was performed in the positive ion ionization mode. Conditions were: laser frequency = 1000 Hz, laser energy = 3.0 μJ, the MALDI and q_0 pressure were of 1 and 8 mTorr (N_2), respectively. The SRM dwell time was 10 ms per transition. Nitrogen was used as the collision gas, at a setting of 5. The monitored transitions were m/z 671.4 → m/z 570.4 (collision energy = 45 eV, declustering potential = 30 eV) for SQV and m/z 676.4 → m/z 575.2 (collision energy = 45 eV, declustering potential = 25 eV) for SQV-d5, m/z 609.2 → m/z 195.1 (collision energy = 55 eV, declustering potential = 25 eV) for RES.

2.9. LC–SRM/MS analysis

The HPLC system consisted of two LC-10 ADvp (Shimadzu, Reinach, Switzerland) pumps operated in high-pressure gradient mode, a SIL-10 ADvp autosampler (Shimadzu, Reinach, Switzerland) and a XTerra MS C18 2.5 μm, 2.1 mm × 50 mm (Waters, Milford, MA, USA) column. The mobile phase A was H_2O + 0.1%

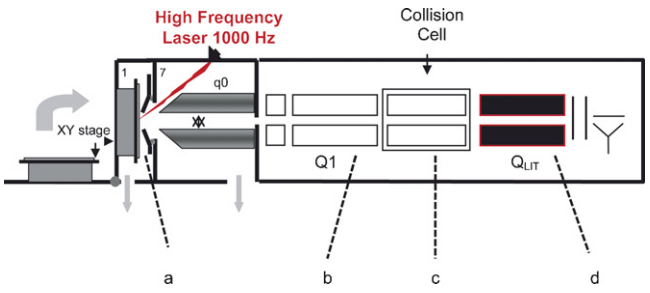

Fig. 3. Schematic representation of the MALDI-QqQLIT instrument, (a) MALDI source, (b) first mass analyzing quadrupole, (c) collision cell, and (d) linear ion trap.

HCOOH and mobile phase B was CH_3CN + 0.1% HCOOH. Saquinavir and its deuterated internal standard were eluted using a water-organic gradient starting with 20% mobile B to 90% mobile phase B in 2.5 min. The total LC cycle time was of 6.0 min. The injection volume onto the column was of 20 μl and the column was operated at a flow rate of 250 μl/min.

Mass spectrometric detection was carried out using a Q TRAP 4000 (AB/MDS Sciex, Concord, ON) equipped with an electrospray source and operated in the positive ionization mode. Conditions were as follows: curtain gas = 20 psi. Gas 1 = 50 psi. Gas 2 = 50 psi. Ion spray voltage = 4800 V. Source temperature = 450 °C, declustering potential = 110 eV. Nitrogen was used as collision gas at a setting of 6. The SRM dwell time was of 100 ms per transition. The monitored transitions were: m/z 671.5 → m/z 570.5 (collision energy = 45 eV) for SQV and m/z 676.5 → m/z 575.4 (collision energy = 45 eV) for SQV-d5.

2.10. Cross validation between MALDI-SRM/MS and LC–SRM/MS assays

Spiked human plasma samples were prepared, including one set of calibration standards (5, 10, 12.5, 25, 100, 250, 500, 1000, 5000 and 10,000 ng/ml) and two sets of QC samples (5, 12.5, 250 and 5000 ng/ml). Patients HIV (+) samples of unknown concentration were heated 60 min at 60 °C to inactivate the virus.

LLE was then performed identically for all samples, according to the previously described LC–SRM/MS protocol (i.e. final reconstitution in 200 μl of CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH). Twenty microliters were injected onto the LC–SRM/MS system. The remaining volume was used for the MALDI-MRM analysis and was evaporated to dryness. Samples were reconstituted in 20 μl (instead of 25 μl) of CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH to take into account the amount of sample used for LC–SRM/MS. After the addition and mixing with 20 μl of CHCA 5 mg/ml in CH_3CN/H_2O (1/1, v/v) + 0.1% HCOOH, 1 μl was spotted manually onto the MALDI plate.

