JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1997, p. 2886–2893 Vol. 35, No. 11
0095-1137/97/$04.00+0

Effects of Specimen Collection, Processing, and Storage Conditions on Stability of Human Immunodeficiency Virus Type 1 RNA Levels in Plasma

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Received 2 May 1997/Returned for modification 24 July 1997/Accepted 19 August 1997

To define the optimal blood collection parameters for plasma human immunodeficiency virus type 1 (HIV-1) viral load testing, plasma HIV-1 RNA levels were quantitated with the NASBA HIV-1 RNA QT System from blood specimens that were collected, processed, and stored under a variety of conditions that might have affected HIV-1 RNA stability. We determined that when whole blood was processed within 2 h of specimen collection the levels of HIV-1 RNA detected in EDTA-, heparin-, and acid citrate dextrose (ACD)-anticoagulated plasma samples were comparable. The levels of HIV-1 RNA in serum specimens (mean = 4.126 log units) were significantly lower ($P < 0.01$) than the levels in corresponding plasma samples (mean = 4.501 log units). One cycle of freeze-thaw (−70°C) did not significantly reduce the level of HIV-1 RNA detected in EDTA-, heparin-, or ACD-anticoagulated plasmas. The EDTA-anticoagulated plasmas showed the smallest decrease in HIV-1 RNA copies (0.050 log units). HIV-1 RNA levels decreased over a 6-month time period in serum as well as in EDTA-, ACD-, and heparin-anticoagulated plasmas stored at −70°C. However, the only significant decreases were for serum (mean decrease = 0.317 log units) and heparin-anticoagulated samples (mean decrease = 0.384 log units). A comparison of the levels of HIV-1 RNA in cell-free plasma collected in VACUTAINER EDTA Plasma Preparation Tubes and in standard VACUTAINER EDTA tubes determined that HIV-1 RNA levels were stable for up to 30 h after collection when stored at either room temperature (mean standard deviation [SD] = ± 0.101 log units) or at 4°C (mean SD = ± 0.102 log units) as cell-free plasma or as EDTA-anticoagulated whole blood (mean SD = ± 0.109 log units). These data indicate that EDTA-anticoagulated plasma is the most suitable and stable matrix for HIV-1 RNA quantitation.

The course of human immunodeficiency virus type 1 (HIV-1) disease progression and the efficacy of antiretroviral therapy are currently monitored by several surrogate markers, including plasma HIV-1 RNA levels (1–9, 11, 14, 17, 20, 23, 28). It has been shown that an increase in plasma HIV-1 RNA levels (viral load) correlates with $CD4^+$ T-cell depletion and disease progression (4–8, 14, 17, 20, 23, 28). In addition, studies indicate that significant decreases in plasma HIV-1 RNA levels correspond to clinical response after the initiation of new antiretroviral therapy (1, 2, 4, 9, 11). Therefore, the quantitation of plasma HIV-1 RNA levels may be the most sensitive indicator of both disease progression and response to antiretroviral therapy and may significantly complement the information provided by additional immunocompetence markers, such as $CD4^+$ T-cell counts. Consequently, the measurement of plasma HIV-1 RNA levels may substantially contribute to improved patient care and the management of HIV disease.

The accuracy and reproducibility of plasma HIV-1 RNA measurements are paramount if this surrogate marker is to be used routinely in patient management. Furthermore, proper design of large clinical trials will require appropriate attention to sample collection and processing. It is critical that blood collection, processing, and storage methods which provide the highest degree of HIV-1 RNA stability are standardized and employed by all sites responsible for the collection, transportation, and testing of such samples. This standardization will not only ensure the accuracy of the test results but allow the comparison of data, especially when specimens are collected and transported from sites which are a part of multicenter clinical trial studies.

Several studies have shown that the stability of cell-free HIV-1 RNA is variably affected by several blood collection parameters, including the types of anticoagulants used (10, 13, 18, 25), the length of time between specimen collection and specimen preparation (13, 18, 19, 21), and plasma storage temperature (3, 13, 18, 19). However, comprehensive data that define additional specimen-handling and storage requirements essential to establishing optimal parameters for viral load testing are not yet available. To address these issues, we quantitated plasma HIV-1 RNA levels using the NASBA HIV-1 RNA QT System (Organon Teknika, Durham, N.C.) from blood specimens collected, processed, and stored under various conditions that might be expected to affect RNA stability. Initially we compared HIV-1 RNA levels in serum and plasma collected with sodium heparin, potassium EDTA, or acid citrate dextrose (ACD) anticoagulants to determine if differences in the collection tubes affected viral titer measurements. We also examined the effects of both a freeze-thaw cycle and long-term storage at −70°C on HIV-1 RNA stability in serum and plasma specimens collected with the various anticoagulants. Finally, we evaluated a new blood tube designed specifically for HIV-1 viral load analysis, the VACUTAINER Plasma Preparation Tube (PPT) with potassium EDTA (Bec-

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ton Dickinson VACUTAINER Systems, Franklin Lakes, N.J.). The EDTA-PPT is a plastic, sterile, evacuated tube which draws 5.0 ml of blood, has a potassium EDTA anticoagulant spray coated on the interior tube wall to avoid dilution of the blood, and a polyester gel barrier to separate the plasma from the cellular blood elements during transport. We compared the performance of the EDTA-PPT to standard VACUTAINER blood collection tubes with EDTA by evaluating the effects of time delay and temperature prior to specimen processing, the effects of freezing-thawing plasma specimens, and the effects of cellular elements in whole blood on HIV-1 RNA stability.

(This study was presented in part at the 4th Conference on Retroviruses and Opportunistic Infections, Washington, D.C., 1997.)

