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Review

High-performance liquid chromatography of HIV protease inhibitors in human biological matrices

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Abstract

Methods for HPLC analysis of protease inhibitors (PIs) in human biological matrices were reviewed. Assays have been developed for analysis of single PIs or for simultaneous measurement of multiple PIs in plasma–serum, saliva, cerebrospinal fluid and semen. Liquid–liquid extraction was most often applied for sample pretreatment, but solid-phase extraction and protein precipitation were used as well. Reversed-phase or ion-pair chromatography have been used to separate PIs. Detection of PIs should be sensitive enough for quantitation of plasma concentrations below trough levels of single PIs, or below proposed therapeutic thresholds for PIs. The large majority of assays employs UV detection. As the potential for interferences is large, the selectivity of every method should be evaluated properly. The available high-performance liquid chromatography (HPLC) methods have been applied in clinical pharmacokinetic studies and for therapeutic drug monitoring of PIs. Participation in an interlaboratory quality control program is recommended for every laboratory engaged in the bioanalysis of PIs.

Keywords: Reviews; Protease inhibitors

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1. Introduction

Contemporary treatment of HIV infection and AIDS is a complex and long-term undertaking, unavoidably entailing polypharmacy. Three therapeutic classes have been developed for inhibition of viral replication: protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). In order to provide optimal efficacy and to prevent viral resistance, available antiretroviral drugs should be administered in combination regimens, which are generally referred to as highly active antiretroviral therapy (HAART). According to current guidelines, HAART should consist of two NRTIs combined with either one or two PIs, or with an NNRTI.

Especially the introduction of PIs (since 1995) has dramatically decreased mortality and morbidity in HIV infection [1]. These drugs interfere with viral replication by inhibiting the HIV protease enzyme [2,3]. This results in production of non-infectious virions and prevents consecutive infection of other cells. To date six PIs have been approved by the Food and Drug Administration (FDA): indinavir, nelfinavir, ritonavir, saquinavir and, more recently, amprenavir and lopinavir.

Since the advent of PIs there has been increasing interest in the bioanalysis of these drugs. Numerous high-performance liquid chromatographic (HPLC) assays have been published for each individual PI [4–24] and for simultaneous determination of several PIs [25–39]. This review describes the rationale for the large interest in bioanalytical methods for PIs and gives a survey of current applications of these methods (Section 2). Essential elements of published methods will be described subsequently (Section 3), followed by a more detailed evaluation of methods for simultaneous measurement of PIs (Section 4). Finally, some conclusions and future perspectives will be described (Section 5).

Published HPLC methods were retrieved using the Medline database (January 1994 to July 2001, keywords ‘‘HPLC’’ or ‘‘high-performance liquid chromatography’’, combined with the names of the individual approved PIs) or Analytical Abstracts (using names of the individual PIs as keywords). Methods cited from these articles were also checked. Only papers written in English and with a full, detailed description (including method validation results) of an HPLC method were included. Methods briefly described in reports of pharmacokinetic studies or in abstracts were therefore not considered. Furthermore, the review confined to methods intended for analysis of PIs (not their metabolites) in human plasma–serum, urine, saliva, cerebrospinal fluid (CSF) and semen. However, a few articles that did not meet the criteria were cited often and contained valuable information; accordingly these articles were included as well [40,41].

2. Application of HPLC analysis of protease inhibitors in pharmacokinetics and therapeutic drug monitoring

Development of new drugs, such as the PIs, inevitably leads to interest in the bioanalysis of the

compounds involved, as drug development traditionally includes various stages that require analytical input. Moreover, research into clinical pharmacology (especially clinical pharmacokinetics) of PIs typically extends beyond the formal approval of these drugs. This may be explained by the accelerated FDA approval conditions for these drugs, which may have called for additional research, but is certainly due to the suboptimal response to PI-based regimens as well. Despite the remarkable antiviral potency of PIs, only about 50% of patients commencing treatment will achieve and maintain adequate antiviral response in the long term. The unfavorable and variable pharmacokinetics of PIs and their large potential for drug interactions are to a large extent responsible for this heterogeneity in antiviral response. Therefore several types of clinical pharmacokinetic studies have been set up in the last years. Accurate and sensitive analytical methods are a prerequisite for all these studies:

2.1. Description of plasma pharmacokinetics of PIs

Many studies have concerned the description of the pharmacokinetics of PIs and combinations of PIs in various populations. These studies have complemented data from the pharmaceutical industry and have shown the unfavorable pharmacokinetics of PIs in regards to their poor and variable bioavailability and short elimination half-lives. Poor bioavailability necessitates food restrictions for intake of most PIs, whereas short half-lives result in inconvenient twice or thrice daily dose regimens. Both may negatively influence compliance to treatment regimens and cause inadequate exposure to the drugs.

2.2. Evaluation of drug interactions

Apart from variable bioavailability and fast elimination, drug interactions are of major concern when PIs are being prescribed. The drugs are primarily metabolized by CYP3A, one of the cytochrome P450 iso-enzymes, and therefore interact with other drugs that inhibit or induce this enzyme. Furthermore, PIs also have capacity to inhibit CYP3A themselves. Especially ritonavir is a potent inhibitor of CYP3A and interacts with a long list of other drugs. The large interaction potential of PIs has been established in numerous drug interaction studies [42].

2.3. Evaluation of PI-based regimens with a better pharmacokinetic profile

New PI-based regimens are being evaluated in order to obtain combinations with pharmacokinetics that are more favorable. More specifically, interactions between PIs are being exploited to overcome the pharmacokinetic shortcomings of PIs as single agents. Combination of low doses of ritonavir with other PIs often leads to higher drug levels of the latter PIs (''pharmacokinetic enhancement''), better bioavailability and a reduction in dose and dose frequency [43–45]. Other pharmacokinetic studies aim at developing once daily PI dosing regimens, again by exploiting interactions with ritonavir [46–48].

2.4. Drug-transporting proteins and sanctuary sites

PIs are to varying degrees substrates for drug-transporting proteins P-glycoprotein and MRP. Affinity for these proteins may prevent penetration of the PIs in some body compartments, such as the central nervous system and semen. As a result, these body compartments could harbor reservoirs of poorly tractable HIV, and are therefore designated as sanctuary sites for HIV [49,50]. Pharmacokinetic studies are being undertaken to study the penetration and retention of PIs in CSF [51] and semen [52] and in other putative sanctuaries. These studies require assays that are validated for these purposes.

2.5. Studies relating pharmacokinetic parameters to observed clinical effect

Over the past 4 years data have emerged demonstrating an important link between PI drug concentrations and efficacy or toxicity of these drugs [53]. As a result, the issue of therapeutic drug monitoring (TDM) for PIs has risen [54]. PIs appear to be appropriate candidates for TDM indeed, as there is also large interindividual variability in their plasma pharmacokinetics, a narrow range between therapeutic and toxic drug concentrations, and no direct measure for the pharmacological effect of PIs applied in combination therapy. However, therapeu-

tic ranges or target values have not been defined unequivocally. Clinical trials have been started to validate such target values and to assess the value of TDM for PIs. So far, only preliminary results of these studies have been presented [55,56]. Nevertheless, TDM for PIs has already been applied and four indications for TDM have evolved from practice. It can be used (1) to prevent treatment failure, (2) to explain or prevent drug toxicity, (3) to manage drug interactions and (4) to document non-compliance to medication schedules. It appears that nonadherent patients can be identified using drug level measurements of PIs in plasma [57,58] or saliva [59,60].

3. HPLC analysis of protease inhibitors: essential elements

3.1. Physico-chemical and pharmacokinetic properties of protease inhibitors

Physico-chemical properties and pharmacokinetic parameters of PIs are valuable clues for the choice of conditions for HPLC separation, as well as for evaluating existing methods. Although the PIs are pharmacologically related, they differ structurally (Fig. 1). PIs are compounds of medium polarity with weak basic properties due to ionizable substituent groups. Table 1 summarizes solubility data, UV absorption maxima and pK_a values, as far as these data could be retrieved [34,35,61–65]. Complete UV spectra of PIs have been depicted in the literature [34,35]. These spectra show that all PIs have high absorbances in the lower wavelength range (200–220 nm). For indinavir, lopinavir, ritonavir and nelfinavir, absorbances in this range are significantly higher than their respective peak absorbances at higher wavelengths. For amprenavir and saquinavir, absorptivities at their maxima approximate those at lower wavelengths [34,35]. The aqueous solubilities of indinavir and nelfinavir are strongly dependent on pH. At pH values above 3.5 these drugs show a sharp decline in solubility [61,64]. Amprenavir, indinavir, nelfinavir, ritonavir and saquinavir appear to be very stable drugs [27]. Whole blood or plasma samples containing these drugs can be kept at room temperature for several days and at −20°C for several months. Repeated freeze–thaw cycles do not affect the stability of the drugs. Stock solutions of these drugs in methanol are stable at −20°C for several months. No such stability data have been published for lopinavir so far, but our own experience with this drug indicates that its stability is comparable to other PIs.