3. Results and discussion

LC–MS allows the analysis of several hundred plasma samples from pre-clinical or clinical studies on a daily basis. High-speed analysis is of interest for very large sets of samples or for on-line analytics where a limited number of samples need to be analyzed very rapidly. A run cycle time of 10 s for LC–MS analysis of a single sample or the analysis of thousand samples within 12 h has been reported [18,19]. Because LC–MS is based on fluidics there is an intrinsic limitation for reducing the analysis time to 1 s. The major goals of liquid chromatography are (i) to allow the injection of large sample volume (analyte concentration), (ii) to further clean-up sample, (iii) to improve analyte selectivity. While sample clean-up remains key to minimize matrix effects during the ionization process, very often the MS sensitivity is sufficient to achieve a limit of quantitation at the ng/ml level without any pre-concentration. Mass spectrometry can be considered as a very selective gas phase separation technique, in particular when tandem MS is used. Under these conditions the chromatographic step can be omitted and the only limitation is metabolite cross-talk, where metabolites can interfere with the quantitation of the analyte. This is particularly a concern for drugs which generate phase II metabolites. Nevertheless, the quantitation of pharmaceutical compounds in biological matrices without chromatographic separation using chip-based infusion has been successfully demonstrated [20,21]. With LC or infusion systems, cycle times below 10–20 s may be challenging to achieve on a routine base. In the case of MALDI the situation is completely different because the analyte is embedded in the matrix and fixed onto a target plate. The laser is operated in the rastering mode and only 4–8% of the spot is consumed. With a fast x, y moving plate (e.g. 5 mm/s) and a high repetition rate laser (1000 Hz), a 1 μl spot can be analyzed in less than a second. Another key advantage is that the same sample can be reanalyzed at any time. Considering a sample analysis time of 5 s, a 384 sample plate can be analyzed within 32 min. Also with MALDI sample preparation remains important, to minimize ionization matrix effects, and automation of sample preparation and spotting becomes mandatory to achieve high sample throughput. In the present study an assay was developed to quantify saquinavir in human plasma by MALDI-SRM/MS. To achieve reproducible and automated deposition of sample and the matrix mixture, electrodeposition was selected.

3.1. Sample preparation

Protein precipitation was the first sample preparation strategy investigated. Indeed, in comparison with other methods, such as solid phase extraction and liquid–liquid extraction, it requires little method development, since it represents a more generic approach. Various means to precipitate proteins can be employed (organic solvents, acids, etc.). In this study, a 1/1 mixture of acetonitrile/ethanol was used for this purpose. The aspect of the spots is illustrated in Fig. 4A, and suggests that crystallization of CHCA did not occur properly for PP. Accordingly, MALDI-SRM/MS analysis did not give rise to any signal. The lack of signal is certainly not due to suppression of ionization by endogenous analytes but from the lack of desorption/ionization of the analyte/matrix. The lack of signal is certainly due to poor crystallization of the matrix/analyte mixture and not by suppression of the ionization by endogenous compounds. These findings are supported by the results published by Kovarik et al. [14] who performed plasma protein precipitation with perchloric acid and reported similar observations. They furthermore showed that a 2 μl on-spot wash with water leads to glossy, white crystals and to a recovery of signals. Nevertheless, since the aim of this work was to emphasize automation, this approach has not been evaluated, because it involves manual steps. As a consequence, liquid–liquid extraction (LLE) was investigated as an alternative procedure. Unlike protein precipitation, spots exhibit homogenous, dense crystals (Fig. 4B) and signals were obtained by MALDI-SRM/MS analysis with sufficient signal to noise ratios to cover a dynamic range from 5 to 10,000 ng/ml.

3.2. Automated sample spotting onto the MALDI target

In the case of MALDI analysis, high-density manual spotting of hundreds of samples would turn into a tedious task. The Xcise was originally developed as a proteomics processing station, but in our case acts as a pipetting station. Advantage was taken of the possibility to control all aspirate/dispense steps and robot arm movements via programmable macros in order to automate sample spotting.

Initially, the spotting was performed by simple contact deposition of the droplet onto the MALDI plate (Fig. 2). The procedure used was similar to those described in the experimental section, except that standard, non-conductive pipette tips were used and the tip to plate distance was optimized to permit the deposition of droplets.

Nevertheless, the samples tend to adsorbed on the tip walls, rather than being focused in a well-defined droplet shape at the end of the tips. This greatly hampered the proper contact deposition of samples onto the MALDI plate and led to spotting failures.

