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Review Article

Quantification of antiretroviral drugs for HIV-1 in the male genital tract: current data, limitations and implications for laboratory analysis

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Abstract

Antiretroviral therapy has reduced the morbidity and mortality associated with HIV-1/AIDS in developed countries. Viral replication in blood plasma is suppressed by antiretroviral drugs, whereas virus in the male genital tract is genetically and phenotypically unique and may not be suppressed. This viral compartmentalization affects antiretroviral drug penetration of the male genital tract and capacity for antiretroviral therapy to reduce sexual transmission. The problem of having two distinct viral populations within any given individual is compounded by the fact that antiretroviral drugs penetrate semen to varying degrees. Incomplete suppression of genital tract virus may yield drug-resistant virus and increase the risk of sexual transmission. This review critically appraises current studies of antiretroviral drug quantification in semen and suggests recommendations to address observed limitations.

Introduction

The goal of antiretroviral therapy (ART) is to inhibit HIV-1 replication and facilitate reconstitution of the immune system. The standard of HIV-1 care is three antiretroviral drugs (ARVs) selected from at least two ARV classes. Viral suppression is achieved when the number of HIV-1 RNA copies per mL of blood plasma, the viral load (VL), falls below the limit of detection of the assay (e.g. VL < 50 copies mL^{-1} by polymerase chain reaction). This is commonly referred to as the 'undetectable VL'. In addition to its health benefits, ART can reduce the sexual transmission of HIV-1 in homosexually active men (Blower et al 2000; Law et al 2001) and in the heterosexual population, where zidovudine is associated with a 50% reduction in male-to-female HIV-1 transmission (relative risk = 0.5, 95% CI 0.1–0.9) (Musicco et al 1994). A Phase III, international, randomized trial (HPTN052) is in progress to evaluate the role of ART in preventing sexual transmission of HIV-1 at the population level (US National Institutes of Health 2006).

There are three potential problems when assessing the role of ART in reducing sexual transmission. First, HIV-1 in blood and the male genital tract (MGT) are genotypically and phenotypically distinct (Wainberg et al 1993; Kroodsma et al 1994; Vernazza et al 1994; Zhu et al 1996; Coombs et al 1998; Delwart et al 1998; Eron et al 1998). There are also pathogenetic differences with regard to syncytium-inducing potential (Gupta et al 2000), and structural differences in functional regions (e.g. the V3 loop and pol amino acid sites associated with ARV resistance) (Curran & Ball 2002). Second, HIV-1 in semen may not originate solely from extravasation or transcytosis (spill-over) from blood, that is, there appears to be local production of unique virus within each compartment. During ART, infectious virus is detectable in semen but not in blood (Vernazza et al 1994; Coombs et al 1998), and, in untreated patients without sexually transmissible infections, semen VL is lower or undetectable VL compared with blood (Vernazza et al 1994, 1996, 1997a, b, 2000). Finally, the ability of ART to reduce the seminal VL rests on ARV penetration of semen (Kashuba et al 1999). The consequence of poor penetration of the MGT by ARVs is the potential for ongoing viral replication, transmissibility and development of drug resistance (Wainberg et al 1993; Kroodsma et al 1994; Merigan 1998; Kashuba et al 1999; Yerly et al 1999; de Ronde et al 2001; Taylor et al 2001b, 2003b; UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance 2001; Barbour et al

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2004; Ghosn et al 2004b). Transmission of drug-resistant virus is a significant concern because it limits ART options in newly infected patients (primary resistance) or patients already infected (secondary resistance), prolongs time to viral suppression and damage to the immune system, and is associated with treatment failure in the future (Grant et al 2002). Although patients with a low or undetectable VL in blood are probably less infectious, they are still capable of transmitting HIV-1.

The compartmentalization of virus in the MGT means there is no guarantee against sexual transmission. The infectious inoculum required for infection to occur has not been established. A proper analysis of ARV penetration of the MGT using appropriately validated assays is needed. Quality assurance programmes of routine monitoring of ART in plasma, however, suggest that laboratory performance is variable or poor. Two of nine laboratories reported most results incorrectly (>20% from the nominal target) in the first round of a quality control assessment; performance did not improve in the third round, where six of 30 laboratories reported every second result incorrectly (Droste et al 2003). The aim of this review is to critically appraise current methods of quantification of ARVs in semen to inform the use of appropriate assays that may facilitate the interpretation of transmission studies and gain an understanding of ARV behaviour in the MGT. We begin with a brief discussion of ARV drug penetration of the MGT, followed by current data on ARV semen levels, a critical appraisal of current assays, and, finally, recommendations addressing the laboratory limitations we have identified.

