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Bioanalysis of HIV protease inhibitors in samples from sanctuary sites

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Abstract

The human immunodeficiency virus (HIV) is present in several sites inside the human body, which are hardly accessible to antiretroviral drugs, the so-called sanctuary sites. The most important sanctuary sites are cerebrospinal fluid (CSF), peripheral blood mononuclear cells (PBMCs) and seminal plasma. The determination of drug concentrations in these sanctuary sites may form an important step in treatment optimisation of HIV-infected individuals. However, bioanalysis in these sites is hampered by several factors with regard to sample preparation, chromatography and detection. In this review, we will discuss these issues and give an overview of published methods using high-performance liquid chromatography (HPLC) for the bioanalysis of HIV protease inhibitors in CSF, PBMCs and seminal plasma.

Keywords: Bioanalysis; HPLC; HIV protease inhibitors; PBMC; CSF; Seminal plasma

1. Introduction

Concentration measurements of antiretroviral drugs are widely used in daily practice to support the treatment of individuals infected with the human immunodeficiency virus (HIV). Therapeutic drug monitoring (TDM) programs are mostly using blood plasma samples. This may be a valid approach since relationships between antiretroviral drug concentrations in plasma, virological response and side effects have been demonstrated [1–7].

The class of HIV protease inhibitors forms an important component of current highly active antiretroviral therapy (HAART) regimens [8]. To date eight protease inhibitors have been approved by the Food and Drug Administration (FDA): amprenavir, indinavir, nelfinavir, lopinavir, ritonavir, saquinavir and more recently, atazanavir and fosamprenavir (a pro-drug of amprenavir). The chemical structures of the compounds are shown in Fig. 1. Numerous methods have been published for the bioanalysis of these protease inhibitors in plasma, which have been excellently reviewed [9].

However, HIV is also present in sites outside the blood compartment. Virus particles in the central nervous system (CNS), for example, are associated with various nervous system pathologies, including opportunistic infections of the CNS, primary CNS lymphoma, neuropathy and HIV-associated dementia [10]. HIV present in seminal plasma is responsible for the transmission of the virus through sexual contact [11]. The CNS and the male genital tract are so-called anatomical sanctuary sites for the virus, since they are scarcely accessible to HAART [12–14]. These sites are protected by the blood–brain and blood–testis barrier, respectively, which form endothelial barriers that hold various drug-transporting proteins [15,16]. Viral rebound from these reservoirs can occur if the antiretroviral therapy is discontinued and therefore, sanctuary sites remain the major obstacles for the eradication of HIV from the body. Whether the male genital tract is a true sanctuary site or just a viral reservoir, is questioned by some authors [17].