Electrodeposition [22] was thus investigated as an alternative procedure. It uses a pulsed electric field to transfer a solution from the pipette tip hanging over the MALDI plate as a non-contact

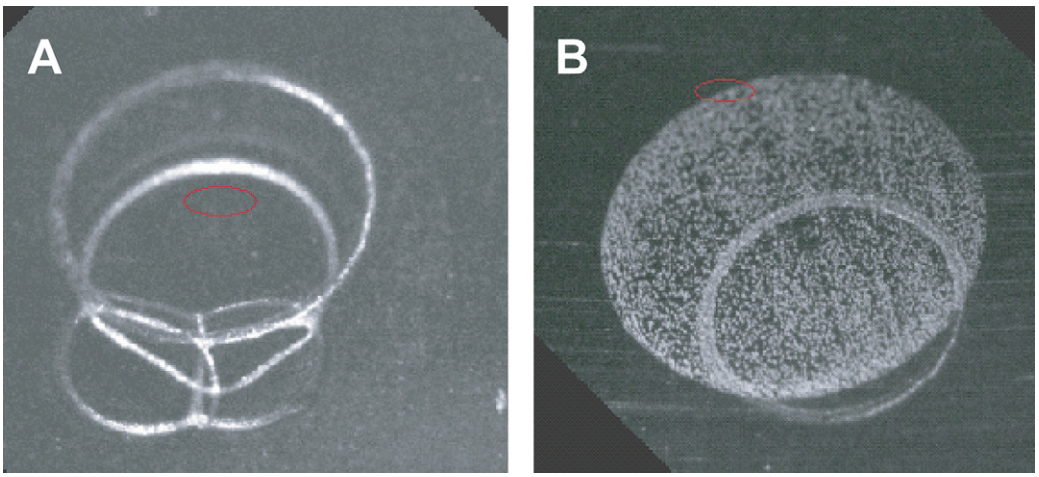

Fig. 4. Pictures of MALDI spots obtained after (A) protein precipitation (PP) and (B) liquid–liquid extraction (LLE).

approach (Fig. 2). The electric field was chosen to be pulsed instead of continuous to prevent putative electrospray of the samples. The overall spotting success rate was found to be between 80% and 100%. It must be furthermore emphasized that a 96-spot plate can be spotted within 20 min without requiring manual intervention, making the process time- and labour-saving compared to manual spotting. Moreover, because the tips are disposable and the matrix is aspirated before the sample, no cross-contamination can occur.

3.3. MALDI-SRM/MS quantitation of saquinavir

The inhomogeneous cocrystallization of an analyte in the matrix lattice gives rise to quantitative variations in signals measured (i) from spot to spot and (ii) between different positions within a given spot. Various sample deposition strategies have been developed to circumvent these issues, such as electrospray deposition of matrix, fast evaporation with vacuum or heating, and the use azeotropic mixtures. In this work, a dedicated strategy has been employed, using the dried-droplet method, which was found the most convenient to cope with the automation requirements.

First, rastering the high repetition rate laser across the entire spot leads to continuous ablation and therefore a continuous signal across the spot. Due to short dwell time 40–50 data points can be collected. Fig. 5 depicts typical MALDI-SRM/MS signals for a plasma 0 sample and the lower limit of quantification (LLOQ).

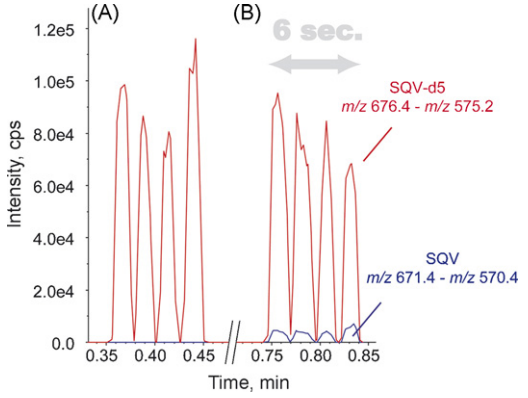

Fig. 5. Representative MALDI-SRM/MS traces (A) plasma blank and (B) LLOQ at 5 ng/ml, LLOQ. The analysis of a sample is performed within 6 s on the basis of four replicates of the same extract.