MATERIALS AND METHODS

Plasma HIV-1 quantitation with the NASBA HIV-1 RNA QT System. Plasma HIV-1 RNA levels were quantitated with the NASBA HIV-1 QT System (Organon Teknika), according to the manufacturer's instructions (26, 27).

In-house measurement of NASBA HIV-1 RNA QT Assay variance and accuracy. Two technologists independently assayed five HIV-1 RNA standards (6.220, 5.610, 4.990, 4.280, and 3.680 log unit copies/100-μl input) in duplicate, over four independent runs with three different kit lots (A, B, and C) for a total of 48 tests per standard. To determine assay variance, the mean HIV-1 RNA log values ± standard deviations per standard were calculated per individual lot and for the three lots combined. For each standard, assay accuracy was determined by percent recovery of expected HIV-1 RNA input.

Patient population and blood collection. Twenty well-characterized HIV-1 seropositive subjects who regularly attend the North Shore University Hospital Center for AIDS Research and Treatment, Manhasset, N.Y., were recruited for two independent investigations. Informed consent was obtained prior to blood collection. Peripheral blood was collected by a single venipuncture from two groups of 10 HIV-1 seropositive patients for the experimental protocols described below.

(i) Patient group 1. Whole blood for studies 1, 2, and 3 was collected from 10 HIV-1 seropositive patients in the following blood collection tubes (Becton Dickinson): 10-ml standard VACUTAINER-ACD (V-ACD), VACUTAINER sodium heparin (V-H), VACUTAINER potassium EDTA (V-E), and VACUTAINER serum (V-S) collection tubes.

(a) Study 1: comparison of HIV-1 RNA titers in serum and cell-free plasma collected with various anticoagulants. An experiment was designed to compare the levels of HIV-1 RNA in serum and cell-free plasma collected with various anticoagulants. All V-ACD, V-H, V-E, and V-S tubes collected from the 10 patients in group 1 were centrifuged at 1,500 × *g* for 20 min in a swinging bucket rotor (RT6000B; Sorvall) immediately after collection. Aliquots (100 μl) of plasma and serum from each tube (time zero) were placed in NASBA HIV-1 QT RNA lysis buffer (Organon Teknika) and vortexed briefly. The lysis buffer tubes were immediately stored at −70°C until they were tested with the NASBA HIV-1 RNA QT System.

(b) Study 2: effect of freezing-thawing on levels of HIV-1 RNA in serum and cell-free plasma collected with various anticoagulants. To determine whether a freeze-thaw cycle would significantly affect the number of HIV-1 RNA copies detected in serum and cell-free plasma, we performed the following experiment. ACD-, EDTA-, and heparin-anticoagulated plasma and serum aliquots from each patient in group 1 were frozen at −70°C immediately after centrifugation as described above for study 1. After 1 week of storage at −70°C the plasma and serum aliquots were quick thawed, and 100 μl of each aliquot was added to NASBA lysis buffer. The NASBA lysis buffer tubes were vortexed briefly. HIV-1 RNA levels were quantitated with the NASBA HIV-1 RNA QT System. HIV-1 RNA levels from study 2 samples were compared to the values obtained from corresponding samples in study 1 (time zero).

(c) Study 3: effect of long-term storage on stability of HIV-1 RNA in serum and cell-free plasma. To determine if the long-term storage of cell-free plasma or serum at −70°C led to a significant decrease in HIV-1 RNA levels, the following experiment was conducted. Six serum and six EDTA-, heparin-, and ACD-anticoagulated cell-free plasma aliquots from each patient in group 1 were frozen in cryovials (Nalge Nunc Intl., Roskilde, Denmark) at −70°C immediately after collection and centrifugation as described for study 1. At 1, 2, 3, 4, and 6 months postcollection, serum and plasma aliquots of each anticoagulant type were removed from −70°C storage and quick thawed. Aliquots (100 μl) of each sample were placed in NASBA lysis buffer. HIV-1 RNA levels from each sample were quantitated with the NASBA HIV-1 RNA QT System, and the results were compared to the HIV-1 RNA levels obtained from corresponding samples in study 1 (time zero).

(ii) Patient group 2. Whole blood for studies 4, 5, and 6 was collected from 10 HIV-1 seropositive patients in the following blood collection tubes: triplicate 5-ml standard V-E tubes (V-E-1, -2, and -3) and duplicate 8-ml VACUTAINER PPTs (PPT 4°C and PPT 23°C). The VACUTAINER EDTA-PPTs contain a gel barrier which, after centrifugation, physically separates the plasma from cellular components including platelets. The EDTA-PPTs are currently experimental and are not commercially available. The following experiments were designed to (i) evaluate the EDTA-PPT for its performance as a new blood tube designed specifically for HIV-1 viral load analysis, (ii) evaluate the effects of time delay and temperature prior to specimen processing, (iii) evaluate the effects of freezing-thawing plasma specimens, and (iv) evaluate the effects of cellular fractions in whole blood on HIV-1 RNA stability.