Antiretroviral drug penetration of semen

A reduction in sexual transmission of HIV-1 is dependent on ARV penetrating the MGT and suppressing HIV-1 replication (Kashuba et al 1999). A schema for the histology of the blood–MGT interface (Figure 1) will assist in the discussion that follows. Assuming that a lipid membrane separates the MGT from the systemic circulation, ARVs accumulate in the MGT via passive diffusion from blood and capillaries (Lewis 1981; Mann & Lutwak-Mann 1982). The interstitial space and adjacent cells of the MGT constitute the extraluminal compartment. ARVs may then enter the prostate, seminiferous tubules and accessory organs, which produce semen (the adluminal compartment), via passive diffusion, active transport and cell mediated mechanisms. The result is an ARV concentration gradient from extraluminal to adluminal compartments (i.e. high to low ARV concentration). The gradient may be increased by the relative differences in penetrability between individual ARVs or ARV classes (see below). Consequently, HIV-1 replication is suppressed in blood but not in semen. To add complexity, genotypically or phenotypically distinct virus with different drug susceptibility may be produced locally within the MGT as discussed previously.

HIV-1 shedding in the genital tract is influenced by the ART regimen, treatment strategy and adherence with pill

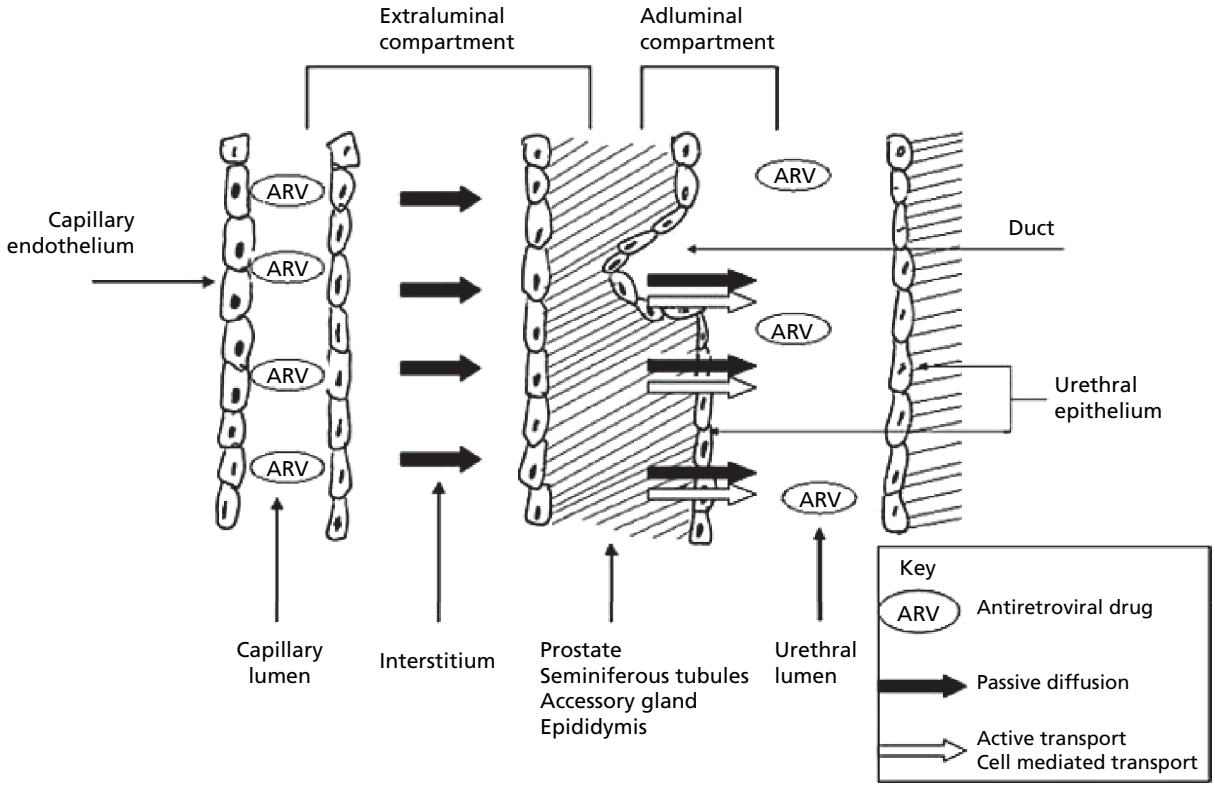

Figure 1 The blood–genital tract interface.

taking. Genital tract shedding is extensive in patients taking one ARV (Eron et al 2000) or two ARVs (Kalichman et al 2001; Taylor et al 2001b), and with ARVs that penetrate the genital tract poorly (Ball et al 1999; Mayer et al 1999), for example protease inhibitors (PIs; see below). Adherence maximizes ARV levels in blood and penetration of the MGT; however, even when adherence is consistent, individual drugs such as PIs may still penetrate poorly (Kashuba et al 1999) and genital tract virus may not be suppressed (Mayer et al 1999; Eron et al 2000). Genital tract shedding is increased during structured treatment interruptions (Liuzzi et al 2003) and when ARVs are taken inconsistently (Barroso et al 2000). Increasing infectivity and transmission of drug resistant virus may result. Although the risk of sexual transmission is lower when blood VL is undetectable, the precise seminal VL required for transmission is not known, and prevention efforts must continue to emphasize condom use irrespective of blood VL and availability of ART.