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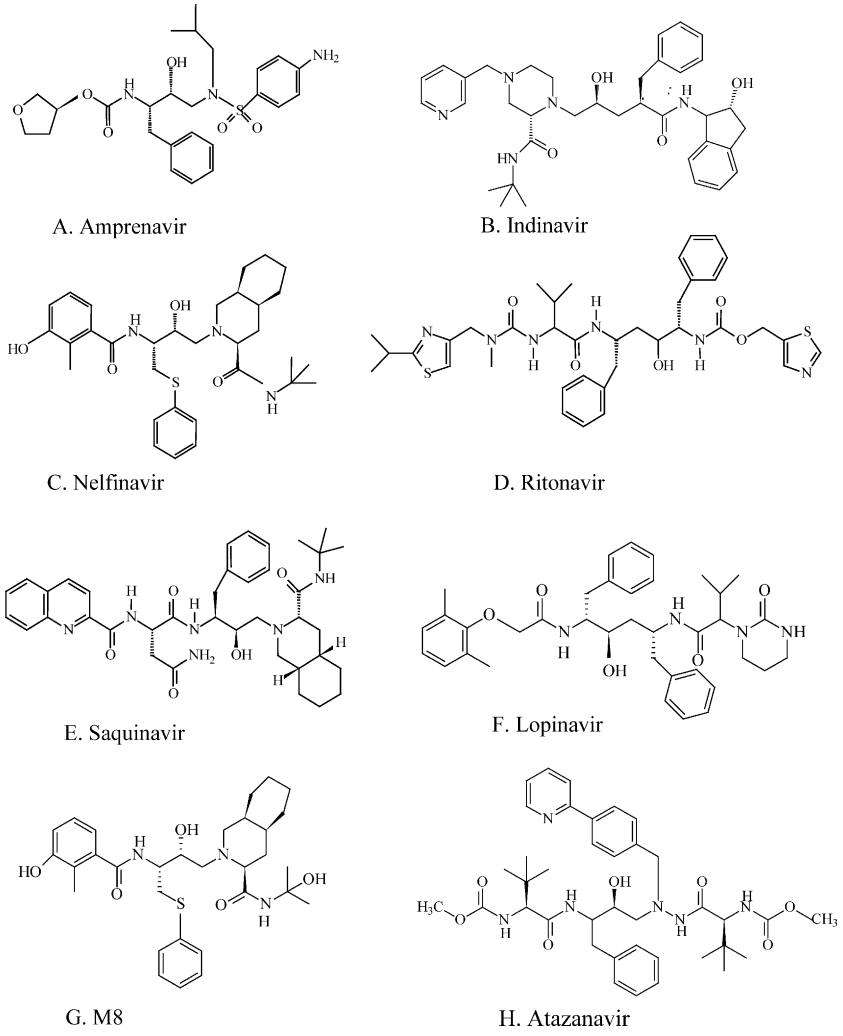

Fig. 1. Chemical structures of the protease inhibitors.

Besides these anatomical sanctuary sites, cellular sanctuary sites can be distinguished. The main cellular viral reservoir is the pool of latently infected resting CD4+ T-cells containing integrated HIV provirus [18,19]. However, in cells that are not in a resting state HIV replicates and therefore, antiretroviral drugs must penetrate into these cells at concentrations sufficiently high to inhibit viral replication.

The penetration of protease inhibitors in sanctuary sites is mainly determined by their affinity for drug-transporting proteins and by the binding to plasma proteins. Protease inhibitors are to varying degrees substrates for the drug-transporting proteins P-glycoprotein (P-gp) and multi-drug resistance associated-proteins (MRPs) [15,16,20,21]. Affinity for these proteins prevents penetration of the protease inhibitors in anatomical and cellular sanctuary sites. Plasma protein binding determines the availability of drugs to cross cell membranes. Most protease inhibitors are lipophilic drugs with a high protein binding to blood plasma proteins (atazanavir 86%, amprenavir 90% and lopinavir, nelfinavir, ritonavir, saquinavir >97%) [11,17]. Indinavir is an exception with a blood plasma protein binding of approximately 60% [11].

Drug concentrations in semen appear to be predominantly determined by passive diffusion since protease inhibitors with a protein binding of less than 90% show moderate to good penetration in the seminal plasma [11,17,22]. The distribution of protease inhibitors to the CNS is, in general, limited [23–28]. Only atazanavir and indinavir reach concentrations that are above their inhibitory concentrations [27,29,30]. The accumulation of protease inhibitors in PBMCs is mostly expressed as the intracellular/plasma drug concentration ratio. Intracellular accumulation hierarchies have been observed in HIV-1 infected lymphocytes in vivo in the order nelfinavir > saquinavir > ritonavir > indinavir [31] and nelfinavir > amprenavir > indinavir [32].

Since it is important to know which drugs reach the viral reservoirs and to which extent, pharmacokinetic studies are undertaken to study the penetration and retention of antiretroviral drugs in sanctuary sites. When measuring concentrations in these matrices it is preferred to use assays that have

been developed and validated for these purposes. Special features apply to these assays with regard to sample preparation, chromatography, detection and validation since sample volumes are often limited and concentrations may be low. In this review, we will discuss the issues that are associated with the bioanalysis of protease inhibitors in samples from sanctuary sites. In addition, we will give an overview of published methods using high-performance liquid chromatography (HPLC) for the bioanalysis of HIV protease inhibitors in CSF, seminal plasma and PBMCs.