(a) Study 4: evaluation of the VACUTAINER EDTA-PPT and effects of whole blood over time on plasma HIV-1 RNA levels. An experiment was designed to evaluate the VACUTAINER EDTA-PPT as a suitable and potentially superior blood collection device for viral load testing compared to the standard V-E blood collection tube. In addition, the experiment was designed to determine if, over time at room temperature, the presence of cellular elements in whole blood would affect the stability of plasma HIV-1 RNA levels. The V-E-1 tube and one EDTA-PPT collected from each of the 10 patients in group 2 were centrifuged at 1,500 × *g* for 20 min in a swinging bucket rotor (RT6000B; Sorvall) at 2 h postcollection. A 100-μl aliquot of cell-free plasma from each sample was placed in NASBA HIV-1 QT RNA lysis buffer, vortexed briefly, and immediately stored at −70°C until tested. The V-E-2 and V-E-3 tubes were stored at room temperature as whole blood until centrifugation, as described above, at 8 and 30 h postcollection, respectively. At the respective times, 100-μl aliquots of cell-free plasma from each tube were placed in NASBA HIV-1 QT RNA lysis buffer and vortexed briefly. The lysis buffer tubes were immediately stored at −70°C until being tested. The centrifuged EDTA-PPT was stored at room temperature (PPT 23°C), and at 8 and 30 h, cell-free plasma aliquots were placed in NASBA lysis buffer, vortexed, and frozen at −70°C until they were tested. The plasma HIV-1 RNA levels from whole blood stored for 2, 8, and 30 h at 23°C prior to processing were compared to the HIV-1 RNA levels in the corresponding 2-, 8-, and 30-h samples collected in the EDTA-PPTs and stored as cell-free plasma at 23°C.

(b) Study 5: effects of temperature and time on stability of HIV-1 RNA in cell-free plasma collected in VACUTAINER EDTA-PPTs. An experiment was designed to assess the effects of both preprocessing storage temperature (refrigeration at 4°C versus room temperature at 23°C) and preprocessing time delays (8 and 30 h) on the stability of HIV-1 RNA in cell-free plasma. Immediately after collection, one EDTA-PPT (PPT 4°C) from each patient was refrigerated at 4°C and one EDTA-PPT (PPT 23°C) from each patient was held at room temperature. At 2 h postcollection the EDTA-PPT 4°C and the EDTA-PPT 23°C tubes were centrifuged at 1,500 × *g* for 20 min in a swinging bucket rotor (RT6000B; Sorvall). Aliquots (100 μl) of cell-free plasma from each tube were placed in NASBA HIV-1 QT RNA lysis buffer and stored at −70°C until they were tested. The centrifuged EDTA-PPT 4°C tubes were held at 4°C and the centrifuged EDTA-PPT 23°C tubes were held at room temperature (23°C). At 8 and 30 h postcollection, 100-μl plasma aliquots from each centrifuged EDTA-PPT 4°C and EDTA-PPT 23°C tube were placed in NASBA lysis buffer and stored at −70°C until they were tested with the NASBA HIV-1 RNA QT System.

(c) Study 6: effects of freezing-thawing on levels of HIV-1 RNA in cell-free plasma collected in VACUTAINER EDTA-PPTs. An experiment was designed to determine if HIV-1 RNA levels in cell-free plasma collected and stored at 4°C in VACUTAINER EDTA-PPTs remained stable after one cycle of freezing-thawing. The EDTA-PPT 4°C tubes collected from the 10 patients in group 2 were placed at 4°C immediately after collection. At 2 h postcollection the EDTA-PPT 4°C tubes were centrifuged, and plasma aliquots from each EDTA-PPT 4°C tube were frozen at −70°C. After 1 week of storage at −70°C, the plasma aliquots were quick thawed. For each sample, 100 μl of plasma was added to NASBA lysis buffer and was vortexed briefly. HIV-1 RNA levels were quantitated with the NASBA HIV-1 RNA QT System. HIV-1 RNA levels were compared to those values obtained from corresponding samples in study 5 (2 h, 4°C).

Data analysis and statistics. All HIV-1 RNA levels are expressed as log unit copies per sample input of 100 μl. Values obtained with ACD-anticoagulated plasmas were adjusted for a dilution effect of the anticoagulant and were based upon total volume of sample collected in each tube and volume of anticoagulant (1.5 ml). Heparin (dried) and EDTA (0.117 ml) anticoagulants did not significantly alter the HIV-1 RNA levels. The correlation of viral load measurements for a subject from serum and plasma samples collected with various anticoagulants was analyzed using the repeated measures analysis of PROM GLM within the SAS/STAT module (SAS Institute Incorporated, Cary, N.C.). An overall multivariant test of differences among the four responses of each subject was performed. This is basically an extension of the paired *t* test from two responses to four responses. In addition, separate contrasts testing each anticoagulant response versus the serum response were also made. These latter contrasts are equivalent to paired *t* tests. The present results were also interpreted relative to in-house assay variance determinations and data obtained previously by other investigators that indicated an expected assay maximum variability range of ±0.300 log units (27).

RESULTS

In-house measurement of NASBA HIV-1 RNA QT Assay variance and accuracy. As shown in Table 1, our in-house

TABLE 1. In-house measurement of NASBA HIV-1 RNA QT Assay variance and accuracy

Standard[a]	Calculated mean (±SD) log values for kit lot[b]:				% Accuracy[d]
	A	B	C	ABC[c]	
I (6.220)	6.077 (0.104)	6.275 (0.104)	6.178 (0.067)	6.177 (0.123)	99.31
II (5.610)	5.557 (0.114)	5.719 (0.064)	5.641 (0.078)	5.639 (0.109)	104.75
III (4.990)	5.000 (0.121)	5.141 (0.131)	4.994 (0.084)	5.045 (0.131)	101.10
IV (4.280)	4.322 (0.136)	4.457 (0.150)	4.351 (0.101)	4.377 (0.141)	102.27
V (3.680)	3.657 (0.235)	3.813 (0.137)	3.661 (0.166)	3.710 (0.194)	100.79

[a] Numbers in parentheses are expected log values of HIV-1 RNA copies per 100 μl of input.
[b] Data are numbers of HIV-1 RNA copies per 100 μl of input expressed as mean log values for 16 tests of each kit lot.
[c] Mean log values for lots A, B, and C combined. Standard deviations indicate mean log assay variation per RNA standard.
[d] Percent HIV-1 RNA recovered relative to expected values for lots A, B, and C combined.

assay variances for the five standards tested with three different lots of the NASBA HIV-1 RNA QT Assay were all within the expected variation of the assay (±0.300 log units) in accordance with the published literature (26). The individual lot variations per standard ranged from ±0.064 to ±0.235 log units, and the combined lot variations per standard ranged from 0.109 to 0.194 log units. The accuracies of the assay, as determined by the percent recovery of the expected HIV-1 RNA input value per standard combined for the three different kit lots, ranged from 99.31 to 104.75%, with a mean of 101.64% (acceptable range, 91.85 to 108%).