All PI plasma concentrations are expressed as the free base. Knowledge of the concentration range of PIs is important for an estimation of the required upper and lower limits of quantitation for their

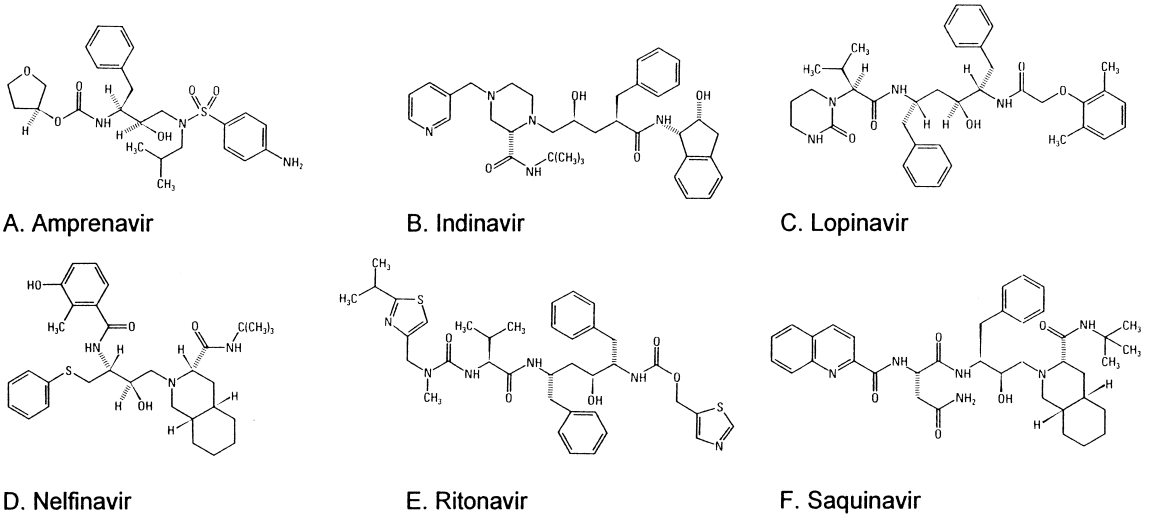

Fig. 1. Chemical structures of amprenavir (A), indinavir (B), lopinavir (C), nelfinavir (D), ritonavir (E) and saquinavir (F).

Table 1
Physico-chemical properties of protease inhibitors[a]

Protease inhibitor	Solubility in water	UV max (nm)	pK_a value	Ref.
Amprenavir	na	265	na	[34,35]
Indinavir	100 mg/ml (sulfate) 60 mg/ml (pH 3.5) 0.3 mg/ml (pH 4.8)	260	3.7	[61,62]
Lopinavir	Practically insoluble	259[b]	na	[63]
Nelfinavir	4.5 mg/ml (mesylate)	252	6.00, 11.06	[62,64]
Ritonavir	Practically insoluble	239	na	[62]
Saquinavir	2.2 mg/ml (mesylate)	239	7.01	[62,65]

[a] See Nomenclature for abbreviations.
[b] Recorded in ACN–phosphate buffer (40:60, v/v).

measurement (see Table 2, [63,66–76]). It should be noted that large variability exists in minimum (trough) and maximum concentrations of PIs in plasma. Furthermore, these concentrations can increase when PIs are combined with ritonavir. PIs exhibit strong (≥90%) binding to plasma proteins, except for indinavir which is about 60% bound to proteins. All PIs are extensively metabolized to numerous metabolites. The major metabolite of nelfinavir (M8) shows in vitro activity and in vivo protein binding comparable to nelfinavir [70,77]. M8 concentrations are roughly about 30% of nelfinavir concentrations [78].

3.2. Protease inhibitor analysis in biological matrices

Bio-analytical assays for PIs have almost exclusively been developed and validated for plasma and serum samples. However, some HPLC methods have been published for analysis of these drugs in other biological fluids that are of interest for clinical pharmacokinetic studies, or TDM; urine, saliva, CSF and seminal fluid [4,6,13,14,19,23,40]. From an analytical point of view these matrices are relatively free of interferences compared to plasma. Authors reporting analytical methods in these fluids do not seem to have experienced large problems in applying or adapting existing methods for measurement of PIs in plasma or serum, provided that these methods are sensitive enough.

3.2.1. Analysis in urine

Analysis in urine may be particularly relevant for indinavir, which is excreted in urine for up to 20% (much more than other PIs), and causes urological complaints by crystallization of indinavir in the urinary tract. Since urine is generally free of protein

Table 2
Concentration range and proposed therapeutic thresholds of protease inhibitors[a,b]

Protease inhibitor	Proposed plasma threshold (ng/ml)	Steady-state C_{min} concentration (ng/ml)	Steady-state C_{max} concentration (ng/ml)	Refs.
Amprenavir	na	280	5360	[66]
Indinavir	100–110	130	6840	[67–69]
Lopinavir/ritonavir	na	5500	9600	[63]
Nelfinavir	0.25–0.45 or 0.77	1000[c], 700[d]	3000[c], 4000[d]	[70–72]
Ritonavir	2100	4000	11000	[73,74]
Saquinavir (HGC)	50	38	198	[75,76]
Saquinavir (SGC)	50	70	2181	[75,76]

[a] See Nomenclature for abbreviations.
[b] All PIs dosed without ritonavir, except for lopinavir which is coformulated with ritonavir.
[c] Regimen: 750 mg three times daily.
[d] Regimen: 1250 mg two times daily.

and lipids we ourselves just acidify urine samples containing indinavir to a pH below 3.5 with orthophosphoric acid, in order to dissolve indinavir that may have precipitated. After centrifugation, urine is then diluted and injected in the HPLC system. Recovery using this methodology is 101%. However, Woolf et al. [6] and Svensson et al. [14] describe a more extensive sample pretreatment method for analysis of indinavir in urine, using the same liquid–liquid extraction procedures and separation conditions for urine and plasma or serum. In the assay by Woolf, recovery of indinavir from urine was less (68%) compared to plasma (81%), but it remained constant over the range of the standard curve.

Interestingly, Woolf et al. also developed another method for analysis of indinavir in plasma, consisting of liquid–liquid extraction followed by HPLC and tandem mass spectrometric (MS) detection with a turbo ion spray interface ([7], see also Table 3). They attempted to apply this plasma method for measurement of indinavir in urine as well, omitting the liquid–liquid extraction step [40]. In theory, the highly specific nature of LC–MS should allow for minimal sample clean up (just dilution) and short chromatographic analysis times. However, after dilution and injection of urine samples, a high degree of variability in MS/MS responses was observed. Both sample clean up and better chromatographic separation (increasing k′) improved the instrument response and reproducibility of ionization, thus potentially improving sensitivity and precision of the method. These findings illustrate that analysis of urine with MS detection may require sample pretreatment and adequate separation of analytes from co-eluting species that are unseen by the detector.

3.2.2. Analysis in saliva

Analysis of PIs in saliva has been studied as an alternative for plasma or serum in TDM, as use of saliva offers several advantages (e.g., easy and non-invasive sample collection, and diminished risk of HIV transmission). Hoetelmans et al. used a special device (Salivette®, a cotton wool swab impregnated with citric acid) as a standardized method to collect stimulated saliva for the measurement of ritonavir and saquinavir [19,23]. They applied the same sample pretreatment as for plasma samples. Only very low concentrations of ritonavir and saquinavir were measured in saliva samples from HIV-infected patients who took these PIs. This is probably due to extensive protein binding of these PIs in plasma, which restricts the amount of drug that can diffuse into saliva. Based on protein binding data, only indinavir can be expected in saliva (see Section 3.1). Hugen et al. analysed indinavir in stimulated saliva using the same separation conditions as for plasma samples [8,59]. Adsorption of indinavir to the Salivette was 40%. Salivary indinavir concentrations correlated well with plasma levels, but a large intra- and inter-individual variation in saliva–plasma concentration ratios was found. It was concluded that salivary indinavir concentrations can not be used to predict plasma concentrations, but may be applied for monitoring of compliance. Wintergerst et al. also found good agreement between indinavir concentrations in plasma and (unstimulated) saliva, particularly at the end of the dose interval [60]. Saliva and plasma samples were analysed using the same LC–MS/MS assay.