2. Methods

A Medline search was performed using the keywords: HIV protease inhibitors (or the names of the individual drugs), concentration, CSF, semen, PBMC and intracellular. Conference abstracts were included if they contained adequate and detailed information on used methods. Two types of publications were distinguished: the first type of reports (Table 1) describes assays that were developed and validated for the purpose of measuring protease inhibitors in CSF, PBMCs or seminal plasma. The second type of publications (Table 2) presents concentrations in CSF, PBMCs or seminal plasma, which were measured with assays developed for and using calibration samples in blood plasma. Publications of the second type were admitted in the table only if the used methods on sample preparation, chromatography and detection were specified with sufficient detail. The presence of a reference to a published method in blood plasma was also sufficient.

3. Bioanalysis of HIV protease inhibitors in samples from sanctuary sites

Bioanalytical quantitative HPLC assays can be divided into three components: sample pre-treatment, chromatography and detection. All are affected by the special features that apply to the quantification of protease inhibitors in CSF, PBMC and seminal plasma. In the next paragraphs, the three components will be separately discussed. In addition, the important issues of method validation with regard to samples from sanctuary sites will be discussed. The pros and cons of different approaches will be considered and a suggestion will be given on how to address the bioanalysis of protease inhibitors in samples from sanctuary sites.

3.1. Sample preparation

The sample volume and the sample pre-treatment are important issues in the preparation of samples from sanctuary sites.

The available sample volume is often very small. This applies especially to CSF that cannot be easily obtained from patients and to PBMCs, which are generally isolated from fairly large volumes of blood. Preferably, the sample pre-treatment should therefore be developed with use of small volumes (e.g. 100 μl). The required sample volumes in the methods summarized in Tables 1 and 2 differed greatly and ranged from 100 μl to 1 ml for CSF, from 100 to 600 μl for seminal plasma and was approximately four million cells for PBMCs.

The sample pre-treatment procedure is dependent on the analyte, on the matrix and on the detection method. Protease inhibitors are lipophilic compounds that can be extracted from CSF, PBMCs and seminal plasma with either solid-phase extraction, protein precipitation or liquid–liquid extraction. Liquid–liquid extraction was used in the majority of assays, likewise plasma assays [9]. However, the procedure for the pre-treatment of samples from sanctuary sites sometimes cannot simply be copied from plasma samples.

Some matrices require a more extensive sample pre-treatment than others since they contain more endogenous substances than others. CSF consists mainly of water and may therefore not require an elaborate sample pre-treatment. Aweeka et al. [28] injected nelfinavir-containing CSF samples onto the analytical column without sample pre-treatment. They did not, however, detect nelfinavir in these samples, which is consistent with other findings [27]. Nelfinavir concentrations in CSF are probably low due to the high plasma protein binding. However, the absence of proteins in CSF could have resulted in adsorption to the used containers as was reported by Herforth et al. [33], and could therefore have caused the failure to detect nelfinavir in CSF. Herforth et al. determined the nelfinavir free drug concentrations in plasma and reported non-specific drug adsorption ranging from 25 to 95% depending on the method used to prepare ultra-filtrate.

The difference in protein content between the different matrices could also give unforeseen results with common pre-treatment procedures such as protein precipitation, solid-phase extraction and liquid–liquid extraction. The effect of the difference in protein content can be diminished by dilution of the samples with blood plasma, as was performed by some authors [34,35].

The preparation of PBMC samples requires specific attention. Following elaborate, strict procedures with the collection of the PBMC samples in the clinic, the preparation of the cell pellet in the laboratory should be performed under stringent conditions, particularly with regard to working temperature and speed of processing. Khoo et al. [31] have shown that significant drug efflux out of cells occurs during laboratory manipulation, which is both time- and temperature-dependent. Therefore, cells should be separated from the blood shortly after venipuncture and cell counting and washing should be performed immediately afterwards. Additionally, ice-cold (4 °C) reagents should be used in combination with refrigerated centrifugation to prevent ex vivo drug efflux.