Nevertheless, due to slightly different spot size and/or possible slight misalignment of the spots, the length of the ablated sections and thus the absolute peak areas are susceptible to significant variation. For this reason, to improve the precision and the accuracy of measurements, the use of an internal standard is almost mandatory to achieve acceptable precision and accuracy. Quantitation was performed on the basis of analyte/IS area ratios.

The second issue is the spot to spot variability, which actually represents the precision of the method if the considered spots are from the same sample (variation observed for multiple determinations of the same sample). From a statistical point of view, a common way to estimate the true value is to use the mean of several determinations per sample instead of just one measurement. This approach is nevertheless time-consuming and therefore not usual in bioanalysis because of the large number of samples to be analyzed. With MALDI-SRM, analysis is achieved within a few seconds, so multiple determinations can be performed without sacrificing the throughput. In this study, each sample was spotted four times. Analysis of a sample (on the basis of four determinations) was performed within 6 s, and the average of the four peak area ratios was used for the linear regression and quantitation.

3.4. MALDI-SRM/MS assay performance with SQV-d5 as an internal standard

On the basis of four determinations (i.e. spots) per sample, an entire series, which includes blank, plasma blank (P00), plasma blank with IS (P0), ten calibration standards, and ten quality control samples can be analyzed within 3 min. A representative MALDI-SRM/MS trace is displayed in Fig. 6.The assay was linear over more than three orders of magnitude. The lower and upper levels of quantification (LLOQ and ULOQ, respectively) have been set *a priori*, in order to fit the clinical needs, and do not represent the limit of the technique. Five batches have been analyzed, accuracies were found to be in the 100–106% range and precisions ($n = 5$) were better than 12% based on QC's samples. These are in agreement with the usual criteria of acceptance for bioanalytical methods (Table 1).

3.5. Selectivity of the MALDI-SRM/MS assay

Nowadays, guidelines recommend combinations of several antiretroviral drugs for the management of HIV infections. From an analytical point of view, this means that a clinical unknown sample will always contain potentialy interfering substances, so the question of the selectivity of the assay has to be addressed. A cocktail

Table 1
Precision and accuracy results of the MALDI-SRM/MS analyses of saquinavir (SQV) with SQV-d5 as IS ($n = 5$)

Sample	Expected concentration (ng/ml)	Calculated concentration (ng/ml)					Mean (ng/ml)	Accuracy ($n = 5$) (%)	RSD ($n = 5$) (%)
		$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$			
CAL 1	5	5.162	5.168	5.233	5.116	5.136	5.163	103	0.9
CAL 2	10	10.04	9.563	9.570	9.193	9.714	9.616	96.2	3.2
CAL 3	12.5	11.91	12.68	11.90	13.15	12.05	12.34	98.7	4.5
CAL 4	25	23.23	23.11	24.26	25.05	25.05	24.14	96.6	3.9
CAL 5	100	96.53	96.03	100.5	93.86	102.0	97.78	97.8	3.4
CAL 6	250	252.6	246.1	237.2	234.8	249.1	243.9	97.6	3.1
CAL 7	500	496.7	492.3	518.5	490.9	504.1	500.5	100	2.3
CAL 8	1,000	1029	1,039	1054	1,065	1011	1,039	104	2.0
CAL 9	5,000	4977	4,925	4623	4,999	5056	4,916	98.3	3.5
CAL 10	10,000	10,883	11,186	11,049	10,791	9886	10,759	108	4.7
QC 1	5	5.554	5.532	5.901	4.506	5.094	5.317	106	10
QC 2	10	11.06	11.48	10.31	8.693	9.436	10.20	102	11
QC 3	12.5	13.49	13.45	11.96	13.53	12.42	12.97	104	5.6
QC 4	25	24.79	24.64	26.28	24.07	26.62	25.28	101	4.4
QC 5	100	100.4	95.92	106.7	97.31	102.9	100.6	101	4.3
QC 6	250	241.8	253.1	243.9	255.9	269.7	252.9	101	4.4
QC 7	500	479.5	506.5	520.4	483.4	520.6	502.1	100	3.9
QC 8	1,000	1012	1,193	984	1,092	1071	1,070	107	7.6
QC 9	5,000	4724	5,490	5,221	4,873	4984	5,058	101	6.0
QC 10	10,000	11,256	10,987	9,728	9,614	9593	10,236	102	8.0
r^2		0.99728	0.99602	0.99544	0.99584	0.99945			
Slope		0.00735	0.00740	0.00764	0.01034	0.00938			
Constant		0.00627	0.00670	0.01096	0.00222	0.00449			