The differences in penetrative capacity between individual ARVs and ARV classes may be determined by drug pharmacokinetics and physicochemical properties (Kashuba et al 1999; Taylor & Pereira 2001) (Table 1). Briefly, maximal drug penetration occurs with a weakly basic, lipophilic and minimally protein-bound ARV; however, the actual degree of drug penetration in semen is not always consistent with pharmacokinetic data, for example zidovudine. Despite a relatively low amount of drug being available systemically after first-pass metabolism (oral bioavailability 60%) (Bartlett & Gallant 2004), the drug concentration in semen is higher than that predicted by pharmacokinetics. Zidovudine may accumulate by ion trapping (Henry et al 1988). Ion trapping is based on the premise that ionization increases hydrophilicity, that is, only non-ionized drugs penetrate biological membranes (Taylor & Pereira 2001). Ionized forms, in contrast, are sequestered on one side of a membrane. Ionization is dependent on the dissociation constant (pK_a) of the drug and the pH of the environment (Kashuba et al 1999). pK_a is the pH at which equimolar concentrations of non-ionized or ionized forms of a drug exist.

Similarly, pharmacokinetic data favour maximal seminal penetration by PIs. Blood plasma pH 7.2 exceeds the pK_a of most PIs and conditions favour increased membrane permeability; PIs are basic molecules that should accumulate in acidic compartments such as prostatic fluid (pH 6.6) by ion trapping (Kashuba et al 1999). Observed drug concentrations in prostatic fluid are, however, lower than predicted (Pichini et al 1994). The pharmacokinetic factors that favour PI penetration may be overridden because PIs bind to plasma proteins such as albumin and α_1-acid glycoprotein. The relative size of the PI–protein complex hinders permeation of biological membranes (Taylor & Pereira 2000).

Much has been made of the blood–testis barrier in impeding drug penetration of the MGT. Located in the seminiferous tubules, the blood–testis barrier is a specialized section of endothelium comprising Sertoli cells connected by tight junctions. These tight junctions hinder diffusion of hydrophilic solutes (including ARVs) 100-fold more than intercellular junctions found elsewhere in the body (Renkin & Crone

Table 1 Physicochemical characteristics of antiretroviral drugs in blood plasma (adapted from Kashuba et al 1999, unless otherwise referenced)

Drug	pK_a	Lipid solubility (K)	T_{max} (h)	t½ (h)	Protein binding in blood (%)
Nucleoside reverse transcriptase inhibitors					
Zidovudine	9.7	Slightly lipophilic (1.15)	0.6–0.8	0.6–1.7	20–38
Lamivudine	4.3	Hydrophilic (NA)	1.0–1.5	3.0–5.0	10–50
Didanosine	9.1	Lipophilic (NA)	0.5–4.6	0.6–2.9	<5
Zalcitabine	4.4	Hydrophilic (0.04)	0.5–2.0	1.0–3.0	<4
Stavudine	10.0	Hydrophilic (NA)	3.8	1.0–1.5	Negligible
Abacavir	0.4, 5.1	Lipophilic (NA)	0.7–1.7	0.9–1.7	50
Tenofovir	3.75[a]	Hydrophilic[b] (NA)	2.3[b]	14.4[b]	< 0.7–7.2[b]
Non-nucleoside reverse transcriptase inhibitors					
Nevirapine	2.8	Slightly lipophilic (1.8)	4.0	25.0–30.0	60
Delavirdine	4.3–4.6	Slightly lipophilic (2.98)	1.0	4.40–11.0	98
Efavirenz	10.2	Lipophilic (NA)	2.0–5.0	40.0–52.0	99.5
Protease inhibitors					
Ritonovir	2.8	Lipophilic (4.0)	2.0–4.0	3.0–5.0	98
Saquinavir	1.1, 7.1	Lipophilic (NA)	NA	6.0	>98
Indinavir	6.2	Hydrophilic (NA)	0.5–1.1	1.0–3.0	60
Nelfinavir	6.0, 11.1	Lipophilic (NA)	2.0–4.0	3.5–5.0	99
Amprenavir	1.9	Lipophilic (NA)	1.2	7.0–10.0	95
Atazanavir[c]	NA	NA (NA)	2.0	6.0	86
Fusion inhibitors					
Enfuvirtide[d]	NA	NA (NA)	4.1	3.8	92

pK_a, dissociation constant; K, partition coefficient; T_{max}, time to reach maximum concentration of drug; t½, elimination half-life; NA, not available.
[a]US Food and Drug Administration, Center for Drug Evaluation and Research 2004; [b]Dando & Perry 2003; [c]Le Tiec et al 2005; [d]Daluge et al 1997.