In the preparation of PBMC samples, a step should be included to provide cell lysis so that the intracellular protease inhibitor concentrations can be quantified. The lysing

Table 1
Overview of bioanalytical methods for protease inhibitors in samples from human sanctuary sites

Compound	Sample preparation	Sample volume	LC		Detection	IS	LLQ	Remarks	Reference
			Mobile phase	Column					
Atazanavir									
PBMC	Automated SPE	5×10^6 cells in 200 μl	ACN:MeOH:H_2O (3:3:4, v/v) + 250 μl 88% HCOOH	YMC Basic (5 μm, 50 mm × 2 mm)	MS/MS	$^{13}C_6$-atazanavir	5 fmol/10^6 cells	Full validation	[36]
Amprenavir									
CSF	LLE	100 μl	Na_3PO_4 25 mM, pH 6.8:ACN (40:60, v/v)	Symmetry C18 (3.5 μm, 100 mm × 4.6 mm)	Fluorimetry at 270 and 340 nm	POB	0.5 ng/ml	+Plasma	[41]
Semen							1 ng/ml	Calibration samples in plasma, quality control samples in CSF and semen	
Semen	PP	100 μl	ACN:H_2O + 0.1% HCOOH (55:45, v/v)	Aquasil C18 (5 μm, 150 × 2.1 mm)	MS/MS	$^{13}C_6$-amprenavir	30 ng/ml	Full validation	[44]
Indinavir									
CSF	LLE	0.5 ml	HAC 50 mM, pH 4.8:CAN (52:48, v/v)	Zorbax S C18 (3.5 μm, 75 mm × 4.6 mm)	UV at 260 nm	–	0.01 μmol/L (LOD)	+Serum/urine	[55]
CSF	SPE*	1 ml	H_3PO_4 10 mM, pH 7.5:ACN (66:34, v/v) (column 1) H_3PO_4 10 mM, pH 7.5:ACN (62:38, v/v) (column 2)	Zorbax SB-CN (5 μm, 80 mm × 4 mm) Inertsil ODS-2 (5 μm, 150 mm × 4.6 mm)	UV at 210 nm	L-738,804	2 ng/ml	+ Plasma	[54]
Ritonavir									
CSF	PP	100 μl	ACN:H_2O containing 25 mM NaAc and 25 mM HSA, pH 4.0 (44:56, v/v)	Zorbax SB C18 (3.5 μm, 75 × 4.6 mm)	UV at 239 nm	–	50 ng/ml (in plasma)	+Plasma and saliva. Part of the validation performed only in plasma	[53]
Saquinavir									
CSF	SPE	600 μl	ACN:H_2O containing 25 mM NaAc and 25 mM HAS, pH 4.0 (40.5:59.5, v/v)	Zorbax SB C18 (3.5 μm, 75 mm × 4.6 mm)	UV at 239 nm	–	2.5 ng/ml (in plasma)	+Plasma and saliva. Part of the validation performed only in plasma	[43]
PBMC	LLE	4×10^6 cells in 500 μl plasma	ACN:H_2O containing 0.1% TFA (55:45, v/v)	Octadecylsilyl	UV at 240 nm	Yes, however not specified	9 ng/ml (in plasma)	Validation in plasma	[52]
Amprenavir/indinavir/nelfinavir/ritonavir/saquinavir									
PBMC	LLE	(3–5) × 10^6 cells	ND	CN RP 18	Fluorimetry (APV) UV (other)	ND	0.5 ng/10^6 cells (APV) 2 ng/10^6 cells (other)	+Plasma	[32]

ACN: acetonitrile; APV: amprenavir; CSF: cerebrospinal fluid; H_2O: water; H_3PO_4: phosphoric acid; HAC: acetic acid; HCOOH: formic acid; HSA: hexane-1-sulfonic acid; IDV: indinavir; PP: protein precipitation; KH_2PO_4: potassium dihydrogen phosphate; LLE: liquid–liquid extraction; LLQ: limit of quantification; LOD: limit of detection; LPV: lopinavir; MeOH: methanol; MS: mass spectrometry; MS/MS: tandem mass spectrometry; NaAc: sodium acetate; Na_3PO_4: sodium phosphate; ND: no data; NFV: nelfinavir; PBMC: peripheral blood mononuclear cell; POB: propyl-*p*-hydroxybenzoate; RTV: ritonavir; SPE: solid-phase extraction; SQV: saquinavir; UV: ultraviolet.