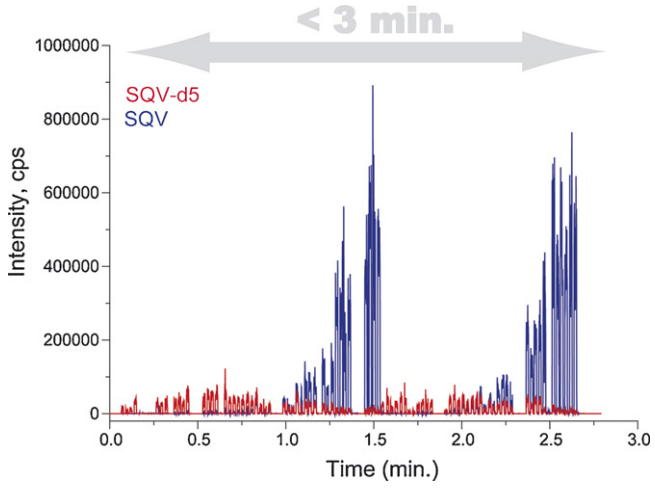

Fig. 6. Representative MALDI-SRM/MS traces of one entire series of STD and QC's. Twenty-three samples (92 spots) analyzed within 3 min.

of eight antiretroviral drugs (six protease inhibitors: amprenavir, atanazavir, indinavir, lopinavir, nelfinavir, ritonavir and two non-nucleosidic transcriptase inhibitors: efavirenz and nevirapine) was spiked with SQV in human plasma. Concentrations of non-SQV antiretroviral drugs were chosen to be superior (or comparable in the case of ritonavir) to their C_{max} (Table 2), while SQV concentration was chosen to be at the LLOQ of the assay.Although ionization suppression was observed, accurate quantitation of saquinavir was possible at the LLOQ (data not shown). Moreover, the simultaneous presence of nine antiretroviral drugs at such concentration levels would not reflect any clinical reality but rather represent a theoretical worst-case situation, so suppression effects due to interfering agents would be expected to be less important with real unknown samples. With quantitative assays without chromatography prior MS detection, metabolites cross-talk can become a significant issue. In humans, saquinavir metabolized rapidly in inactive mono- and dihydroxlated metabolites [15]. In humans, in the case of SQV, it is unlikely that these metabolites generate the parent drug by collision-induced dissociation in the source or in the collision cell. Because it is difficult to obtain reference compounds and in order to address properly this issue a cross validation was performed between the MALDI-SRM/MS assay and a LC–SRM/MS

Table 2
Pharmacokinetic parameters according to manufacturer's prescribing information (vide infra) and concentration in sample of the antiretroviral drugs used in the selectivity study

Drug	Regimen and reference	C_{max} (ng/ml)	C_{min} (ng/ml)	Concentration in sample (ng/ml)
Saquinavir	600 mg tid (a)		79	5
Amprenavir	1200 mg bid (b)	7,660	320	15,000
Atanazavir	400 mg sid (c)	3,152	273	10,000
Efavirenz	600 mg sid (d)	4,072	1768	10,000
Indinavir	800 mg tid (e)	7,744	154	10,000
Lopinavir	400 mg + 100 mg ritonavir bid (f)	9,800	2900	10,000
Nelfinavir	1250 bid (g)	4,000	700	10,000
Nevirapine	400 sid (h)	4,500	–	10,000
Ritonavir	600 mg bid (i)	11,200	3700	10,000

sid: one time a day; bid: two times a day; tid: three times a day.

assay with real clinical samples. Both methods provided similar results. To really benefit from MALDI-SRM/MS approaches without chromatography the metabolism of the drug needs to be well characterized. A strategy to improve the selectivity of chip-based infusion assay was proposed by Leuthold et al. [21] where the authors calculated a ratio between the SRM and MS^3 results. A similar set-up could also be used with the present MALDI-SRM/MS assay to check the selectivity of the analysis.