3.2.3. Analysis in CSF and semen

Analysis of PIs in CSF and semen requires higher sensitivity than measurement of PIs in plasma. Limits of quantitation for plasma assays are often in the 10–50 ng/ml range, but drug levels behind blood–brain and blood–testes barriers can be significantly lower. Furthermore, only small volumes of CSF are mostly available.

Sparidans et al. extracted amprenavir from small samples (100 μl) of CSF or semen using liquid–liquid extraction [4]. Recovery of amprenavir was more than 95%. Fluorescence detection enabled measurement of amprenavir in the low nanogram range (see Table 3). In order to facilitate the use of calibration samples in plasma for measurement of CSF and semen samples, plasma was added to these matrices prior to further treatment.

Zhong et al. report a method for measurement of indinavir in both plasma and CSF samples (see Table 3, [13]). Recovery of indinavir from CSF was more than 90% and the lower limit for quantitation of indinavir in CSF was 2 ng/ml. Likewise, Svensson et al. [14] developed a method that can be applied for both serum and CSF, as well as urine.

The methods reported by Hoetelmans et al. for

Table 3
Summary of published HPLC methods: measurement of single PIs[a]

PIs[b]	Matrix	Sample pretreatment	Column	Mobile phase	Run time[c]	Limit of quantitation[d]	Detection[e]	Refs.
APV	Plasma, CSF, semen	LLE chloroform	C_{18} (100×4.6 mm, 3.5 μm) 50°C	Isocratic 25 m*M* sodium phosphate buffer pH 6.8–ACN (60:40 v/v) 1.5 ml/min	18.5	1 (plasma semen) 0.5 (CSF)	FL Ex 270 nm Em 340 nm	[4]
APV	Serum, plasma	LLE at basic pH diethylether	C_{18} (50×2.0 mm, 3 μm)	Isocratic ACN–water (1:1, v/v)+0.1% formic acid 0.15 ml/min	<5	50	MS/MS	[5]
IDV	Plasma, urine	LLE at pH 9.5 MTBE Backextr. in 10 m*M* HCl Re-extr. MTBE pH 9.5	Column switching I: cyano (80×4 mm, 5 μm) II: C_{18} (150×4.6 mm, 5 μm)	Isocratic I: ACN–water (34:66 v/v) II: ACN–water (38:62 v/v) Both in 10 m*M* orthophosphoric acid, pH 7.5 1.2 ml/min	20	5	UV 210	[6]
IDV	Plasma	LLE at pH 9.5 MTBE	C_8 (50×2 mm, 3 μm)	Isocratic ACN–water (40:60 v/v) +7 m*M* ammonium acetate, pH 4.9, 0.2 ml/min	6	1 (at least)	MS/MS	[7]
IDV	Plasma	Protein precipitation ACN	C_{18} (150×4.6 mm, 5 μm)	Isocratic ACN:50 m*M* phosphate buffer pH 6+4 g/l TMACl (40:60 v/v) 1 ml/min	12	50	UV 210	[8]
IDV	Plasma	LLE at pH 9.0 diethylether	C_4 (150×3.9 mm, 5 μm)	Isocratic 10 m*M* $NH_4H_2PO_4$+1 m*M* 1-heptanesulfonic acid sodium pH 4.8–ACN (65:35 v/v) 0.6 ml/min	30	10	UV 210	[9]
IDV	Urine	Dilution with ACN ACN *or* LLE at pH 9.5 MTBE	C_8 (50×2.0 mm, 3 μm)	Isocratic ACN–7 m*M* ammonium acetate (40:60 v/v), pH 4.9 *Or* Isocratic ACN–7 m*M* ammonium acetate (30:70 v/v), pH 4.9 Both 0.2 ml/min	6 12	na	MS/MS	[40]
IDV	Plasma	SPE (C_{18})	C_8 (250×4.6 mm, 5 μm)	Isocratic ACN–10 m*M* KH_2PO_4 pH 3.1 (40:60 v/v) 1.5 ml/min	19	25	UV 210	[10]
IDV	Plasma	LLE pH 10.4 Dichloro-methane Hexane wash	C_{18} (150×4.6 mm, 5 μm)	Isocratic ACN–25 m*M* phosphate buffer+0.2% triethylamine in water, pH 7 (34.5:65.5 v/v) 2 ml/min	15	25	UV 210	[11]

Table 3. Continued

PIs[b]	Matrix	Sample pretreatment	Column	Mobile phase	Run time[c]	Limit of quantitation[d]	Detection[e]	Refs.
IDV	Plasma	SPE (Oasis® HLB)	C_8 (150×4.6 mm, 5 μm)	Isocratic Water–ACN–5.9 *M* orthophosphoric acid–triethylamine (73:27:0.5:0.02 v/v), pH 3.2 0.8 ml/min	12	10	UV 210	[12]
IDV	Plasma CSF	SPE (strong cation-exchange, SCX benzene sulfonic acid)	Column switching I: cyano (80×4 mm, 5 μm) II: C_{18} (150×4.6 mm, 5 μm) Columns at 28°C (see [6])	Isocratic I: ACN–water (34:66 v/v) II: ACN–water (38:62 v/v) Both in 10 m*M* orthophosphoric acid, pH 7.5 1.2 ml/min	20	5 (plasma) 2 (CSF)	UV 210	[13]
IDV	Cell culture	Protein precipitation (ACN)	C_{18} (250×3 mm, 5 μm)	Isocratic 10 m*M* NaH_2PO_4 pH 6.3: ACN (65:35 v/v) 0.6 ml/min	19	4	ED First el.:+400 mV Second el.: +750 mV	[41]
IDV	Serum, urine, CSF	LLE at basic pH Diethylether Back extr. in acid aqeous phase	C_{18} (75×4.6, 3.5 μm)	Isocratic 50 mmol/l acetic acid buffer (pH 4.8)–ACN (52:48 v/v) 1.5 ml/min	3.5	na (LOD: 6 ng/ml)	UV 260	[14]
IDV	Plasma	96-well SPE (mixed phase cation-exchange)	C_{18} (30×3.0 mm, 3 μm) 35°C	Isocratic ACN–10 m*M* ammonium acetate+TFA 0.5 ml/l (42.5:57.5 v/v) 0.6 ml/min	Very short (high throughput)	1	MS/MS	[15]
NFV	Plasma	LLE at pH 10 Ethyl acetate-ACN (90:10 v/v)	C_{18} (250×4.6 mm, 5 μm)	Isocratic 25 m*M* monobasic sodium phosphate buffer pH 3.4–ACN (58:42 v/v) 1.3 ml/min	12	50	UV 220	[16]
NFV, M8	Plasma	LLE at pH 9.5 MTBE–hexane (90:10) Hexane wash	C_{18} (250×4.6 mm, 5 μm)	Isocratic 0.1% TFA–ACN–MeOH (49:46:5 v/v), pH 5 1.5 ml/min	15	25 (NFV, M8)	UV 220	[17]
RTV	Plasma	LLE Ethyl acetate–hexane (9:1 v/v) Hexane wash	C_{18} (50×4 mm or 50×4.6 mm, 3 μm)	Isocratic ACN–MeOH–0.01 *M* TMAP in 0.1% aqueous TFA (40:5:55 v/v) 1 ml/min	15	12	UV 205	[18]
RTV	Plasma, saliva, CSF	Protein precipitation (ACN)	C_{18} (75×4.6 mm, 3.5 μm)	Isocratic ACN:25 m*M* sodium acetate +25 m*M* hexane-1-sulfonic acid, pH 4 (44:56 v/v) 1 ml/min	20	50	UV 239	[19]