* Column switching was applied to further purify the extracts.

Table 2
Overview of bioanalytical methods for protease inhibitors in samples from human sanctuary sites

Compound	Matrix	Sample preparation	Sample volume	LC		Detection	IS	LLQ	Remarks	Reference
				Mobile phase	Column					
Indinavir	CSF	SPE	100 μl	ACN:KH_2PO_4 buffer, pH 3.1 (40:60, v/v)	Microsorb MV-C8 (5 μm, 250 mm × 4.6 mm)	UV at 210 nm	Verapamil	25 ng/ml		[30,35,56]
Indinavir	Semen	SPE	600 μl	ACN:H_2O containing 25 mM NaAc and 25 mM HAS, pH 6.0 (40.5:59.5, v/v)	Zorbax SB-C18 (3.5 μm, 75 mm × 4.6 mm)	UV at 210 nm	–	25 ng/ml	Seminal plasma was 1:1 diluted with blank human heparinized plasma	[34,35,57]
Indinavir	Semen	LLE	200 μl	ACN:H_2O (57:43, v/v)	Megellen 5C8 (5 μm, 250 mm × 4.6 mm)	UV at 205 nm	Ritonavir	20 ng/ml [58]		[58,59]
Indinavir/lopinavir	CSF/semen	PP	ND	NH_4HCOO 20 mM:ACN (30:70, v/v)	Hypurity Elite 5C18 (5 μm, 250 mm × 4.6 mm)	MS/MS	Ro 31-9564	10 ng/ml [26]	No data on pre-treatment of CSF/semen	[26,60]
Lopinavir	PBMC	PP	ND	MeOH:NH_4Ac 10 mM, pH 5 (35:65, v/v); MeOH gradient elution	Inertsil ODS3 C18 (5 μm, 50 mm × 2.0 mm)	MS/MS	D_5-SQV	100 ng/ml	PBMCs reconstituted in 100 μl plasma	[37,46]
Lopinavir	Semen	LLE	500 μl	ACN:$KH_2PO_4$50 mM, pH 6.53 (40:60, v/v) linear gradient elution	Inertsil ODS2 C18 (5 μm, 150 mm × 4.6 mm)	UV at 215 nm	A-86093	ND	No data on lopinavir	[50,61]
Nelfinavir	CSF	No	ND	ACN:phosphate buffer	C4 RP	ND	Saquinavir	25 ng/ml	+Plasma. Calibration samples in synthetic CSF	[28]
Nelfinavir	CSF/semen	LLE	250 μl	ACN:$NaH_2PO_4$25 mM, pH 3.4 (42:58, v/v)	Symmetry C18 (5 μm, 250 mm × 4.6 mm)	UV at 220 nm	DPX	50 ng/ml [27]	+Lymphoid tissue	[27,62]
Ritonavir/saquinavir	Semen	LLE	0.2–0.5 ml	ACN:H_2O (63:37, v/v)	Megellen 5C8 (5 μm, 250 mm × 4.6 mm)	UV at 238 nm	31-9564	20 ng/ml (SQV), 25 ng/ml (RTV) [58]	No data on ritonavir	[58,63]
Ritonavir/saquinavir	CSF	SPE	0.25 ml	ACN:NH_4Ac 2.5 mM, pH 6.5 (7:3, v/v)	Phenomenex ODS Luna (3 μm, 30 mm × 2.0 mm)	MS/MS	D_5-SQV	1 ng/ml (RTV), 0.2 ng/ml (SQV)	CSF ultrafiltrate + plasma ultrafiltrate. Quality control samples prepared in CSF	[24]
Ritonavir/saquinavir	CSF	LLE	0.5 ml	ACN:MeOH:TMAP in 0.1% TFA (40:5:55, v/v)	ODS-AQ (3 μm, 50 mm × 4.0 mm)	UV at 205 nm	A-86093	0.2 ng/ml [25]	No data on saquinavir	[25,64]
Indinavir/lopinavir/ nelfinavir/M8/ ritonavir/saquinavir	PBMC	LLE	ND	NH_4HCOO 10/20 mM:ACN (30:70, v/v)	Hypurity Elite 5C18 (5 μm, 250 mm × 4.6 mm)	MS/MS	Ro 31-9564	20 ng/ml (SQV), 10 ng/ml (RTV), 40 pg/10 × 10^6 cells (IDV), 3.1 ng/ml (NFV/M8)	Value of LLQ varies per application. PBMCs reconstituted in 100 μl water	[31,65–70]
Indinavir/lopinavir/ ritonavir	CSF/semen	LLE	300 μl	ACN:$NH_4H_2PO_4$10 mM plus HSA 1 mM, pH4.8 (35:65, v/v)	Inertsil ODS2 C18 (5 μm, 150 mm × 4.6 mm) [27]	UV at 210 nm	L 738,804	20 ng/ml (IDV), 50 ng/ml (LPV and RTV) [27]	+Lymphoid tissue. No data on lopinavir/ritonavir	[27,71]