3.6. MALDI-SRM/MS assay performance with reserpine as internal standard

The use of an internal standard is essential for MALDI-SRM/MS analysis to achieve acceptable precision and accuracy. Uniform distribution and thus identical crystallization pattern of the analyte and of the internal standard within the spot is then required. Obviously, stable isotope-labeled compounds would represent the ideal choice, but, and especially in drug discovery or clinical environments, are not always available. Sleno and Volmer investigated some physico-chemical properties susceptible to predict potential matches, and emphasized the key role of structure analogy, molecular weight and log *D* for the selection of the IS [23].

As an alternative to isotopically labeled IS for SQV, one option would be to select a structural analogue. Nevertheless in the case of SQV, those are potential co-administrated substances, which, in the absence of prior separation, are not appropriate candidates. Alternative substances were investigated for their closeness of Mr, pK_a and log *D* with SQV. Reserpine was found to be the most suitable. The assay for saquinavir was linear ($r^2 > 0.99$) from 5 to 1000 ng/ml (Table 3). Quantitation without a deuterated internal standard was therefore possible, without compromising the LLOQ but with a smaller dynamic range. The smaller dynamic range may be related to competition in the ionization process at high concentration.

3.7. Analyte extraction recovery and matrix effects

Matrix effects and LLE extraction recovery have been investigated at two SQV concentration levels (12.5 and 1000 ng/ml). This estimate relies on the use of three sets of samples, each set comprising three samples. In this section, calculations were performed on the basis of the SQV area, without any internal standard due to the experimental design. Indeed, since SQV and SQV-d5 are stable isotope analogues, they share the same ionization properties and will then undergo equal matrix effects. Thus, using area ratio will not be informative for determining matrix effects.

Table 4 summarizes the results, where the individual value of a given sample was calculated as the average measurements of four spots. Inter-spot variability (RSD) was less than 20%. In addition, the individual value of each set was calculated by taking the average of three samples. Inter-sample variability (RSD) was between 11% and 26%, suggesting that an internal standard is crucial for accurate quantitation. The three sets were composed as follows: 1st set: standard solutions of SQV; 2nd set: blank plasmas extracted and then spiked with SQV; 3rd set: plasmas spiked with SQV before LLE extraction.

Matrix effects (%) were calculated as: $100 \times [(\text{2nd set}/\text{1st set}) - 1]$. Positive values correspond to matrix enhancement, whereas negative values correspond to matrix suppression. They were found to be of −7.9% and −7.7% at 12.5 and 1000 ng/ml, respectively. LLE recovery was estimated as the area ratio of the 3rd set/2nd set, and was found to be of 105% and 81.9% at respectively 12.5 and 1000 ng/ml. Similar values were obtained using a LC–MS/MS-based determination (data not shown), and are also in agreement with those Frerichs et al. [24] who used similar sample preparation and experimental design for recovery calculation.

3.8. LC–SRM/MS assays for saquinavir

An LC–SRM/MS assay was established using the basic framework of the MALDI-SRM/MS method, namely same sample preparation and same dynamic range. Only the reconstitution volume of the sample had to be greater (200 μl instead of 25 μl) to permit an effective 20 μl sample injection.

A representative SRM chromatogram for a plasma 0 and at the LLOQ is shown in Fig. 7. The assay was linear for 10–10,000 ng/ml. Five batches were analyzed, and accuracies were in the 99–103% range and precisions ($n = 5$) better than ±9% (Table 5).

In comparison with LC–MS/MS approaches, the first evident advantage of MALDI-SRM/MS is its analysis speed. In the present case, the LC–SRM/MS analysis of a sample took 6 min. Under these

Table 3
Precision and accuracy results of the MALDI-SRM/MS analyses of saquinavir with reserpine as IS ($n = 3$)