Comparison of HIV-1 viral titers in serum and cell-free plasma collected with various anticoagulants. HIV-1 RNA was detected with the NASBA HIV-1 RNA QT System in all plasma and serum specimens from the 10 subjects in group 1 (Table 2). Overall, the plasma matrix specimens contained a higher HIV-1 RNA copy number than did the serum specimens, as indicated by the average log unit RNA copy numbers for each matrix type (EDTA = 4.477; heparin = 4.538; ACD = 4.487; serum = 4.126). The average HIV-1 RNA log unit copy number for all plasma specimens (4.501 log units) was significantly greater than that determined for the serum specimens (4.126 log units) ($P < 0.01$).

When the results for each of the plasma specimen types were compared to the serum results, the serum results were less than those obtained for the corresponding plasmas, with the exception of specimen no. 6, which had been collected with a V-E tube. For specimen no. 6, equivalent results of 1,500,000 copies/100 μl were obtained for both the serum sample and the plasma sample collected with EDTA anticoagulant. These results were shown to be statistically significant for each pair of serum and plasma specimens by using paired *t* tests (EDTA, $P < 0.01$; ACD, $P < 0.02$; heparin, $P < 0.01$).

Effect of freezing-thawing on stability of HIV-1 RNA in serum and cell-free plasma collected with various anticoagulants. We compared the number of HIV-1 RNA copies detected in serum and plasma specimens added directly to NASBA lysis buffer at the time of processing to the number of copies detected from serum and plasma specimens which had been frozen at −70°C and then thawed prior to testing. The mean log variations in the HIV-1 RNA copies quantitated from the serum and the ACD-, EDTA-, and heparin-anticoagulated plasmas stored at −70°C for 1 week compared to those quantitated from the serum and plasma specimens placed directly in NASBA lysis buffer at the time of collection are shown in Table 3. The serum samples showed an increase in HIV-1 RNA copies (+0.073 log units), with three samples having significant variations from the mean (no. 1 = +0.398 log units, no. 7 = +0.967 log units and no. 10 = −0.301 log units). Plasma samples collected with EDTA anticoagulant were the least affected by the freeze-thaw cycle (mean variation, −0.050 log units) compared to plasma collected with ACD (mean variation, −0.219 log units) or with heparin (mean variation, −0.240 log units). Mean variations were not significant.

When examining the effects of a freeze-thaw cycle on individual samples, EDTA-collected samples were again the most stable, with only one specimen displaying a significant RNA loss (no. 7, −0.412 log units), followed by two ACD-collected samples (no. 3, −0.846 log units; no. 9, −0.376 log units) and

TABLE 2. Comparison of HIV-1 RNA levels in serum and cell-free plasma collected with EDTA, ACD, and heparin anticoagulants

Specimen no.	No. of HIV-1 RNA copies/100 μl of input (log)					Plasma-serum variation[b]
	Cell-free plasma collected with:			Mean plasma value[a]	Serum	
	EDTA	Heparin	ACD			
1	4.114	4.255	4.014	4.128	4.000	−0.128
2	4.613	4.663	4.585	4.620	4.491	−0.129
3	3.505	3.708	3.758	3.657	3.230	**−0.427**
4	4.845	5.146	4.886	4.959	4.623	**−0.336**
5	5.000	4.914	5.053	4.989	4.740	−0.249
6	6.176	6.230	6.405	6.270	6.176	−0.094
7	4.491	4.146	4.173	4.270	3.431	**−0.839**
8	4.613	4.778	4.702	4.698	4.113	**−0.585**
9	3.740	3.833	3.939	3.837	3.204	**−0.633**
10	3.672	3.708	3.352	3.577	3.255	**−0.322**
Mean	4.477	4.538	4.487	4.501	4.126	**−0.375**

[a] Mean plasma HIV-1 RNA copies per 100 μl of EDTA-, heparin-, and ACD-collected samples combined per patient.
[b] Numbers in boldface reflect log variances greater than the variability (±0.300 log units) normally expected for the assay.

TABLE 3. Effects of one freeze-thaw cycle on HIV-1 RNA levels in serum and plasma collected with EDTA, ACD, and heparin anticoagulants

Sample no.	Log variation in HIV-1 RNA levels in samples frozen prior to processing compared to time zero samples[a]			
	Cell-free plasma collected with:			
	EDTA	ACD	Heparin	Serum
1	−0.195	−0.125	−0.176	+0.398
2	−0.136	−0.213	−0.584	−0.093
3	+0.158	−0.846	−0.386	0.000
4	+0.076	−0.118	+0.030	−0.118
5	−0.244	−0.201	−0.051	−0.032
6	−0.062	−0.222	−0.054	−0.217
7	−0.412	0.000	−0.347	+0.967
8	−0.057	−0.160	−0.548	−0.035
9	+0.224	−0.376	−0.220	+0.158
10	+0.148	+0.067	−0.065	−0.301
Mean	−0.050	−0.219	−0.240	+0.073

[a] Time zero samples were processed immediately after collection. −, decrease in HIV-1 RNA copies from time zero values; +, increase in HIV-1 RNA copies from time zero values. Underlined values indicate changes in HIV-1 RNA copies from time zero values which are outside of the expected variance of the assay.

four heparin-collected samples (no. 2, −0.584 log units; no. 3, −0.386 log units; no. 7, −0.347 log units; and no. 8, −0.548 log units). Although these data suggest that there is generally a loss of plasma HIV-1 RNA copies as the result of a freeze-thaw cycle, overall the mean loss for the 10 plasma samples per anticoagulant is insignificant and falls within the expected variability range of the assay itself. The RNA loss was variable and dependent upon both the specimen and the type of anticoagulant present. These data indicate that EDTA provides a more stable matrix than ACD or heparin, with no significant loss of HIV-1 RNA copies when plasma is stored at −70°C prior to testing.