ACN: acetonitrile; APV: amprenavir; CSF: cerebrospinal fluid; DPX: 6,7-dimethyl-2,3-di-(2-pyridyl)quinoxaline; H_2O: water; HSA: hexane-1-sulfonic acid; IDV: indinavir; PP: protein precipitation; LLE: liquid–liquid extraction; LLQ: limit of quantification; LOD: limit of detection; LPV: lopinavir; MeOH: methanol; MS: mass spectrometry; MS/MS: tandem mass spectrometry; NaAc: sodium acetate; NaH_2PO_4: sodium phosphate monobasic; NH_4HCOO: ammonium formate; ND: no data; NFV: nelfinavir; PBMC: peripheral blood mononuclear cell; RTV: ritonavir; SPE: solid-phase extraction; SQV: saquinavir; TFA: trifluoroacetic acid; TMAP: tetramethylammonium perchlorate; UV: ultraviolet.

solution is expected to lyse the cells and then extract the drug contained within the cells [36]. Examples of used lysing solutions are hypotonic buffers and organic solvents as methanol or chloroform, eventually mixed with water [36,37]. An advantage of the use of organic solvents is that they are able to denature proteins, thereby causing drug release from the cell components. In addition, protease inhibitors are very soluble and stable in these solvents. Since the composition and volume of the lysing solution affects the lysing efficiency and recovery of the drugs, this process should be thoroughly studied.

The method of detection also influences the choice of the sample pre-treatment. Due to the selectivity of mass spectrometric (MS) detectors it was believed that extensive sample pre-treatment for LC–MS/MS assays was redundant. Although sample pre-treatment for LC–MS/MS assays does not need to be as elaborate as for LC-based assays utilizing spectrophotometric ultraviolet (UV) detection, it remains pivotal to remove matrix components that may contaminate the system or cause ion suppression, especially when high sensitivity is required [38]. With UV detection, at wavelengths close to 200 nm, the sensitivity for the quantification of protease inhibitors is increased. However, since specificity is highly decreased at these wavelengths, it is very important to remove endogenous substances co-eluting with the compound of interest. In general, liquid–liquid extraction and solid-phase extraction might be more appropriate than protein precipitation for sample pre-treatment of sanctuary sites samples since they generally yield cleaner extracts.