Table 3. Continued

PIs[b]	Matrix	Sample pretreatment	Column	Mobile phase	Run time[c]	Limit of quantitation[d]	Detection[e]	Refs.
RTV	Plasma	LLE Ethyl acetate–hexane (9:1 v/v) Hexane wash	C_{18} (300×3.9 mm, 10 μm)	Isocratic ACN–0.05 *M* monobasic ammonium phosphate (pH 3) (52.5:47.5 v/v) 2.0 ml/min	12	na	UV 210	[20]
SQV	Plasma	SPE (C_2)	ODS (20) guard column (30×4.6 mm, 5 μm)	Isocratic Aqueous ACN (80:20 v/v) with 0.0025 *M* ammonium acetate pH 6.5 1.5 ml/min	1.5	0.4	MS/MS	[21]
SQV	Plasma	Hexane wash, LLE with diethylether	C_8 (125×3 mm) 45°C	Isocratic 5 m*M* sulfuric acid–ACN (75.5:24.5 v/v) containing 10 m*M* TBA pH 3.5 1 ml/min	10	10 (at least)	UV 240	[22]
SQV	Plasma, saliva, CSF	SPE (C_2)	C_{18} (75×4.6 mm, 3.5 μm)	Isocratic ACN–25 m*M* sodium acetate +25 m*M* hexane-1-sulfonic acid, pH 4 (40.5:59.5 v/v) 1 ml/min	35	2.5	UV 239	[23]
SQV	Plasma	Protein precipitation (monochloracetic acid) SPE (C_8)	Phenyl (4 μm)	Isocratic methanol–0.01 *M* ammonium acetate–glacial acetic acid (90:9.75:0.25) 2 ml/min	15	1.0	UV 239?	[24]
SQV	Plasma	LLE at basic pH diethyl ether Hexane wash	C_8 (250×4.6 mm, 5 μm)	Isocratic aqueous ACN (37:63) 1 ml/min	30	20 (at least)	UV 239?	[24]

[a] See Nomenclature for abbreviations.
[b] References listed by PI, then chronologically.
[c] Run time in minutes.
[d] Limit of quantitation in ng/ml.
[e] Wavelength of detection in nm.

measurement of ritonavir and saquinavir in plasma and saliva can also be applied to CSF samples [19,23]. Recoveries from CSF were 99–101% for ritonavir and 60–61% for saquinavir.

3.3. Heat treatment to inactivate HIV

Samples from HIV-infected persons obviously pose a health hazard. Chemical treatments can be used to inactivate HIV, but such treatments may influence the HPLC analysis, or degrade the PIs to be measured. For example, Marzolini et al. evaluated viral inactivation by Triton X-100, but this detergent perturbed UV detection at 201 nm and influenced the peak shape of the PIs [33].

Heat treatment is another effective means for deactivation of HIV. Deactivation has been performed at 56–60°C, using a variety of heat treatment durations (from 30 min to 4 h). Somewhat conflicting data have been reported with regard to stability of PIs under these circumstances. Whereas several authors have assessed that 30 min to 1 h at 56°C or

60°C did not affect concentrations of amprenavir [4,32,33,37], indinavir [12,15,28,32,33,37], nelfinavir [28,32,33,37], ritonavir [19,28,32,33,37] and saquinavir [28,32,33,37], others have found slight degradation (less than 15%) for indinavir [9] and ritonavir [18], larger decreases for saquinavir after 2 h at 60°C (mean decrease 18% [24]), and even an increase in indinavir concentrations after 4 h at 58°C [10]. Therefore, heat treatment may have a slight affect on PI concentrations. This implicates that calibration and quality control samples should be heat treated as well, or that heat treatment should be avoided.

If sample pretreatment consists of an extraction step using organic solvents, this may be sufficient to deactivate biological hazards such as HIV [79].

Irradiation has been suggested as an approach for deactivation of HIV in biological samples [79], but this methodology has not been applied in any of the described analytical methods.

3.4. Sample pretreatment

Sample pretreatments that have been applied for analysis of PIs in liquid biological matrices include protein precipitation, liquid–liquid extraction and solid-phase extraction.

3.4.1. Protein precipitation

Protein precipitation reagents used in the analysis of PIs are acetonitrile and monochloracetic acid. Protein precipitation with acetonitrile has been used as the sole sample pretreatment method in the analysis of indinavir in plasma [8] and cell cultures [41] and in the analysis of ritonavir in plasma [19]. Monochloracetic acid has been used as a prelude to solid-phase extraction of saquinavir from plasma [24].

3.4.2. Liquid–liquid extraction

Liquid–liquid extraction of PIs has been used in the majority (56%) of the assays, both for extraction of single PIs as well as for simultaneous extraction of several PIs. Most extractions were performed in one single step. Before extraction, samples have been alkalinized, thereby allowing PIs to exist in an uncharged form, being more readily extracted by organic solvents. Using this methodology, PIs have been extracted using methyl *tert.*-butyl ether (MTBE) [6,7,27,29–31,36,40], MTBE–hexane [17], diethylether [5,9,14,22,24,26], ethylacetate–hexane [18,20,28,38], ethylacetate–acetonitrile [16], chloroform [4] or dichloromethane [11]. MTBE and diethylether have been used most often. As a consequence of their low densities, these solvents can be easily collected after extraction, as the upper layer in a tube. Freezing the lower aqueous layer in a dry ice–acetone bath may facilitate collection of the organic solvent. The solvents are then evaporated to dryness and the residue is reconstituted for injection in the chromatographic system [5,7,9,29,31,40]. Alternatively, the reconstituted aqueous phase can be washed with hexane if lipophilic co-elutants have to be removed or if quantitation in the lower range is desired [17,22,24,26,27,30,36]. Hexane washing has been applied in the same way after extraction with other solvents [11,18,20,38]. As an alternative to hexane washing, the extraction step into MTBE or another solvent may be followed by back extraction of PIs into acid [14], if necessary with subsequent pH adjustment to high values and re-extraction into MTBE. Woolf et al. demonstrated the latter re-extraction strategy for indinavir [6]. However, this procedure resulted in low recoveries of nelfinavir and M8 compared to washing with hexane [17].

3.4.3. Solid-phase extraction

Solid-phase extraction of PIs was first applied by Knebel et al., who extracted saquinavir on C_2 solid-phase cartridges, obtaining more than 95% recovery [21]. Hoetelmans et al. modified this procedure slightly and applied it for extraction of saquinavir [23] and for simultaneous extraction of five PIs [32].

C_8 columns have been used for extraction of saquinavir [24], whereas C_{18} cartridges have been applied for extraction of indinavir [10] and for simultaneous isolation of multiple PIs [25,33,35]. Using C_{18} columns, Marzolini et al. [33] and Simon et al. [39] extracted PIs together with NNRTIs, and Aymard et al. used C_{18} cartridges to isolate PIs, NNRTIs as well as NRTIs from one single plasma sample [35].

Oasis® HLB cartridges (Waters) have been applied to extract indinavir alone [12] or five PIs simultaneously [34,37]. Poirier et al. chose this polymeric sorbent because its hydrophilic properties prevent the wettability problem encountered with C_{18} packings,

and because reproducible results can be obtained even when the cartridges run dry [12,34].

The potential for separation of PIs based on cation-exchange is suggested by the presence of nitrogen in multiple functional groups in the PI molecules. Zhong tested cartridges with weak cation-exchange functional groups (carboxylic acid) for extraction of indinavir, but strong cation-exchange functional groups (benzenesulfonic acid) showed the best separation and recovery [13].

Rose et al. developed an assay for high throughput analysis of indinavir in plasma, using semi-automated 96-well solid-phase extraction in the mixed phase cation-exchange format (MPC), in conjunction with LC–MS/MS [15]. This allowed for analysis of 288 samples (three 96-well plates) in one overnight run.

3.5. Separation conditions

Reversed-phase or ion-pair chromatography appear to be the most appropriate HPLC methods for analysis of ionizable drugs such as PIs in an aqueous biological matrix. Separation conditions described in most publications are fairly straightforward, derived from initial conditions that have been proposed for systematic HPLC method development [80].

Therefore frequently chosen stationary phases were C_8 or C_{18}, whereas other columns (C_4 [9,29], cyano [6,13], phenyl [24,30]) have been applied occasionally. Woolf et al. [6] and Zhong et al. [13] used a column switching system for measurement of indinavir in plasma and urine [6] or plasma and CSF [13]. The column switching configuration was designed to separate indinavir from endogenous interferences. By combining the different selectivities provided by the first (cyano) and second (C_{18}) column, the analytes could be detected under interference-free conditions at 210 nm.

Some authors have thermostatted column temperature above ambient temperature, apparently in order to influence selectivity, to reduce the variation in retention times, or to improve peak efficiency [4,13,15,22,29,34,35,39].

Mobile phase frequently consisted of acetonitrile and a buffer, most often a phosphate buffer. Because of the ionic character of PIs, buffering the aqueous phase of the solvent is imperative. When the mobile phase pH is close to the pK_a values of one of the PIs, small pH changes can have a major impact on band spacing. Variation in mobile phase pH is thus a powerful way to influence selectivity when separating PIs. However, the exact pH conditions that favor maximum resolution of PIs may not favor method ruggedness. Several authors stress the exact setting of mobile phase pH for adequate and reproducible separation [17,23,27,30,33].