1996). Although there is probably some impedance of drug penetration, the barrier does not comprise the entire surface area of the MGT and cannot be the sole reason for poor ARV penetration (Lowe et al 2004).

Available data for ARV penetration of semen is presented below. To our knowledge there is no current data for seminal levels of integrase inhibitors, CCR5 inhibitors, or newer drugs in current classes.

Nucleoside reverse transcriptase inhibitors (NRTIs)

NRTIs target the virally coded reverse transcriptase enzyme, thereby terminating the viral DNA chain and viral replication. NRTIs ought not to penetrate the MGT because of lipid insolubility, yet NRTIs are the most penetrable ARV class, suggesting the involvement of drug sequestration or active transport (Reddy et al 2003; Taylor et al 2003a) (Table 2). Seminal zidovudine and lamivudine levels are twice that in blood (Henry et al 1988; Pereira et al 1999, 2000; Taylor et al 2000; Reddy et al 2002). The observed seminal zidovudine level may result from drug binding to seminal protein (Anderson et al 2000). Seminal abacavir exceeds the inhibitory concentration (70 ng mL^{-1}) for maximally susceptible HIV-1 strains by 10–20 fold (Daluge et al 1997); however, unlike zidovudine and lamivudine (van Praag et al 2001b), abacavir is eliminated from semen more rapidly (plasma half-life 0.8–1.5 h) (Foster & Faulds 1998), potentially limiting its antiviral potency. Didanosine also appears to penetrate semen well (Gatti et al 2001). Seminal tenofovir exceeds the expected blood concentration of 22 ng mL^{-1} (Ghosn et al 2004a). This may be explained by active drug transport, a small molecular size, a relatively long half-life or a partitioning effect due to the dissociation constant of the drug. Data on seminal stavudine concentrations is very limited (Taylor et al 2000). There is no data for zalcitabine. A limitation of NRTI studies, apart from being small, is correlating the degree of viral suppression with seminal ARV concentration; NRTIs undergo intracellular phosphorylation and thus most of the active drug is intracellular and immeasurable throughout a dosing interval. Therefore, high seminal NRTI levels may not equate to maximal viral suppression if intracellular NRTI levels are actually low (and vice versa).

Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs block reverse transcriptase by non-competitive inhibition. Studies of NNRTI penetration are small (Table 3). The data for efavirenz is conflicting: seminal efavirenz penetration is probably poor (Reddy et al 2002), reaching only 10% of concurrent blood concentration in one study (Taylor et al 2001a), but there was no efavirenz in another study despite optimal blood levels (Ghosn et al 2004a). Seminal nevirapine may be dependent on the time of measurement after dosing (van Praag et al 2001a), although blood concentrations in another study reached 60–100% independent of time after dosing (Taylor et al 2000). In four patients, the semen/blood concentration ratio (degree of seminal penetration) for delavirdine (no longer used in routine clinical practice due to inferior efficacy and toxicity) is very low (0.16) (Taylor et al 2001a).

Table 2 Concentrations of nucleoside reverse transcriptase inhibitors in semen

Drug	Sample size, n (reference)	Median concentration ng mL^{-1} (range)[a]	Median semen/blood concentration ratio (range)	T (h)
Zidovudine	n = 79 (Pereira et al 1999)	292 (194–438)	15.7 (9.4–27)	~6
	n = NA (Anderson et al 2000)	NA	AUC 2.2 (1.6–2.8)	0–12
	n = NA (Reddy et al 2003)	NA	AUC 3.31	0–12
Lamivudine	n = 3 (Taylor et al 2000)	1224 (391–1606)	8.7 (4–16.3)	8–12
	n = 82 (Pereira et al 1999)	2701 (1460–4320)	12.4 (6.8–18.8)	0–12
	n = NA (Reddy et al 2003)	NA	AUC 6.6 (4–8.1)	0–21
Abacavir	n = 4 (van Praag 2001b)	1223 (925–2051)	NA	2
Stavudine	n = 5 (Taylor et al 2000)	<25 (<25–388)	3.5 (1–6)	8–12
Didanosine	n = 5 (Gatti et al 2001)	430 (<50–1240)	NA	4–12
Tenofovir	n = 4 (Ghosn et al 2004a)	NA	NA	1–6

n, number of samples; NA, not available; AUC, area under the curve; T, time from dose ingestion. [a]Approximate trough concentration (in relation to T).