Sample	Expected concentration (ng/ml)	Calculated concentration (ng/ml)			Mean (ng/ml)	Accuracy ($n = 3$) (%)	RSD ($n = 3$) (%)
		$n = 1$	$n = 2$	$n = 3$			
CAL 1	5	5.066	5.095	5.074	5.078	102	0.3
CAL 2	10	9.983	9.297	9.948	9.743	97.4	4.0
CAL 3	12.5	11.88	12.87	11.68	12.14	97.1	5.3
CAL 4	25	26.04	26.11	26.64	26.26	105	1.2
CAL 5	100	97.69	90.45	101.9	96.67	96.7	6.0
CAL 6	250	254.1	241.9	252.4	249.4	99.8	2.6
CAL 7	500	507.8	527.4	518.2	517.8	104	1.9
CAL 8	1000	988.0	1050	926.2	988.2	98.8	6.3
QC 1	5	4.548	5.437	4.959	4.981	99.6	8.9
QC 2	10	8.553	11.27	8.944	9.590	95.9	15
QC 3	12.5	11.88	11.77	12.56	12.07	96.6	3.5
QC 4	25	24.80	25.93	23.40	24.71	98.8	5.1
QC 5	100	87.63	105.3	88.18	93.71	93.7	11
QC 6	250	221.1	238.0	239.6	232.9	93.2	4.4
QC 7	500	536.2	500.3	542.8	526.4	105	4.3
QC 8	1000	969.0	1079	1184	1077	108	10
r^2		0.99882	0.99496	0.99659			
Slope		0.03074	0.03595	0.03190			
Constant		0.01828	−0.01547	−0.00091			

Table 4
Matrix effects and LLE extraction recovery at SQV concentration levels of 12.5 and 1000 ng/ml.

ng/ml	Set	Sample #	Area, mean of 4 spots	RSD of 4 spots (%)	Area, mean of 3 samples	RSD samples (%)	Matrix effects (%)	LLE recovery (%)
		1	10,6167	5.0				
	Set 1	2	121,875	5.7	128,839	20.8		
		3	158,475	14.9				
		1	108,000	6.3				
12.5	Set 2	2	134,075	7.5	139,017	24.3	−7.9	105
		3	174,975	8.4				
		1	122,125	15.1				
	Set 3	2	127,325	14.3	146,175	25.6		
		3	189,075	9.5				
		1	6,197,050	17.2				
	Set 1	2	8,125,250	4.6	791,8342	20.6		
		3	9,432,725	4.7				
		1	7,410,250	8.6				
1000	Set 2	2	8,937,925	4.0	8,526,267	11.5	−7.6	81.9
		3	9,230,625	3.6				
		1	6,064,475	14.7				
	Set 3	2	7,441,500	6.1	6,981,683	11.4		
		3	7,439,075	7.5				

Table 5
Performance of the LC–SRM/MS method with SQV-d5 as internal standard: precision and accuracy results of the QC samples

Sample	Expected concentration (ng/ml)	Calculated concentration (%)					Mean (ng/ml)	Accuracy ($n=5$) (%)	RSD ($n=5$) (%)
		$n=1$	$n=2$	$n=3$	$n=4$	$n=5$			
QC 2 (LLOQ)	10	9.774	10.60	9.033	11.35	10.70	10.29	103	8.7
QC 4	25	25.31	26.56	23.67	24.97	24.18	24.94	99.8	4.5
QC 6	250	245.1	235.2	259.8	255.6	256.7	250.5	100	4.1
QC 9	5000	5324	4897	5067	5349	5188	5165	103	3.6