3.2. Chromatography

Reversed-phase or ion-pair chromatography appear to be the most appropriate HPLC methods for analysis of protease inhibitors in biological matrices as can be read from Tables 1 and 2. Most applied are C8 and C18 columns, with eluents consisting of a mixture of methanol or acetonitrile and a buffer at neutral to acidic pH (pH 3–7.5). Run times of the assays are usually dependent on the number of analytes that are measured simultaneously in a single run and on the method of detection. For LC–UV assays, complete separation of analytes is necessary, whereas for LC–MS/MS assays chromatography is mainly used to separate the analytes from matrix components whereby analytes may co-elute [39]. For assays that measure protease inhibitors in samples from sanctuary sites, less requirements are set to the analytical run time in comparison to assays used for TDM purposes. In the development of a high-throughput bioanalytical assay for TDM purposes, a short run time in combination with a quick and simple sample pre-treatment procedure are of paramount importance and sensitivity may be a secondary item. Since drug concentrations in sanctuary sites are only measured in a limited number of samples in clinical studies and in most cases not in routine daily practice, run times may be less important. Especially for assays that apply mass spectrometric detection, longer run times could yield a higher sensitivity due to reduced ion suppression [39,40]. The degree of ion suppression can also be influenced by the choice of the mobile phase composition. Polson et al. observed that the extent of ionisation varied drastically with mobile phase (−20 to 93% ion suppression) [38]. From the point of ion suppression, a methanol:aqueous 0.1% formic acid mixture was most optimal as mobile phase. When using spectrophotometric UV detection, the run time of the assay should compromise between specificity and sensitivity. The specificity usually increases with longer run times since the compound of interest is better separated from matrix constituents. However, the sensitivity may decrease since longer run times are associated with broader peaks.

3.3. Detection

Due to the requirements regarding sensitivity, specificity and selectivity, fluorescence and tandem mass spectrometry are by far preferred methods of detection for samples from sanctuary sites over UV detection. UV detection can be performed at low wavelengths to increase sensitivity, however, at the expense of specificity. All protease inhibitors have high absorbances in the lower wavelength range (200–220 nm) [9]. Anyhow, tandem mass spectrometry and fluorescence provide better sensitivity and selectivity than UV detection. Tandem mass spectrometry, in addition, is applicable to a significantly larger group of compounds than fluorescence since it does not require the presence of fluorescent groups or otherwise a derivatization procedure. Amprenavir is the only protease inhibitor that has been measured with use of fluorescence detection [32,41]. Indinavir was reported not to exhibit fluorescence [42], whereas saquinavir demonstrated only minor fluorescence [43].

Tandem mass spectrometric detection was applied only in some of the assays summarized in Tables 1 and 2 [24,36,44–46]. Single MS detection has been used for the quantification of protease inhibitors in plasma [47]. However, it is not the most suitable detection method to measure protease inhibitors in sanctuary sites because of its reduced specificity and sensitivity in comparison to MS/MS. Spectrophotometric detection at wavelengths between 210 and 260 nm has been the most applied mode of detection for protease inhibitors in the different matrices, presumably because this detection method is more readily available. Overall, the assays were sensitive with lower limits of quantification in the ng/ml range, however, sometimes with use of relatively large sample volumes. In recent years, the number of publications on the quantitative bioanalysis of protease inhibitors in plasma using LC–MS has increased tremendously. In the near future, further application of this detection method to quantify protease inhibitors in samples from sanctuary sites can be foreseen.

3.4. Validation

The fundamental parameters of a bioanalytical method validation according to the Food and Drug Administration's (FDA) guideline include accuracy, precision, selectivity, sensitivity and stability [48,49]. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The FDA guideline requires that a full validation be executed when a bioanalytical method is developed and implemented for the first time. However, when a previously validated method is modified for measurement in a different matrix (e.g. plasma → CSF), a partial validation may be sufficient. Moreover, limited sample volumes and rare matrices may necessitate and allow a partial validation, as is the case for samples from sanctuary sites. Such a partial validation should at least provide data on the accuracy and precision of the measured concentrations. In addition, sensitivity is a key parameter. The sensitivity of an assay is determined by the sample pre-treatment, chromatography and most of all the choice of detection method, as was discussed before.

When measuring antiretroviral drug concentrations in samples from sanctuary sites, it is preferred to use assays that have been developed and validated for those purposes with calibration and/or quality control samples in the specific matrix. However, the major part of presented results has been obtained with use of blood plasma assays and has been only sparsely validated. Many of the methods summarized in Table 2 have not been validated at all in this respect.