Table 3 Concentrations of non-nucleoside reverse transcriptase inhibitors in semen

Drug	Sample size, n (reference)	Median concentration ng mL^{-1} (range)[a]	EC50, ng mL^{-1} (MEC ng mL^{-1})	Median semen/blood concentration ratio (range)	T (h)
Nevirapine	n = 6 (Taylor et al 2000)	3900 (1300–9100)	39.9 (3400)	0.61 (0.59–0.78)	8–12
Efavirenz	n = 13 (Taylor et al 2001a)	238 (49–1256)	404 (1100)	0.09 (0.03–0.43)	24
	n = 147 (Reddy et al 2002)	NA	NA	AUC 0.034 (0.02–0.05)	0–24
Delavirdine	n = 4 (Taylor et al 2001c)	1025 (819–1210)	NA	0.16 (0.12–0.21)	6–9

n, number of samples; NA, not available; AUC, area under the curve; EC50, 50% effective concentration corrected for protein binding; MEC, minimum effective concentration; T, time from dose ingestion. [a]Approximate trough semen concentration (in relation to T).

PIs

PIs block the virally coded protease enzyme and cleavage of the gag/pol polyprotein. Mature virions cannot be produced and infectious virus is less likely to bud from the cell. The antiviral effect of PIs continues within the cytoplasm after virus buds from the cell (Table 4). PIs penetrate semen least well of all ARV classes. Indinavir is the exception. Seminal indinavir concentration is time-dependent, with maximum levels achieved 2–5 h after dosing (van Praag et al 2001a). Unlike other PIs, the trough concentration in semen is 100 ng mL^{-1} (Burger et al 1998), and this exceeds the concurrent indinavir level in blood (Taylor et al 1999, 2001b; van Praag et al 2001a; Lafeuillade et al 2002). This apparent accumulation in semen may result from the low level of indinavir–protein binding (60%), yielding more free drug. When indinavir is boosted with low-dose ritonovir, supra-maximal

Table 4 Concentrations of protease inhibitors in semen

Drug	Sample size, n (reference)	Median concentration ng mL^{-1} (range)[a]	EC95, ng mL^{-1} (MEC)	Median semen/blood concentration ratio (range)	T (h)
Indinavir	n = 7 (Taylor et al 2001b)	558 (272–3178)	42	1.44	6–8
	n = 12 (Lafeuillade et al 2003)	Mean 714	NA	1.9	NA
Indinavir + ritonovir	n = 9 (van Praag et al 2000)	1634 (1109–2431)	NA	NA	8–12
	n = 39 (van Praag et al 2001a)	448 (240–1015)	NA	NA	8
Amprenanvir	n = 43 (Pereira et al 2002)	319 (73–929)	102 (400)	0.2 (0.1–0.39)	0–12
Ritonovir	n = 9 (Taylor et al 2001b)	295 (<25–870)	1514 (2100)	< 0.04 (< 0.02–0.11)	9–12
Saquinavir + ritonovir	n = 7 (Taylor et al 2001b)	<20	278 (100)	< 0.04 (< 0.02–< 0.061)	9–12
Nelfinavir	n = 7 (Solas et al 2003)	159	<630 (MEC50)	0.08	12
	n = 7 (Lafeuillade et al 2002)	mean 156 ± 48	567 (800)	0.07	NA
Lopinavir + ritonovir	n = 14 (Sankatsing et al 2002)	230 (46–3900)	EC50 = 64.2 (1000)	0.034 (0.02–0.07)	1–12
	n = 23 (Lafeuillade et al 2003)	191	NA	0.07	NA
Atazanavir	n = 15 (van Leeuwen et al 2007)	221 (70–350)	NA	0.10 (0.08–0.17)	NA[b]

n, number of samples; NA, not available; EC95, 95% effective concentration corrected for protein binding; MEC, minimum effective concentration; T, time from dose ingestion. [a]Approximate trough semen concentration (in relation to T); [b]atazanavir concentrations plotted against T, single estimate of T not supplied.

seminal concentrations are achieved. Drug transporter activity or inhibition of P-glycoprotein may be enhanced by the ritonovir (van Praag et al 2000). Amprenavir achieves higher levels in semen than ritonovir and saquinavir (Kashuba et al 1999; Pereira et al 2002), despite being 90% protein-bound in blood (Kashuba et al 1999). This is another example where pharmacokinetics are inconsistent with the degree of observed drug penetration. The vitamin E component of amprenavir capsules (Yu et al 1999) is thought to increase overall drug solubility and penetration. Amprenavir, unlike indinavir, does not inhibit cellular transporters when boosted with low-dose ritonovir in semen (Chaudry et al 2002), which may explain static seminal amprenavir levels with boosting (Ghosn et al 2004a). Seminal levels of full-dose ritonovir (no longer used in clinical practice) and saquinavir reach 2–4% of concurrent blood levels (Taylor et al 1999). The seminal nelfinavir concentration is lower than the minimum effective concentration of 630 ng mL^{-1} required to suppress 50% of viral isolates (Molla et al 1998). Lopinavir penetrates semen poorly, with a median concentration of 0.23 ng L^{-1} (range 0.046–3.9 ng L^{-1}) (Sankatsing et al 2002). Even when boosted with ritonovir, lopinavir concentration in semen still only averages 6% (Lafeuillade et al 2003) or less (Fiore et al 1997) of concurrent blood levels. A small study of 15 patients showed that seminal penetration by atazanavir is variable, with a low median blood/semen ratio of 0.10 (van Leeuwen 2007). Data for fosamprenavir, tipranavir and duranavir are unavailable.