Besides changes in pH, several other mobile phase characteristics have been varied in order to optimize band spacing and peak shape. Apart from changes in solvent type selectivity, several authors have used additives (diethylamine, triethylamine, trifluoracetic acid) in the mobile phase, presumably to improve the peak shape of the basic PIs (less tailing) or to act as weak ion-pairing reagents [11,12,15,17,18,31,34]. Furthermore, 10 methods describe the addition of strong ion-pair reagents to the mobile phase as an additional way to vary band spacing [8,9,18, 19,22,23,32,33,35,38]. As mobile phases were generally slightly acidic, alkylsulfonates have been applied to provide retention of the basic PIs in their protonated form [9,19,23,32,33,35]. Tetra-alkylammonium salts have been used in slightly acidic mobile phases to avoid tailing [8], probably by blocking silanols, or to obtain a clean baseline by retention of negatively charged interferences [22].

All HPLC methods for single PI assay involve isocratic separation conditions. If isocratic conditions are applied for simultaneous chromatography of several PIs, this may result in a wide retention range of the drugs. This may be reflected in inadequate resolution of the most polar PIs from early eluting interferences on the one hand, and peak broadening of the late-eluting (less polar) PIs on the other. Such problems may be present in some of the methods for simultaneous measurement of PIs [31,32]. Gradient elution has been applied in seven out of 15 methods for simultaneous measurement of PIs [26,27,33, 34,37–39].

Figs. 2–5 show typical separations that have been obtained in methods for simultaneous analysis of PIs.

3.6. Sensitivity

Most of the HPLC methods for PIs have been developed for application in pharmacokinetic studies and TDM. Pharmacokinetic studies require lower limits of quantitation below expected trough levels of

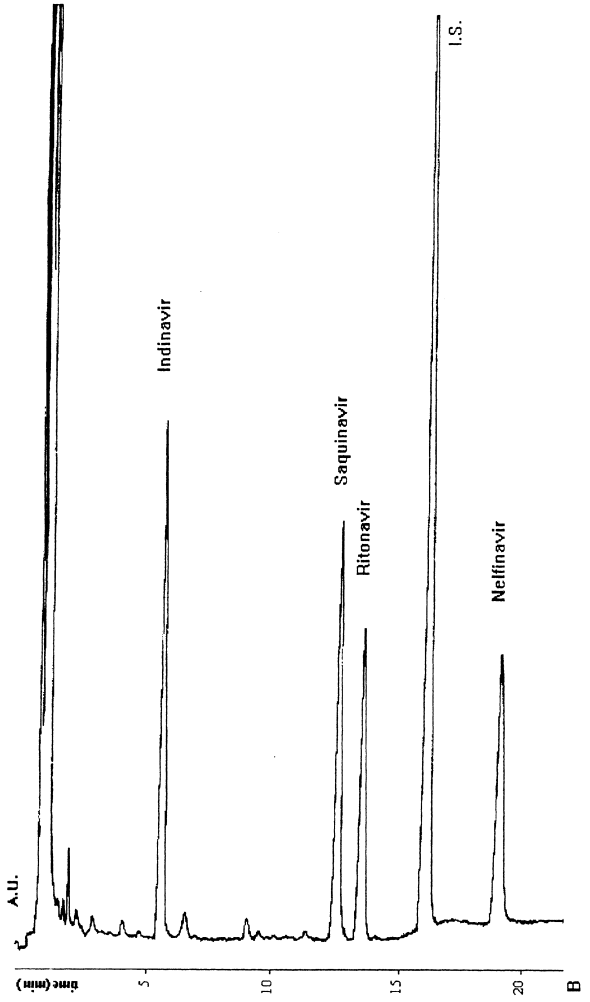

Fig. 2. Typical chromatogram of a spiked plasma sample containing 1050 ng/ml of indinavir, nelfinavir, ritonavir, and saquinavir, and internal standard (IS) (from Hugen et al. [27]).

PIs, in order to be able to accurately calculate important pharmacokinetic data (e.g., half-life) from the terminal phases in drug elimination. TDM demands a similar or better sensitivity; limits of quantitation should be below population trough levels for single PIs, or preferably below threshold values that have been proposed for PIs (Table 2). Data in Table 2 refer to trough levels after administration of PIs as single agents. Co-administration of two PIs results in higher plasma levels and is becoming increasingly popular. Analytical methods that have been validated to measure such higher drug levels only (thus being less sensitive) have restricted applicability, as many patients still take single PIs.

Clearly, trough levels for saquinavir are relatively low compared to other PIs (Table 2), whereas ritonavir trough and threshold levels are in the μg/ml range. Therefore, ritonavir will not pose sensitivity problems. Moreover, quantitation of this drug is not indicated when it is applied in low doses, as pharmacokinetic enhancer for other PIs (which is most often the case nowadays).

There is a large variation amongst assays in lower limits of quantitation for measurement of PIs (see Tables 3 and 4). A number of assays are certainly not sensitive enough to measure concentrations below population trough levels or below proposed threshold limits (Table 2).

3.7. Selectivity

The selectivity of the methods for PI assay is another major item in method validation. Apart from endogenous substances, the potential for drug interferences is enormous, due to the large number of co-administered drugs used by HIV-infected patients, as well as the formation of a large number of PI metabolites. Interferences are especially troublesome to the development of methods for simultaneous analysis of PIs. To assure selectivity, many authors have analyzed several samples of blank plasma. Furthermore, co-administered drugs have been tested for interference. The number of drugs tested varied from antiretroviral drugs only to a tremendous amount of co-medications. Metabolites have generally not been available so far and their influence has been evaluated in a small number of studies only, by analysis of samples from treated subjects. Some authors apply peak purity techniques built into diode array UV systems to show that the spectra of peaks are consistent [12,17,34,38]. It should be noted that methods developed before 1998 (mainly for measurement of single PIs) have not been evaluated for interference by new antiretroviral compounds.

3.8. Detection

Detector type and operation can affect the response of PIs and interferences, thus influencing both sensitivity and selectivity. Concerning sensitivity,