Effects of long-term storage at −70°C on stability of HIV-1 RNA in serum and cell-free plasma collected with various anticoagulants. To determine if long-term storage (up to 6 months) at −70°C affected viral RNA titers, we quantitated the HIV-1 RNA levels at 1, 2, 3, 4, and 6 months postcollection in serum and EDTA-, ACD-, and heparin-anticoagulated cell-free plasmas stored at −70°C. These results were compared to the HIV-1 RNA levels detected in comparable serum and cell-free plasma specimens placed in lysis buffer immediately after specimen collection (time zero).

As shown in Table 4, the mean HIV-1 RNA levels in EDTA-, ACD-, and heparin-anticoagulated cell-free plasmas decreased during 6 months of storage at −70°C. The decreases in viral titers were variable and due to a combination of RNA loss from one cycle of freeze-thaw (Table 3) and long-term storage over time. The decreases in viral titers were significant (≥0.300 log units) for heparin-anticoagulated plasmas, based upon the assumption of an expected ±0.300 log unit variation of the assay. For the 10 heparinized plasmas the mean loss due to a freeze-thaw cycle was 0.240 log units, the mean loss due to long-term storage was 0.144 log units, and the total mean RNA loss over 6 months was 0.384 log units or 8.46 log% of time zero RNA copies. The decreases in RNA levels were not significant for samples collected with EDTA and ACD. For the 10 EDTA-anticoagulated plasmas, the mean loss due to a freeze-thaw cycle was 0.050 log units, the mean loss due to long-term storage was 0.192 log units, and the total mean loss over 6 months was 0.242 log units or 5.4 log% of time zero RNA copies. For the 10 ACD-anticoagulated plasmas, the mean loss due to a freeze-thaw cycle was 0.219 log units, the mean loss due to long-term storage was 0.052 log units, and the total mean loss over 6 months was 0.271 log units or 6.04 log% of time zero RNA copies. The HIV-1 RNA levels in the 10 serum samples had a mean decrease over 6 months of storage of 0.317 log units or 7.68 log% of time zero and was related to RNA loss during storage.

Evaluation of the VACUTAINER EDTA-PPT and comparison of HIV-1 viral titer stability over time in whole blood versus cell-free plasma. Baseline RNA levels were determined for samples (patient group 2) collected in V-E tubes and EDTA-PPTs which had been centrifuged and processed at 2 h postcollection. A 2-h time point was chosen for baseline levels, since this time frame most closely reflects the minimal time necessary to collect, transport, and process clinical samples. We compared the 2-h baseline plasma HIV-1 RNA levels to HIV-1 RNA levels measured at 8 and 30 h for matching plasma samples stored as whole blood collected in V-E tubes or as cell-free plasma collected and stored in EDTA-PPTs.

As shown in Table 5, the mean log values of plasma HIV-1 RNA detected at 2-, 8-, and 30-h time points were comparable for the 10 samples stored at room temperature as EDTA-anticoagulated whole blood (3.861 log units) or as EDTA-anticoagulated cell-free plasma (3.859 log units). The standard deviations of the mean log values at 2, 8, and 30 h for the 10 cell-free plasmas (range, 0.045 to 0.156 log units) and for the 10 whole-blood samples (range, 0.035 to 0.269 log units) were all within the expected variation of the assay itself (±0.300 log units). The variation in the number of HIV-1 RNA copies as a

TABLE 4. Effects of long-term storage at −70°C on serum and plasma HIV-1 RNA levels

Time (mo)	Mean no. of HIV-1 RNA copies/100 μl of input (log [log %])[a]			
	Cell-free plasma collected with:			Serum
	EDTA	Heparin	ACD	
0	4.477	4.538	4.487	4.126
1	4.373 (97.68)	4.188 (92.29)	4.213 (93.90)	3.840 (93.07)
2	4.237 (94.63)	4.251 (93.68)	4.197 (93.55)	3.826 (92.73)
3	4.195 (93.70)	4.305 (94.86)	4.332 (96.56)	3.946 (95.64)
4	4.160 (92.92)	4.067 (89.62)	4.148 (92.45)	3.697 (89.60)
6	4.210 (94.04)	3.962 (87.31)	4.190 (93.38)	3.735 (90.52)
Mean loss[b]	0.242 (5.4)	0.384 (8.46)	0.271 (6.04)	0.317 (7.68)

[a] Data are mean log values of HIV-1 RNA copies per 100 μl of 10 specimens per time point and specimen matrix. Data in parentheses are HIV-1 RNA levels expressed as log percentage of time zero HIV-1 RNA values.