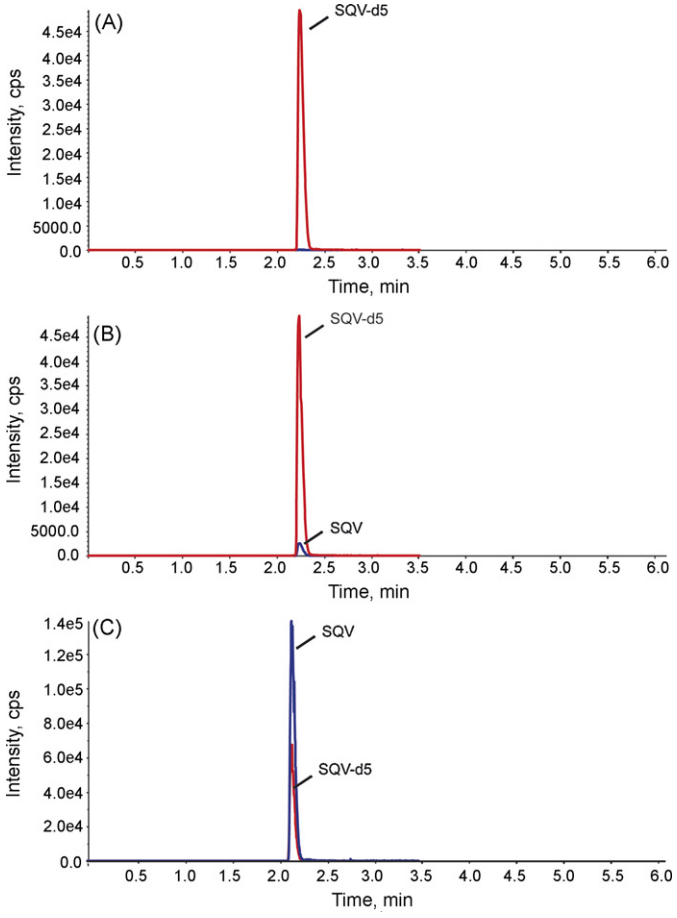

Fig. 7. Representative SRM chromatograms for LC–SRM/MS analysis (A) plasma 0, (B) LLOQ at 10 ng/ml, and (C) patient sample containing 400.3 ng/ml saquinavir.

conditions, the analysis of a whole series (ten calibrations standards, ten QC samples and various blanks) takes more than 2 h. This has to be compared with the time of 3 min for complete calibrations and QC samples set for MALDI-SRM/MS. Moreover, due to the broad dynamic range, the LC–SRM/MS method has been hampered by autosampler carryover effects. If analyzed immediately after a high concentration sample (i.e. close to ULOQ), low concentration samples (i.e. close to LLOQ) give rise to over-evaluated signals, because of the improper cleaning of the injector needle from one injection to another. To cope with such situations, one common strategy is to insert blank samples between high and low concentration level samples, with some loss of throughput as a drawback. In contrast, with MALDI-SRM/MS, carryover is not an issue. LLOQ samples can

Table 6
Cross-validation results of clinical samples

Sample	Calculated concentration (ng/ml)		Bias (%)
	MALDI-SRM/MS	LC–SRM/MS	
Patient 01	1761	1722	2.3
Patient 02	651.5	679.0	−4.1
Patient 03	1059	1088	−2.7
Patient 04	382.5	400.3	−4.5
Patient 05	971.7	1008	−3.6
Patient 06	577.6	603.9	−4.4
Patient 07	997.6	996.7	0.1
Patient 08	88.85	98.01	−9.3
Patient 09	2895	2884	0.4
Patient 10	684.4	695.9	−1.6
Patient 11	78.11	81.85	−4.6
Patient 12	154.2	159.2	−3.2
Patient 13	1521	1524	−0.2
Patient 14	409.3	414.1	−1.2
Patient 15	1166	1192	−2.2
Patient 16	1243	1247	−0.3
Patient 17	841.9	840.1	0.2
Patient 18	2173	2195	−1.0

be analyzed immediately after ULOQ samples without jeopardizing accuracy of measurements.

3.9. LC–SRM/MS and MALDI-SRM/MS cross validation with clinical samples

Samples, including calibration standards, QC and unknowns were extracted, analyzed by LC–SRM/MS first and then by MALDI-SRM/MS. The results for the patient's samples are summarized in Table 6. Saquinavir concentrations of 18 unknown samples were determined with both methods, using their corresponding linear regression equations. To demonstrate that both assays provide similar results the bias was calculated for each sample from the LC–SRM/MS versus the MALDI-SRM/MS methods. As illustrated in Table 6 the bias was found to be less than 10% for all samples. Acceptance criteria would be for QC's ±15% which would result in a maximum 30% bias between both assays.

4. Conclusions

MALDI-SRM/MS was successfully applied for the high-speed quantitative analysis of saquinavir in human plasma samples without prior chromatographic separation. The method was found to be precise and accurate within the requirement of bioanalytical work using a labeled internal standard. The methodology also offers also the possibility of using non-isotopically labeled internal standard. As for most mass spectrometric-based methods, sample preparation is critical. To achieve high sample throughput, the spotting and crystallization processes need to be automated and are one of the main sources of analytical variation. Compared to LC–SRM/MS or infusing approaches, MALDI-SRM/MS can analyze samples within a few seconds allowing thousand of measurements on a daily basis. Considering that method development remains relatively time consuming, the methodology could be particularly attractive for late clinical trials, for therapeutic drug monitoring or for quantitation of biomarkers considering that the sample workup can be performed at a different location than the analysis.

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