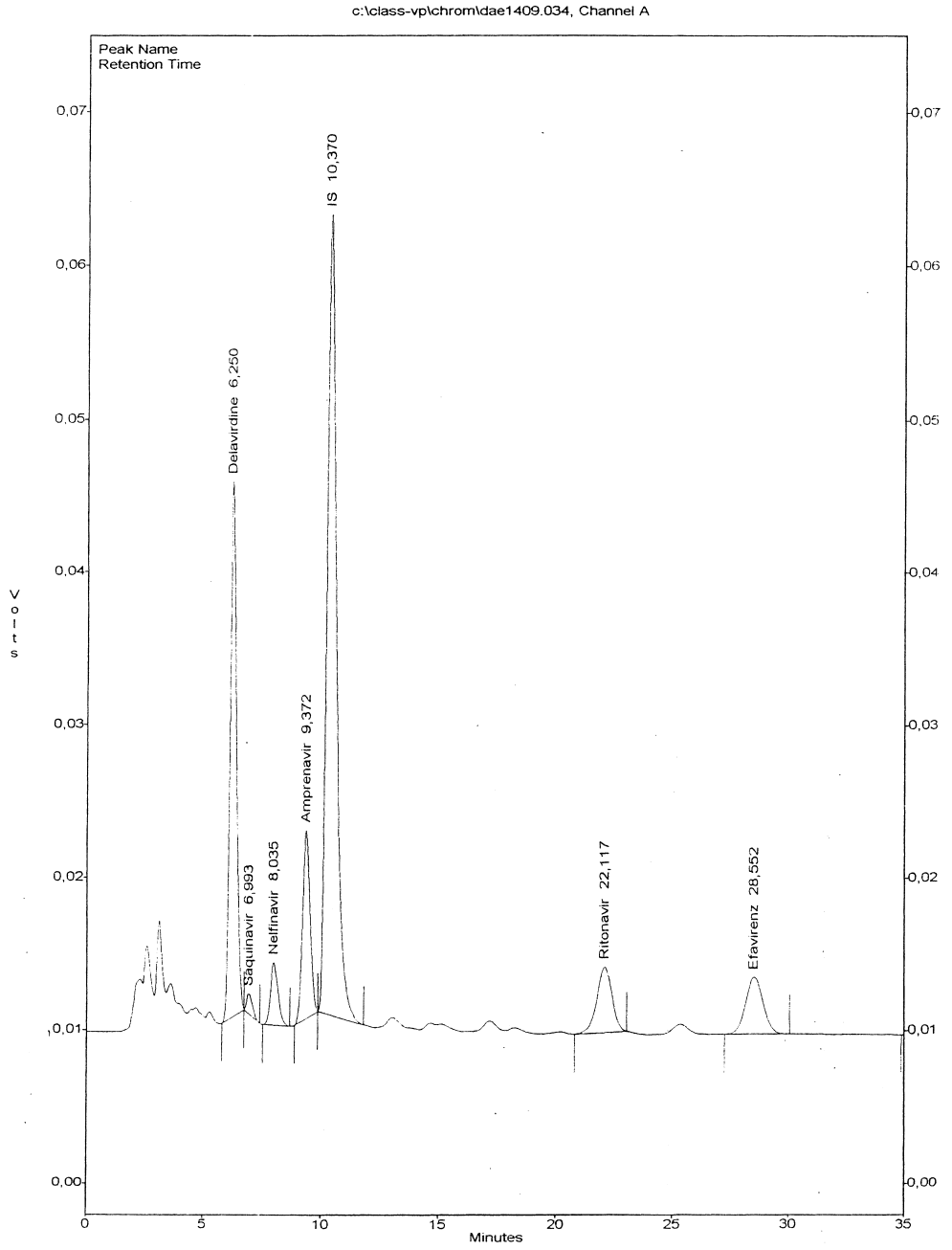

Fig. 3. Typical chromatogram of a spiked plasma sample containing four PIs, delavirdine, efavirenz and internal standard (from Proust et al. [31]).

molar absorptivities of PIs appear to be sufficiently high to meet the sensitivity requirements for measurement of PIs in plasma. Therefore the large majority of methods use UV-detection. In order to obtain maximum sensitivity, many authors have chosen the lower wavelength range for detection of

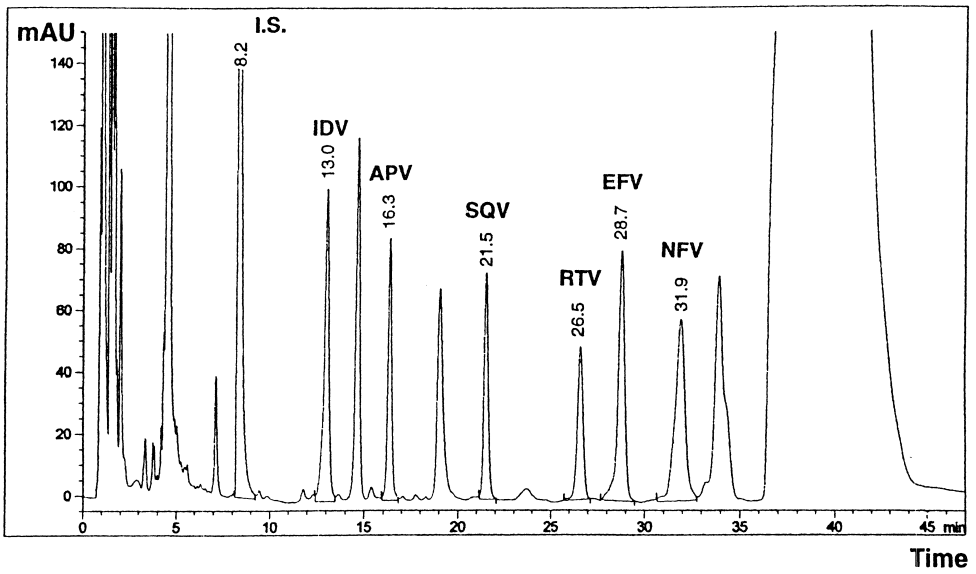

Fig. 4. Typical chromatogram of a spiked plasma sample containing 3000 ng/ml of PIs, efavirenz and internal standard (IS) (from Marzolini et al. [33]).

single or multiple PIs. As a consequence of increased sensitivity, this may also permit the use of small plasma volumes. However, low wavelengths are rather non-specific and many endogenous interferences or drugs will absorb in this region. Accordingly, detection at lower wavelengths demands careful investigation of selectivity. As an alternative, PIs can be measured at higher wavelengths in order to minimize interference rather than maximize response. For example, Poirier et al. measured four PIs

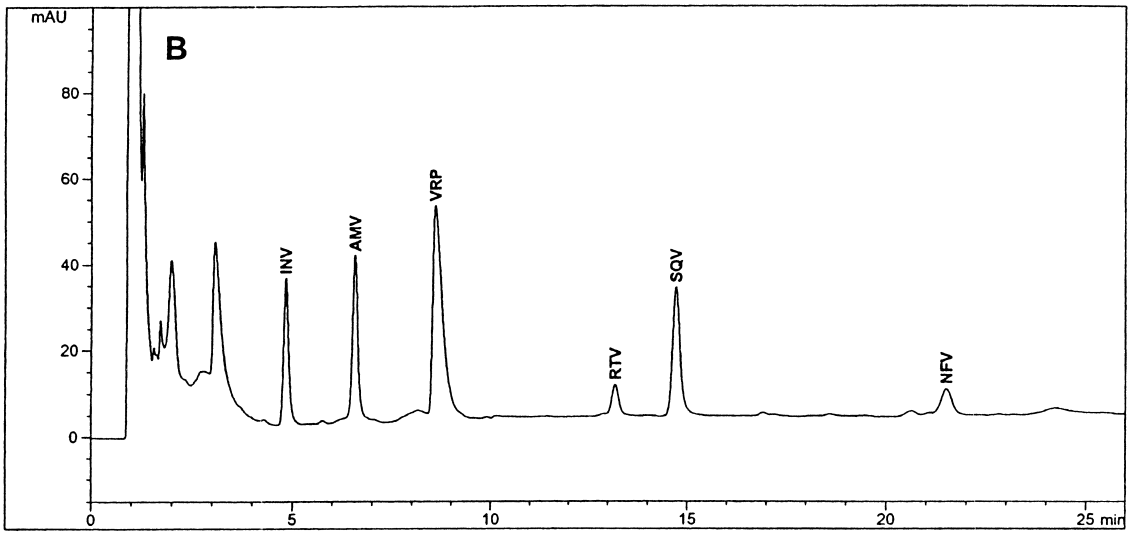

Fig. 5. Typical chromatogram of a spiked plasma sample containing 400–500 ng/ml of amprenavir (ANV), indinavir (INV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and internal standard (VRP) (from Sarasa-Nacenta et al. [37]).

Table 4
Summary of published HPLC methods: simultaneous measurement of PIs[a]

PIs	Matrix	Sample pretreatment	Column	Mobile phase	Run time[b]	Limit of quantitation[c]	Detection[d]	Ref.
RTV, SQV	Serum	SPE (C_{18})	C_8 (150×4.6 mm, 5 μm)	Isocratic ACN–5 m*M* potassium phosphate monobasic buffer pH 8 (55:45 v/v), 1 ml/min	11	RTV: 800 SQV: 50	UV 240	[25]
IDV, RTV SQV	Plasma	LLE at pH 9.4 diethyl ether Hexane wash	C_{18} (150×2 mm, 5 μm)	Gradient A. 67 m*M* KH_2PO_4 pH 4.6 B. ACN 0.2 ml/min	49	IDV: 75 RTV: 45 SQV: 10	UV 258 (IDV) 240 (RTV, SQV)	[26]
IDV, NFV RTV, SQV	Plasma	LLE at basic pH MTBE Hexane wash	C_{18} (150×4.6 mm, 5 μm)	Gradient A. ACN B. 50 m*M* KH_2PO_4 (pH 5.63) 1.5 ml/min	30	all PIs: 40	UV 215	[27]
IDV, NFV RTV, SQV (+DLV)	Serum	LLE at basic pH Ethyl acetate–hexane (1:1)	C_8 (150 mm)	Isocratic ACN–MeOH–15 m*M* phosphate (pH 7.5) (45:5:50 v/v) gradient flow-rate 0.8–1.5 ml/min	25	IDV, NFV, RTV: 100 SQV: 10	UV 254	[28]
IDV, NFV RTV, SQV	Plasma	LLE at basic pH MTBE	C_4 (250×3 mm, 5 μm) 40°C	Isocratic ACN–50 m*M* sodium formate buffer (52:48, v/v) pH 4.10, 0.5 ml/min	16	IDV: 49 NFV: 43 RTV: 50 SQV: 22	UV 218 (IDV, NFV, RTV) 235 (SQV)	[29]
IDV, NFV RTV, SQV	Plasma	LLE at pH 10.8 MTBE Reconstitution in TMAP-sol. Hexane wash	Phenyl (250×4.6 mm, 5 μm)	Isocratic 0.04 *M* ammonium acetate–ACN (48:52 v/v), pH 7.5 1.0 ml/min	15	IDV, NFV: 100 RTV: 250 SQV: 25	UV 260	[30]
APV, NFV, RTV, SQV (+DLV, EFV)	Plasma	LLE at pH 10 MTBE	C_{18} (5 μm)	Isocratic Sodium phosphate 25 m*M*–ACN (55.2:44.8 v/v)+ diethylamine 0.9% + THF 1%, pH 3.0 0.5 ml/min	35	APV: 50 NFV: 150 RTV, SQV: 100	UV 260	[31]
APV, IDV NFV, RTV SQV	Plasma	SPE (C_2)	C_{18} (75×4.6 mm, 3.5 μm)	Isocratic ACN–25 m*M* sodium acetate +25 m*M* hexane-1-sulfonic acid, pH 6.0 (40.5:59.5 v/v) 1.5 ml/min	20	APV, IDV, SQV: 25 NFV, RTV: 50	UV 210 (APV, IDV, NFV) 239 (RTV, SQV)	[32]
APV, IDV, NFV, RTV, SQV (+EFV)	Plasma	SPE (C_{18})	C_{18} (125×4 mm, 5 μm)	Gradient A. ACN B. H_3PO_4+sodium heptane sulfonate in water, pH 5.15 C. 0.3% acetic acid in ACN 1 ml/min	47	APV, SQV: 100 IDV, RTV, NFV: 250	UV 201	[33]