[b] Mean HIV-1 RNA loss over 6 months expressed as log values. Data in parentheses are mean HIV-1 RNA losses expressed as log percentage of time zero HIV-1 RNA values

TABLE 5. Stability of plasma HIV-1 RNA over time when stored as EDTA-anticoagulated whole blood versus EDTA-anticoagulated cell-free plasma

Sample no.	Mean no. of HIV-1 RNA copies/100 μl of input (log ± SD)		
	Cell-free plasma[a]	Whole blood[b]	Mean variation (whole blood vs plasma)
666	2.894 ± 0.082	2.961 ± 0.232	+0.067
667	3.385 ± 0.045	3.329 ± 0.131	−0.056
668	3.376 ± 0.149	3.425 ± 0.269	+0.049
669	4.245 ± 0.102	4.249 ± 0.075	+0.004
811	4.304 ± 0.120	4.320 ± 0.053	+0.016
812	4.810 ± 0.062	4.823 ± 0.035	+0.013
813	4.206 ± 0.094	4.295 ± 0.048	+0.089
842	3.329 ± 0.093	3.466 ± 0.125	+0.137
843	4.127 ± 0.111	4.053 ± 0.046	−0.074
845	3.915 ± 0.156	3.691 ± 0.079	−0.224
Mean[c]	3.859 ± 0.101	3.861 ± 0.109	+0.002

[a] Mean log values of HIV-1 RNA copies for the 2-, 8-, and 30-h time points combined for plasma collected in PPTs.
[b] Mean log values of HIV-1 RNA copies for the 2-, 8-, 30-h time points combined for plasma collected in V-E tubes.
[c] Mean log values of HIV-1 RNA copies for 10 patients per collection tube and time point combined.

result of collection and storage conditions between the whole-blood and plasma samples ranged from −0.224 to +0.137 log units, with a mean variation of +0.002 log units. These data indicate that the levels of HIV-1 RNA in cell-free plasma, separated from cellular components by the gel barrier of the EDTA-PPT, and the levels of HIV-1 RNA in whole blood are highly comparable and stable at room temperature for up to 30 h postcollection.

Effects of temperature and time on stability of HIV-1 RNA in cell-free plasma. A comparison of the stability of HIV-1 RNA levels in cell-free plasma stored at either room temperature (23°C) or under refrigeration (4°C) is presented in Table 6. The mean log values of individual specimens stored for 2, 8, and 30 h at 23°C were found to be equivalent at all time points to the corresponding specimens stored at 4°C. The combined mean log values at 2, 8, and 30 h for all 10 patients were equivalent at 23°C (3.860 log units) and 4°C (3.804 log units). The standard deviations of the mean log values of the RNA

TABLE 6. Effects of processing temperature over time on HIV-1 RNA levels in EDTA-anticoagulated plasma from PPTs

Sample no.	Mean no. of HIV-1 RNA copies/μl of input (log ± SD) for plasma stored in PPTs at[a]:		
	23°C	4°C	Mean variation (23 vs 4°C)
666	2.894 ± 0.082	2.869 ± 0.090	+0.025
667	3.385 ± 0.045	3.318 ± 0.170	+0.067
668	3.376 ± 0.149	3.249 ± 0.174	+0.127
669	4.245 ± 0.102	4.202 ± 0.039	+0.043
811	4.304 ± 0.120	4.272 ± 0.093	+0.032
812	4.810 ± 0.062	4.845 ± 0.089	−0.035
813	4.206 ± 0.094	4.189 ± 0.092	+0.017
842	3.329 ± 0.093	3.225 ± 0.079	+0.104
843	4.127 ± 0.111	4.071 ± 0.102	+0.056
845	3.915 ± 0.156	3.803 ± 0.096	+0.112
Mean[b]	3.860 ± 0.101	3.804 ± 0.102	+0.056

[a] Mean log values of HIV-1 RNA copies for the 2-, 8-, and 30-h time points combined for plasma collected in PPTs and stored at 23 or 4°C.
[b] Mean log values of HIV-1 RNA copies for 10 patients per collection tube and time point combined.

TABLE 7. Effects of one freeze-thaw cycle on HIV-1 RNA levels in plasma collected in EDTA-PPTs

Sample no.	No. of HIV-1 RNA copies/ 100 μl of input (log)[a]		Variation (freeze-thaw vs 2-h storage)[b]
	2-h storage (4°C)	Freeze-thaw	
666	2.934	2.954	+0.020
667	3.505	3.398	−0.107
668	3.205	3.176	−0.029
669	4.146	4.114	−0.032
811	4.342	4.255	−0.087
812	4.908	4.716	−0.192
813	4.230	4.114	−0.116
842	3.176	3.176	0.000
843	4.079	4.041	−0.038
845	3.806	3.690	−0.116
Mean	3.833	3.763	−0.070

[a] HIV-1 RNA levels were compared in EDTA-anticoagulated plasma samples collected in PPTs and stored either as whole blood for 2 h at 4°C (2-h storage) or as plasma aliquots frozen at −70°C and thawed prior to RNA quantitation.
[b] Log increase or decrease in RNA levels after one cycle of freeze-thaw.

copies, combined for the 2-, 8-, and 30-h time points, for plasma stored at 23°C (range, 0.045 to 0.156 log units) and at 4°C (range, 0.039 to 0.174 log units) were all within the expected variation of the assay (±0.300 log units). The mean variation due to differences in collection and storage conditions between the two groups of specimens was 0.056 log units. These data indicate that HIV-1 RNA levels are highly comparable and stable in cell-free plasma stored at either room temperature (23°C) or under refrigeration (4°C) for up to 30 h prior to testing.