Fusion inhibitors

Enfuvirtide is an injectable ARV given twice daily to patients with multi-class viral resistance (so-called 'salvage therapy'). The drug blocks the gp41 glycoprotein on the cell membrane, thereby preventing viral fusion. Enfuvirtide suppresses virus when blood levels exceed 1000 ng mL^{-1}; however, a higher concentration is needed to inhibit R5 quasispecies than X4 quasispecies (Derdeyn et al 2000, 2001). Most of the virus that is sexually transmitted is R5, a quasispecies that is tropic for the CCR5 co-receptor on macrophages and T lymphocytes. R5 predominates in the early HIV-1 infection but may develop into X4 by unknown mechanisms during chronic infection. X4 appears in advanced HIV-1 disease and is more pathogenic than R5. X4 is tropic for the CXCR4 co-receptors on T lymphocytes, causing the cells it infects to coalesce into giant cells or syncytia that are visible under light microscopy. There are also viruses that are dual-tropic.

The reason more enfuvirtide is required to inhibit R5 is unknown. We suggest three reasons. First, more drug may be required because R5 infects relatively more cell types (i.e. macrophages and T lymphocytes) in different anatomic sites. Second, it may be that syncytia express fewer functional binding sites for enfuvirtide because cell receptors are distorted when cells coalesce and the surface/volume ratio of the giant cells is less than that of individual cells. Finally, rapid viral replication within syncytia during advanced disease may be more error prone due to the disruption of cellular components from individual cells being thrown together, producing less fit X4 progeny. Larger studies are needed to analyse reasons for the difference in inhibitory enfuvirtide concentration.

Enfuvirtide does not accumulate appreciably in seminal plasma (concentrations are below 50 ng mL^{-1}) (Ghosn et al 2004a). There are two possible reasons. First, enfuvirtide is a large molecule with a small volume of distribution, and its relative degree of ionization may hinder penetration of semen in-vivo. A potential problem with a concentration gradient is the emergence of viral rebound and drug-resistant virus within the MGT. This is particularly problematic in patients with advanced infection or multi-class resistance, in whom seminal VL is high (Musicco et al 1994; Nicolosi et al 1994a, b; O'Brien et al 1994) and X4 virus predominates; enfuvirtide may not reduce sexual transmission of X4 from these patients. Second, non-specific binding of enfuvirtide to sample tubes and the chromatographic column (Lawless et al 1998) reduces the apparent concentration in semen. Whether the low enfuvirtide concentration in semen is determined by physicochemical properties per se or laboratory limitations is unknown. Nevertheless, current data suggests that there may be insufficient drug to effectively suppress viral replication in the MGT.

Critical appraisal of assays measuring antiretroviral drug concentration in semen

The low concentrations observed for some ARVs may be explained by inherent physiochemical properties (e.g. lipid solubility), pharmacokinetics (e.g. time to maximal plasma concentration), and nature of biological mechanisms (e.g. active transport). Nonetheless, as discussed previously, these factors do not entirely account for the drug penetration observed. Unreliable or unvalidated assays may also yield inaccurate data (Aarnoutse et al 2002). Indeed, a recent audit found that 20% of laboratories reported every second result incorrectly (Droste et al 2003).

We assessed 15 procedures for the quantification of ARV concentrations in blood plasma and semen against published acceptance criteria from the literature (Shah et al 2000) and regulatory bodies (US Department of Health and Human Services 1998). The main methods were: radioimmunoassay, high-performance liquid chromatography (HPLC) using UV or fluorescence detection and HPLC dual mass spectrophotometry. The assessment criteria were: compliance with assay precision, accuracy, linearity, limit of quantification; recovery, specificity, stability and purity checks other than retention time (for assays quantifying multiple PIs at once); method validation in plasma and semen, and abnormal analytical issues (e.g. non-specific binding to plastic tubes causing loss of drug during analysis such as occurs with enfuvirtide). Papers were selected from the literature using PubMed (National Library of Medicine, USA; www.pubmed.gov). Connectors for drug class, seminal plasma and individual ARVs were searched in order to capture the majority of relevant literature.