Since the quantification of protease inhibitors in blood plasma is extensively applied in clinical trials and for TDM purposes, these methods are readily available [9]. Moreover, the lack of availability of blank CSF, PBMCs and seminal plasma often precludes the preparation of calibration and quality control samples in these matrices. With minor modifications, blood plasma assays have been used to measure concentrations in CSF, PBMCs and seminal plasma. Nevertheless, these modifications have not always been clearly stated and the influence of these modifications on the results has not been investigated in detail. In many of the publications in which blood plasma samples were used for quantification, details on the sample pre-treatment in the second matrix, and to a lesser extent the lower limit of quantification (LLQ) were not specified [26,50]. The use of different matrices for clinical and calibration samples (e.g. CSF samples with a plasma calibration line) could yield problems with regard to sample recovery and matrix effects. It is pivotal to account for differences in recovery from the different matrices that might occur during the sample pre-treatment. During chromatography and detection different matrix effects may occur. For example, with tandem mass spectrometric detection, the degree of ion suppression of the analyte may differ for different matrices [51].

Therefore, it is necessary to test whether the use of applied procedures is justified. The accuracy, precision and reproducibility of the measured concentrations need to be guaranteed. Alternative procedures to demonstrate the suitability of the method may be the use of calibration samples in plasma combined with quality control samples in the special matrix [24,41], or the use of a synthetic matrix for the preparation of calibration samples, as done by Aweeka et al. [28].

The preparation of calibration and quality control samples in special matrices requires specific attention. With PBMCs, for example, spiking the protease inhibitors intracellularly is not possible. In the published methods, protease inhibitors were either spiked to a stock solution of lysed PBMCs [36] or were added to the cells followed by overnight incubation [52]. An advantage of the first method is that the drug concentration of the calibration and control samples is exactly known, whereas in the second method the intracellular drug concentration is dependent on the amount of drug that enters and retains in the cells, and on the efficiency of the washing steps. Therefore, the second method yields relative concentrations while with the first method absolute concentrations can be measured. Concentrations in PBMC-samples are expressed as amount per 10^6 cells or as amount per ml, derived from a measured volume of each PBMC of 0.4 pL [31]. Both expressions require accurate counting of the number of cells in the samples.

In general, the validation of assays specifically developed for special matrices was more elaborate than the validation of procedures derived from blood plasma assays. Most of the methods summarized in Table 1 were partly validated in the special matrix [32,41,43,53,54]. Nevertheless, only two of the methods in Table 1 were fully validated in PBMCs [36] and seminal plasma [44], respectively.

4. Conclusions

It is important to have insight into the penetration of antiretroviral drugs in sanctuary sites since HIV present in these reservoirs can lead to viral rebound, even when the viral load in plasma is undetectable. In recent years, several methods using HPLC have been published that describe the quantification of protease inhibitors in CSF, semen and PBMCs. Concentrations in these matrices have been measured with assays that were specifically developed and validated for those purposes or with assays that were validated in plasma and used with minor modifications for another matrix of interest. However, on account of the special features that apply to the quantification of protease inhibitors in these matrices it is questionable whether the use of such assays is justified.

Preferably, an assay for the quantification of protease inhibitors in a special matrix is developed and validated for its intended use in the matrix of interest. Quality control and preferably calibration samples should be prepared in the same matrix as the clinical samples. In the further development of the method, attention should be paid to the special features that apply to the matrix. The procedure for the sample preparation should be able to handle small sample volumes

and provide clean extracts. Solid-phase and liquid–liquid extractions usually are most suitable. Sensitivity is a key parameter since drug concentrations in samples from sanctuary sites may be low and sample volumes are often small. Tandem mass spectrometry may be the most suitable detection method since it provides high sensitivity and specificity and is generally applicable.

The suitability of an assay to quantify concentrations of protease inhibitors in samples from sanctuary sites should be demonstrated, at least by means of a partial validation.

In conclusion, the quantification of protease inhibitor concentrations in samples from sanctuary sites is associated with several pitfalls and particularities that should be addressed in the development of the assay. Therefore, it is incorrect to use an assay that was developed in plasma for the analysis of protease inhibitors in sanctuary site samples without proper proof (validation) that the procedure leads to adequate results. An issue as important as the penetration and retention of antiretroviral drugs in sanctuary sites requires this accurate and careful approach.

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