Effects of freezing-thawing on levels of HIV-1 RNA in cell-free plasma collected in VACUTAINER EDTA-PPTs. Table 7 compares the HIV-1 RNA levels in EDTA-anticoagulated plasmas which had been stored as whole blood for 2 h at 4°C with those of plasma aliquots from the same samples which had been frozen at −70°C and thawed prior to HIV-1 RNA quantitation. The mean variation for the 10 samples was −0.070 log units (range, −0.192 to +0.020 log units) and was not significant. All individual sample deviations from time zero were also nonsignificant. These results are in agreement with study 3 which demonstrated that EDTA-anticoagulated plasma specimens stored at room temperature prior to freezing at −70°C did not have a significant reduction in the number of detectable HIV-1 RNA copies after one cycle of freeze-thaw.

DISCUSSION

Quantitative HIV-1 RNA testing by using various amplification techniques (9, 11, 22–27) has become an essential component in evaluating patient prognosis and in assessing the efficacy of antiretroviral therapy for all HIV-positive patients (1–9, 14, 17, 20, 23, 28). In addition, quantitative viral load measurements are critical for clinical trial studies of new antiretroviral agents (1, 2, 3, 9, 11). Therefore, it is imperative that blood collection, processing, and storage methods be optimized so as to assure accuracy and reproducibility of viral load test results not only within a specific laboratory but among different laboratories and across different test methodologies.

Several studies have focused on identifying the blood collection and processing parameters which will ensure accuracy and reproducibility of quantitative HIV-1 RNA viral load testing. Mole et al. (18), using both a reverse transcription–PCR–enzyme-linked immunosorbent assay (RT-PCR-ELISA) (9)

and a Quantiplex bDNA (branched DNA) HIV-RNA assay (Chiron Corporation, Emeryville, Calif.) (22, 24), examined the effects of both preprocessing time delays and blood collection tubes on the stability of plasma HIV-1 RNA. Using the RT-PCR-ELISA they found that after 24 and 72 h HIV-1 RNA levels in cell-free plasma stored in VACUTAINER cell preparation tubes (CPTs) are consistently higher and more stable than HIV-1 RNA levels in plasma stored as whole blood in standard VACUTAINER tubes. The viral loads in CPTs and VACUTAINER tubes declined significantly over time (72 h) as measured with the bDNA assay.

A multicenter study by Holodniy et al. (13) compared the recovery and stability of HIV-1 virion-associated RNA levels in plasma from whole blood collected in VACUTAINER CPT, VACUTAINER PPT, VACUTAINER serum separation tubes, and the standard VACUTAINER tubes with sodium heparin, ACD, sodium citrate, and potassium EDTA used as anticoagulants. Using the bDNA signal amplification method (22, 24), they demonstrated a nonsignificant decrease in plasma HIV-1 RNA when stored as EDTA-anticoagulated whole blood for up to 30 h. In addition, HIV-1 RNA levels did not decrease as significantly in cell-free plasma stored at room temperature in either citrate PPTs or citrate CPTs as they did in standard VACUTAINER citrate tubes.

Since the methodology for NASBA-based RNA quantitation is significantly different from that of the RT-PCR and bDNA assays, it was essential to determine if blood collection procedures and sample storage conditions would similarly affect HIV-1 RNA quantitation by the NASBA method. In addition, several parameters, such as long-term blood storage, have not been examined in past studies. The results derived from these studies would in most cases be applicable to specimen-handling protocols independent of the test methodology. Therefore, to assure optimal testing conditions for HIV-1 RNA quantitation, we designed experiments which evaluated the effects of different blood collection protocols, temperature, time to specimen processing, and specimen storage conditions on the stability and reproducibility of HIV-1 RNA measurements. Included in these studies was an evaluation of a blood collection tube designed specifically for viral load analysis, the PPT tube.

In-house testing proficiency and assay variance were determined to ensure that our test results and the comparison of data between test parameters were accurate and in agreement with the expected assay maximum variability of ± 0.300 log units (27). Five HIV-1 RNA standards of known concentrations were assayed independently by two technologists over multiple test runs and three kit lots. The individual lot variations per standard, based upon 16 repeats per standard, ranged from ± 0.064 to ± 0.235 log units, with the greatest variance demonstrated for the standard with the lowest number of HIV-1 RNA copies. These data confirm that a variation of ≥ 0.300 log units is an acceptable criterion for determining significant differences in HIV-1 RNA copy number when comparing the test parameters in the study. The in-house accuracy of the assay, as determined by the percent recovery of the expected HIV-1 RNA input value per standard, for the three kit lots combined, ranged from 99.31 to 104.75%, with a mean of 101.64%. These data clearly indicate that in our laboratory the NASBA HIV-1 RNA QT Assay has excellent accuracy and precision.

Our initial studies determined the effects of various blood collection tubes on HIV-1 RNA levels. Cell-free plasma separated from whole-blood samples collected in VACUTAINER tubes with ACD, heparin, or potassium EDTA anticoagulants had comparable levels of HIV-1 RNA copies when processed within 2 h of collection time. However, V-ACD tubes contain 1.5 ml of anticoagulant, which could result in a dilution effect on the measurement of viral load when small volumes of blood are drawn. V-H tubes contain dried anticoagulant and V-E tubes contain only 117 μl of anticoagulant, and therefore, there is no significant dilution effect when viral load is measured in plasma collected in these tubes. However, heparin anticoagulant can have an inhibitory effect on PCR-based HIV-1 amplification assays (10).

Although HIV-1 RNA titers can be detected in serum samples, the levels of HIV-1 RNA copies were significantly lower in serum than in matched EDTA-, ACD-, or heparin-anticoagulated plasma samples (P, <0.01, <0.02, or <0.01, respectively). The reduced levels of HIV-1 RNA in serum samples may result from the entrapment of either free viral particles or virion-immune complexes during the clotting process. The variation in the number of HIV-1 RNA copies in serum versus that in matched plasma samples ranged from -0.094 to -0.839 log units, and the RNA loss was significant (>0.300 log units) in 5 of the 10 serum samples. These results are in agreement with those of Holodniy et al. (13), who found that HIV-1 RNA levels measured by the bDNA assay are significantly lower in serum specimens than in plasma specimens. These data indicate that the loss of HIV-1 RNA copies in serum samples can be highly variable from one patient to another. In addition, the loss may also vary from one sample to another for the same patient. These data suggest that although HIV-1 RNA levels can be quantitated in serum samples, serum is not a suitable matrix for accurate and reproducible results due presumably to intrinsic variations in the clotting process.