Our review has led us to question the quality and precision of the assays used in these studies for the following reasons (Table 5):

Table 5 Review of the analytical methods used to quantitate antiretroviral drugs in seminal plasma

Reference	Antiretroviral drug	Strengths	Limitations
Pereira et al (1999)	Zidovudine and lamivudine		Procedure unpublished and no validation data presented at time of publication (see Pereira et al 2002).
Chaudry et al (2002)	Amprenavir		Method cited from a conference abstract; no validation data presented.
Lafeuillade et al (2003)	Lopinavir		Incorrect reference cited. No validation data presented.
Sankatsing et al (2002)	Lopinavir		Analytical method cited was not validated for the drug studied.
Reddy et al (2002)	Efavirenz	Limit of quantitation, recovery and interassay variability presented.	Commercial laboratory cited for assay validation. Minimal validation data cited. No methodology presented
Ghosn et al (2004a)	Enfuvirtide, efavirenz and protease inhibitors	Cited article for efavirenz and protease inhibitors offers complete validation for human plasma only.	A review paper is cited for protease inhibitor analysis (deemed inappropriate because procedure used in this study cannot be identified). Cited article for efavirenz and protease inhibitors not validated for semen and limit of quantitation (100 ng mL^{-1}) may not be appropriate for semen. Minimal validation data provided for tenofovir assay in dog plasma only. Partial validation of T20 assay in human plasma.
Pereira et al (2002)	Amprenavir, zidovudine and lamivudine	Near complete validation of amprenavir, zidovudine and lamivudine assays in appropriate matrix (seminal plasma).	No recovery experiment, no testing of ion-suppression effects and no validation of compound stability after heat inactivation in pre-treatment step.
Sparidans et al (2000	Amprenavir	Near complete validation in appropriate matrix. Heat stability in matrix tested, interference from other agents tested. Excellent sensitivity reported.	Appears relative recovery experiment performed (absolute recovery preferred method) and recovery results in semen unclear.
Anderson et al (2000)	Zidovudine and zidovudine glucuronide	Radioimmunoassay that appears to have been developed in-house and adapted by a commercial company (unclear).	Very limited validation data reported in original method cited. Appears that method was commercialized but validation data unpublished.
Isaac et al (2004)	Lopinavir and indinavir		Cited article in abstract form only. Validation data limited for human plasma only.
Solas et al (2003)	Indinavir, nelfinavir, lopinavir and ritonavir		Assays cited were validated for indinavir and nelfinavir only in human plasma.
Taylor et al (2001b)	Indinavir ritonavir and saquinavir		Very limited validation data presented in human plasma only.
van Praag et al (2000)	Indinavir	Complete validation performed for indinavir in human plasma. Heat stability in plasma tested, interference from other agents tested.	Seminal plasma diluted 1:1 with human plasma but this practice not validated. Cited method only validated in human plasma.
van Praag et al (2001a)	Nevirapine and indinavir	Complete validation performed for both compounds in human plasma. Heat stability in plasma tested, interference from other agents tested.	Cited methods validated for human plasma only. Seminal plasma diluted 1:1 with human plasma but not validated.
van Praag et al (2001b)	Abacavir	Complete validation performed for assay in human plasma only.	Cited method validated for human plasma only. Seminal plasma diluted 1:3 with human plasma but not validated.
van Leeuwen et al (2007)	Atazanavir	Complete validation including ion-suppression effects and use of best available internal standard.	Cited assay not validated for seminal plasma.

- Method of test validation was not reported or the cited method was unpublished (Pereira et al 1999; Chaudry et al 2002; Sankatsing et al 2002; Lafeuillade et al 2003);
- There was inadequate evidence of test validation or an inappropriate reference was cited (Reddy et al 2002; Ghosn et al 2004a);
- Appropriate validation of the assay was reported but important parameters such as recovery experiments were not performed (Pereira et al 2002; Sparidans et al 2000). We appreciate that full validation is sometimes limited by the availability of adequate semen volume;

- The stability of ARVs after heat inactivation of HIV-1 in plasma or semen (3 h at 58°C) for safe handling was not discussed (Pereira et al 1999, 2002; Anderson et al 2000);
- Validated methods were reported for plasma analysis but not semen. Some authors diluted semen with plasma (1:1), presumably to reduce matrix effects; however, evidence for the appropriateness of this practice was not presented (van Praag et al 2000, 2001a, b; Taylor et al 1999, 2001b; Sankatsing et al 2002; Solas et al 2003; Ghosn et al 2004a; Isaac et al 2004);
- One assay was validated for multiple PIs but did not appear to include the PI examined in the study (Sankatsing et al 2002);
- One HPLC assay was used to quantify multiple drugs and to test for peak purity with retention time only. The chance of interference from metabolites or other drug therapy increases under these conditions, producing false positive results (van Praag et al 2001a; Sankatsing et al 2002; Solas et al 2003); and
- No testing for ion-suppression in HPLC mass spectrophotometry. Ion-suppression effects are caused by matrix components from blood and other substances and vary between biological matrices (Taylor 2005). Only one assay performed a complete validation by assessing ion-suppression effects (van Leeuwen 2007). Recent data suggests stable isotopically labelled internal standards do not overcome this limitation (Wang et al 2007).