Table 4. Continued

PIs	Matrix	Sample pretreatment	Column	Mobile phase	Run time[b]	Limit of quantitation[c]	Detection[d]	Ref.
APV, IDV, NFV, RTV, SQV	Plasma	SPE (Oasis® HLB)	C_{18} (150×2.1 mm, 4 μm) 24°C	Gradient A. 0.5% 5.8 mol orthophosphoric acid+ 0.02% triethylamine, pH 5.0 B. ACN C. MeOH 0.4 ml/min	45	APV, IDV: 5 NFV, RTV, SQV: 10	UV 265 (APV) 210 (IDV, NFV, RTV, SQV)	[34]
APV, IDV, NFV, RTV, SQV (+EFV)	Plasma	SPE (C_{18})	C_{18} (250×4.6 mm, 5 μm) 37°C	Isocratic 0.04 *M* Na_2HPO_4+4% v/v OSA–ACN (50:50 v/v) 1.3 ml/min	32	APV: 25 IDV, NFV, RTV: 50 SQV: 5	UV 261 (APV, IDV) 241 (RTV, SQV) 254 (NFV)	[35]
APV, IDV, NFV, RTV, SQV	Plasma	LLE at basic pH MTBE Hexane wash	C_{18} (150×4.6 mm, 5 μm)	Isocratic ACN–50 m*M* KH_2PO_4+50 m*M* $NaHPO_4$ (pH 5.6) (43:57 v/v) 1.5 ml/min	40	all PIs: 50	UV 215	[36]
APV, IDV, NFV, RTV, SQV	Plasma	SPE (Oasis®)	C_{18} (150×3.9 mm, 5 μm)	Gradient A. 15 m*M* potassium phosphate pH 5.75 B. ACN 1 ml/min	25	APV: 50 IDV: 40 NFV: 85 RTV: 100 SQV: 44	UV 210 (APV, IDV) 240 (RTV, SQV) 220 (NFV)	[37]
APV, IDV NFV, RTV, SQV (+NVP)	Plasma	LLE Ethyl acetate–hexane (9:1 v/v) Hexane wash	C_{18} (250×4.6 mm, 5 μm)	Gradient A. ACN+0.025 *M* TMAP in 0.2%TFA B. MeOH+0.025 *M* TMAP in 0.2%TFA 0.9 ml/min	35	APV: 50 IDV, NFV, SQV: 200 RTV: 400	UV 239 (RTV) 254 (APV, NFV, SQV) 259 (IDV)	[38]
IDV, NFV, RTV, SQV, (+DLV, EFV, NVP)	Serum	SPE (C_{18})	C_{18} (150×4.6 mm, 5 μm) 60°C	Gradient A. ACN B. 0.004 *M* sulphuric acid 0.85 ml/min	52	IDV: 210[e] NFV: 400 RTV: 510 SQV: 100	UV 265 (IDV) 240 NFV, SQV RTV)	[39]

[a] See Nomenclature for abbreviations.
[b] Run time in minutes.
[c] Limit of quantitation in ng/ml.
[d] Wavelength of detection in nm.
[e] Limits of detection instead of limits of quantitation.

at 210 nm and amprenavir at 265 nm [34]. The latter PI eluted together with an endogenous peak, which was detected at 210 nm, but not 265 nm, the wavelength of maximum absorbance for amprenavir.

Alternative detectors may be selected when samples are to be analyzed with low PI concentrations, such as in CSF and semen (Section 3.2), or when high sensitivity and specificity are required for other reasons.

Sparidans et al. used fluorescence detection to

enable them to measure low concentrations of amprenavir (lower limit of quantitation: 0.5 ng/ml) in small sample volumes of semen and CSF [4]. No derivatization was required. Other PIs have not been measured using fluorescence detection. Indinavir was reported not to exhibit fluorescence [6], whereas saquinavir demonstrated only minor fluorescence (wavelengths of excitation and emission are 325 and 375 nm. respectively [23]).

Fizzano et al. used electrochemical detection for sensitive and specific measurement of low indinavir concentrations (lower limit of quantitation 4 ng/ml) in cell cultures [41]. A hydrodynamic voltammogram of indinavir showed a voltage-dependent increase starting from +500 mV before reaching a final plateau after +750 mV. The first electrode potential was set at +400 mV, to remove compounds with lower oxidation potentials than indinavir. For detection, the second electrode was set at +750 mV. Under these conditions detection of indinavir was twice more sensitive than that obtained with an UV detector set at 210 nm.

MS detection has been applied for measurement of amprenavir [5], and it allowed for measurement of indinavir at 1 ng/ml in plasma [7,15]. Likewise, measurement of saquinavir in plasma can be performed with a lower limit of quantitation of 0.4 ng/ml, using HPLC with MS detection [21].

Quantitation of PIs has mostly been performed with use of internal standard calibration. Some methods did not use an internal standard, for reasons of inavailability of a suitable internal standard, or because satisfactory validation results were obtained without the use of one [14,19,23,32,35,36,39].

3.9. Intralaboratory and interlaboratory quality control

Important decisions are taken based on data obtained with bioanalytical methods for PIs. Therefore application of these methods requires quality control (QC) procedures, usually including intralaboratory method validation, intralaboratory QC procedures (e.g., use of internal QC samples), and participation in an interlaboratory QC program.

Concerning interlaboratory QC, only two methods were tested against reference methods [24,29]. Furthermore, interlaboratory QC programs for measurement of antiretroviral drugs have not been available until recently. However, an international interlaboratory QC program for both PIs and NNRTIs was initiated lately [81], and two national programs have been started in France [82,83]. Results of the three programs have been similar, demonstrating that intralaboratory QC procedures need to be improved in a substantial number of laboratories participating in these programs. For example, 17 laboratories in the USA, Canada, Europe and Australia participated in the second round of the international program and measured varying concentrations of four PIs and two NNRTIs [81]. Twenty percent limits around the weighed-in concentrations of the drugs were considered to be appropriate thresholds for a satisfactory measurement. Measurements of indinavir, nelfinavir, ritonavir and saquinavir yielded satisfactory results in 69%, 78%, 78% and 94% of the analyses, respectively. Only two laboratories performed all measurements (including those of NNRTIs) within 20% limits [81].

The findings of the three QC programs demonstrate both the need for and utility of ongoing QC programs in this area of bioanalysis. Inaccurate analysis of patient samples within the scope of TDM may result in inappropriate dose adjustments, or the advice not to adjust doses where it might be desirable. Both may lead to unnecessary toxicity or to inadequate drug levels, causing resistance development. Inaccurate measurements in pharmacokinetic studies may, for example, lead to incorrect evaluation of drug interactions, which may affect the treatment response of many patients. However, by participating in a QC program, laboratories are being alerted to possible undetected problems in their QC procedures. This enables them to optimize their methods.