A lack of systematic sampling is a limitation of studies of ARV penetration (Kashuba et al 1999), and there have been calls for new collection strategies (Reddy et al 2002). A proper understanding of ARV penetration is hampered by the lack of a separate therapeutic target in semen with which to compare effective blood plasma concentrations. The seminal target must be established because semen is different to blood and thus drug pharmacokinetics cannot be directly extrapolated from blood. There is also confusion whether one should measure free or bound drug and when to make such measurements (Taylor et al 2001b). In order to overcome these limitations, the protein-adjusted concentration required to inhibit 90% of viral replication (IC90) is frequently used as the therapeutic target in semen (Taylor et al 1999; Reddy et al 2002; Isaac et al 2004); however, the choice of this target to evaluate in-vivo drug potency is controversial (Montaner et al 2001). The in-vitro IC90 metric only measures compound potency (Drusano et al 2002) and protein binding to serum albumin at non-physiological concentrations, while ignoring other types including serum and semen proteins. The IC_{90} does not account for drug concentrations that vary with time in-vivo (Drusano et al 2002).

The limitations of the IC_{90} are borne out in the following examples. The IC_{90} for efavirenz is 25 ng mL^{-1} (Reddy et al 2002), but the effective concentration in-vivo to suppress viral replication in most patients is reported to be 1000 ng mL^{-1} (Marzolini et al 2001). A similar discrepancy is reported for the PIs. The in-vitro IC50 for amprenavir is 15 ng mL^{-1} (Sadler et al 2001), while a study investigating a concentration–response relationship in HIV-1-infected patients has identified a therapeutic target of 320 ng mL^{-1} (Sadler et al 2001). These examples suggest that the IC50 or IC90 is inappropriate. Furthermore, parameters may be poorly defined or used interchangeably, for example IC50–95 with EC50–95 (effective concentration), and MEC (minimum effective concentration) with C_{min} (minimum drug concentration, trough level after dosing).

The semen/blood ratio (SBR) compares total seminal ARV concentration and blood ARV concentration. SBR is not ideal, however, because it is dependent on the time of measurement after dosing. ARV blood levels are calculable using the dose and time of ingestion, volume of distribution and area under the curve, whereas seminal drug levels, as discussed earlier, must be estimated. SBR is also a single-point measure and cannot reflect adherence with ART (Reddy et al 2003). Serial SBRs are needed to elucidate seminal ARV concentration in steady-state conditions, but obtaining serial samples from patients in a timely manner is not practical.

Some workers suggest non-human primate models to overcome the problems identified with quantifying ARV levels in human semen, however the literature does not support using these models. Although there are similarities between HIV-1 and simian immunodeficiency virus (Milman & Sharma 1994), and between the MGT and non-human primate genital tracts (de Sousa et al 1995), non-human primate studies are difficult and costly to perform. Moreover, unlike human ejaculate, which coagulates in minutes, non-human primate ejaculate coagulates within seconds (Kashuba et al 1999), making analysis of seminal plasma impractical. Measuring ARV levels in the small amount of residual plasma is technically difficult (Lanzendorf 1990). Canine and feline models are unsuitable because there is no biological or virological analogy with the human system (Kashuba et al 1999).

Hitherto, studies have focused on ARV concentrations in seminal plasma. To our knowledge there are no published studies that quantify intracellular ARV concentration. ARV metabolism to active drug and antiviral activity occur within cells (except for enfuvirtide); effective intracellular ARV concentrations and thus viral suppression may therefore be attained despite low ARV concentrations in seminal plasma (van Praag et al 2001b). In effect, ARVs may first penetrate cells in the extraluminal compartment, with any 'spill-over' taken up by other cells (e.g. seminal leukocytes) or organs (e.g. prostate). This argument is based on the premise that ARVs enter semen via passive diffusion.

Conclusion

The effectiveness of ART in reducing sexual transmission of HIV-1 depends on adequate ARV penetration of the MGT. There is a concentration gradient from blood to MGT by virtue of passive diffusion, but within this gradient exists differences in individual ARV and ARV class penetration. The differential penetration of ARVs is not readily predictable with pharmacokinetics and physicochemical properties. Moreover, current assays differ in quality, precision and validation, and these factors should be considered when interpreting data on seminal ARV levels. Current problems include obtaining serial semen samples from the same individual, non-standardized semen collection methods, single-point random measurements of drug concentrations in semen (that do not predict steady-state concentrations or

drug exposure to the entire MGT), the effect of drug–protein binding in semen, inability to fractionate semen into its organ-specific parts (to quantify ARV penetration in different parts of the MGT), and the expense of mass spectrometry and processing of specimens. We recommend that: (i) quantification of seminal ARV concentration be improved by validating tests for seminal drug concentration, using serial time-point measurements of semen and/or blood plasma VL to calculate steady-state drug concentrations and area under the curve; (ii) calculating SBRs that are the best indicator of drug exposure within each compartment; (iii) standardizing collection and storage of semen specimens; (iv) reporting quantitative analytical procedures; and (v) validating tools to measure adherence with ART.

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