4. HPLC methods for simultaneous measurement of protease inhibitors

Development and use of one HPLC method for measurement of several PIs saves time and costs compared to several methods for single PIs. Fifteen methods for simultaneous analysis of PIs have been published so far. All these methods have been developed (and will most likely be employed widely) for pharmacokinetic studies and TDM. Therefore

criteria for deciding on which method to choose should be derived from these applications.

Firstly, the method of choice should be applicable for as many PIs as possible, since a large variety of HAART schemes is being prescribed in every patient population. The possibility to simultaneously measure other antiretroviral drugs (especially NNRTIs, such as efavirenz and nevirapine) is an advantage.

Secondly, a method for simultaneous measurement of PIs should be sensitive enough to measure both trough levels of single PIs and proposed threshold limits (Section 3.6, Table 2).

Thirdly, high specificity should be ensured because of the large number of co-administered drugs in HIV infection (Section 3.7).

Concerning the choice of sample pretreatment and separation conditions, it seems that available HPLC equipment and expertise, as well as personal preferences, may well direct the choice of these method characteristics.

With regard to the choice between liquid–liquid and solid-phase extraction, the costs of disposable cartridges may be an additional criterium [36,38].

With respect to separation conditions, it appears advantageous to choose conditions that are as simple as possible, thus avoiding the use of ion-pair reagents as well as amine modifiers. Generally, ion-pair chromatographic methods are more complicated to use and are subject to additional experimental problems [80]. Furthermore, the use of strongly retained additives in the mobile phase (ion-pair reagents, amine additives) can complicate the use of gradient elution [84]. Gradient elution may also appear more complicated than isocratic separation, but it is often required or preferred for samples with many analytes and a wide retention range. Some may have a strong bias against the use of gradient elution for several reasons [84]. Certainly, gradient methods do not always transfer well to other laboratories. This may be particularly true for non-linear gradients.

Run time is another important criterion when there is pressure on the laboratory to perform large numbers of assays. This may even be relevant when samples are being processed automatically. Run times for simultaneous analysis of PIs varied from 11 [25] to 52 min [39].

With regard to detection conditions, it should be noted that all published methods for simultaneous measurement of PIs use UV detection. The (in)availability of a switchable UV-detector or photodiode array detector may influence the choice of a method.

The published methods for simultaneous measurement of PIs are summarized in Table 4 and will be briefly commented upon below.

Frappier [25] and Langmann [26] measured only few PIs. The lower limit of quantitation for ritonavir in the method by Frappier is high. The method applied by Langmann et al. may be considered lengthy (49 min).

The method by Hugen [27] has recently been extended (and slightly modified) for measurement of amprenavir, lopinavir and nelfinavir metabolite M8 as well (data in press). This method demands very pure HPLC quality water and extra pure MTBE. Peroxides in MTBE can cause decomposition of nelfinavir. Furthermore, pH of the mobile phase should be set exactly at the desired level.

Moyer [28] applied an unusual gradient, not for mobile-phase strength, but for flow-rate. Flow-rate increased during the run, apparently aiming at a shorter run time. The limit of quantitation for indinavir (100 ng/ml) is high. Several significant interferences were noted among a very large amount of drugs evaluated for interference.

Remmel et al. [29] report adequate limits of quantitation, but inspection of the chromatogram of a low concentration QC sample shows only small peaks for indinavir, nelfinavir and ritonavir at concentration levels far above their limits of quantitation.

Bouley et al. [30] separated PIs in only 15 min. However, from the chromatograms it appears that there is just baseline resolution between bands of the internal standard and ritonavir, whereas saquinavir and nelfinavir elute close together as well. The limit of quantitation for indinavir is high (100 ng/ml), despite a relatively large volume of plasma (1 ml) to be used in this method. Possible interferences by other than antiretroviral drugs were not reported.

Proust et al. describe a method which includes efavirenz, but unfortunately not indinavir [31]. The limit of quantitation of saquinavir is high for measurement of this drug when administered without ritonavir.

In the isocratic method by Van Heeswijk et al. [32], indinavir and amprenavir elute early, on the solvent front and close to endogenous interference peaks. The last band of nelfinavir shows peak broadening and is non-symmetric, which may result in sensitivity problems at lower concentrations.

The assay by Marzolini [33] uses a rather complex, non-linear gradient and requires careful control of one of the solvents' pH for reproducible separation. These features may complicate the transfer of this method to other laboratories. Reported limits of quantitation for indinavir and saquinavir are high. However, as the authors mention, accuracy and precision at the lower limits of quantitation are well below 20% allowances. Therefore, it may be possible to decrease these limits.

Poirier et al. [34] use a photodiode array detector for measurement of amprenavir at 265 nm and other PIs at 210 nm. An automatic switchable UV detector may not be applicable as an alternative, since the small difference in retention time between amprenavir and indinavir may not allow for programmed wavelength changes.

Aymard et al. developed a method for measurement of 12 antiretroviral drugs, PIs, NNRTIs and NRTIs [35]. One solid-phase extraction procedure was coupled with two separate reversed-phase HPLC systems, one for five PIs and efavirenz, and one mainly for NRTIs.

Yamada et al. used a sample preparation procedure identical to that described by Hugen [27], but they chose isocratic (instead of gradient) separation conditions [36]. The method was not tested for interference by other antiretroviral drugs or other medications.

In the method by Sarasa-Nacenta et al., good separation of five PIs depends on accurate setting of pH and ionic strength of the mobile phase [37].

Dailly et al. measured nevirapine together with five PIs [38]. Separation is achieved by gradient elution, combined with a hydrophobic ion-pair reagent in the mobile phase. Limits of quantitation for indinavir, ritonavir and saquinavir are high.

Simon et al. were able to measure four PIs and three NNRTIs in one run [38]. Run time may be considered long (52 min). Possible interference by other drugs was not reported. The limits of detection are high.

5. Conclusions

Since the introduction of PIs for treatment of HIV infection, numerous HPLC methods have been developed for analysis of these drugs in plasma and serum, saliva, CSF and semen. Fifteen methods described so far have concerned the simultaneous analysis of several PIs in one run. Heat treatment for deactivation of HIV may lead to slight degradation of PIs in plasma samples. Liquid–liquid extraction was most often applied for sample pretreatment, but solid-phase extraction and protein precipitation were used as well. Reversed-phase or ion-pair chromatography have been used to separate PIs. Isocratic conditions have been applied for measurement of single PIs, and gradient elution has been used in seven of the 15 methods for simultaneous measurement of PIs. Detection of PIs should be sensitive enough for quantitation of concentrations below trough concentrations of single PIs, or below presumed therapeutic thresholds for PIs. The large majority of assays employs UV detection. As the potential for interferences is large, the selectivity of every method should be evaluated properly.

The available HPLC methods have been applied in clinical pharmacokinetic studies with PIs and have provided the basis for important developments in the clinical pharmacology of antiretroviral drugs. New interests, such as in free (non-protein bound) plasma concentrations and intracellular PI drug levels, also require application and development of reliable assays. Furthermore, studies relating pharmacokinetics to clinical effects have raised large interest in TDM for PIs. The promising perspective of TDM to optimize the clinical use of PIs may really spread the use of HPLC methods, as TDM requires that measurement of PIs is not confined to a small number of research laboratories, but can be applied in hospital laboratories as well. Fortunately, most HPLC assays for PIs are quite straightforward and can be performed with equipment that is available or affordable in most hospitals. Assays for simultaneous measurement of PIs appear to be most convenient for the purpose of TDM. It is recommended that any laboratory engaged in the analysis of PIs, whether as a routine service or as part of a research project, joins an interlaboratory QC program in addition to establishing its own QC procedures.

6. Nomenclature

ACN	acetonitrile
APV	amprenavir
C_{max}	maximum (peak) concentration
C_{min}	trough concentration
CSF	cerebrospinal fluid
DLV	delavirdine
ED	electrochemical detection
EFV	efavirenz
El.	electrode
Em	wavelength of emission
Ex	wavelength of excitation
FL	fluorescence
HAART	highly active antiretroviral therapy
HGC	hard-gelatin capsule
IDV	indinavir
LLE	liquid–liquid extraction
LOD	limit of detection
MeOH	methanol
MTBE	methyl *tert*.-butyl ether
na	not available (or not reported)
NFV	nelfinavir
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NVP	nevirapine
OSA	octane sulphonic acid
PI	protease inhibitor
QC	quality control
RTV	ritonavir
SGC	soft-gelatin capsule
SPE	solid-phase extraction
SQV	saquinavir
TBA	tetrabutylammonium hydrogen sulphate
TDM	therapeutic drug monitoring
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMACl	tetramethylammonium chloride
TMAP	tetramethylammonium perchlorate

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