Blood samples for HIV-1 plasma viral loads are often collected at physicians' offices or blood-drawing sites which are not capable of separating plasma from cellular blood components prior to shipment to off-site laboratories for testing. As a result samples may be stored as whole blood at room temperature for as long as 24 to 30 h prior to plasma separation and storage. Several conflicting studies have examined the possibility that the presence of cellular fractions of whole blood may result in a decrease of measurable HIV-1 RNA (12, 13, 15, 16, 21, 23). It has been suggested that HIV-1 virion absorption to platelets could result in significantly lower plasma HIV-1 RNA levels (16). However, studies by Piatak et al. (23) and Holodniy et al. (13) found that $<5\%$ of the RNA level was platelet associated. The loss of viral RNA may also be enhanced by the type of anticoagulant present in the plasma sample (13, 18, 25). Preliminary studies conducted in our laboratory indicated that plasma HIV-1 RNA levels had variable stability over time (unpublished data). We detected up to a 1 log unit decrease in plasma HIV-1 RNA levels when samples were left for 18 h at room temperature as whole blood with ACD anticoagulant. These results are in agreement with data from Holodniy et al. (13) and Mole et al. (18) that demonstrated a decrease in RNA copies over time in ACD-anticoagulated plasma held as whole blood at room temperature. Holodniy et al. found that HIV-1 RNA levels are the most stable in samples collected with EDTA anticoagulant (13).

Based upon the previous studies and our preliminary data we compared plasma HIV-1 RNA levels in whole blood collected in standard V-E blood collection tubes to RNA levels in cell-free plasma collected in VACUTAINER EDTA-PPTs. The variables examined which could affect HIV-1 RNA stability included the effects of cellular fractions, preprocessing storage temperature, and time delays to processing. We found that plasma HIV-1 RNA levels were comparable and stable for up to 30 h at room temperature both in whole blood stored in the V-E collection tube and in cell-free plasma stored in the

EDTA-PPT. The mean variation in HIV-1 RNA copies due to storage and collection conditions was 0.002 log units. In addition, equivalent results were found when cell-free plasma was stored in EDTA-PPTs held at room temperature or refrigerated prior to specimen addition to NASBA lysis buffer. These data suggest that HIV-1 RNA is stable in cell-free plasma or in whole blood for up to 30 h and that refrigeration of samples did not offer any significant advantage. These results correlate with those obtained by Vandamme et al. (25), who found that HIV-1 RNA, quantitated by NASBA, is efficiently protected by the virus particle in whole blood for up to 48 h. In addition, Holodniy et al. (13) found an insignificant decrease in HIV-1 RNA titers for EDTA-anticoagulated plasma stored as whole blood for 30 h at room temperature. The stability of plasma HIV-1 RNA titers at room temperature beyond 30 h needs to be evaluated in order to determine the maximum length of time samples can be held as either whole blood or cell-free plasma prior to testing or storage at −70°C.

The EDTA-PPT was found to be a convenient and simple blood collection tool which assured the separation of plasma from cellular components. A major benefit of the PPT is that it allows blood samples to be centrifuged at the blood collection site and the entire tube to be transported at controlled room temperature to laboratories for viral load quantitation. This method may prove extremely beneficial and cost saving for clinical trial studies or for sites which do not have the capabilities to separate plasma specimens or ship frozen plasma on dry ice. However, it must be stressed that care should be taken to guarantee the temperature of transport and prevent exposure of the samples to severe temperature fluctuations. PPTs may be particularly beneficial for those circumstances where processing is delayed beyond 30 h. The effects of using PPTs in situations for which longer processing delays are unavoidable need to be further investigated.

The majority of blood specimens submitted for HIV-1 viral load testing are generally transported frozen and are stored at −70°C as cell-free plasma until the time of testing. Specimens must then be thawed prior to HIV-1 RNA quantitation. This was a concern since previous studies using limiting dilution cultures had suggested that there is significant loss in plasma viral titers after just one round of freeze-thaw (19). Therefore, it was important for us to determine if the freezing and thawing of plasma samples would significantly reduce the number of HIV-1 RNA copies detected. Of the 20 EDTA-anticoagulated plasma samples tested in study 2 (preprocessing storage at 23°C) and study 6 (preprocessing storage at 4°C) we found no significant decreases in RNA levels after one round of plasma freeze-thaw. Overall the mean loss for the 20 EDTA-collected samples was only 0.0614 log units. Viral RNA loss was greater in the ACD- and heparin-anticoagulated plasmas than in the EDTA-collected samples. However, the losses were still not significant and were within the expected variation of the assay. The greatest variability within a group of plasma specimens was detected in the heparin-anticoagulated plasmas. The serum samples had an overall mean increase in viral titers after a freeze-thaw cycle. The increase was not significant but the range of the variation among the serum specimens was the greatest among all matrices tested. These data suggest that EDTA-anticoagulated plasma is the least variable matrix for HIV-1 RNA testing when specimens must undergo a round of freeze-thaw prior to quantitation.

Our studies which examined RNA stability in serum and plasma after long-term storage at −70°C determined that the decreases in the mean viral loads over 6 months in both EDTA- and ACD-anticoagulated plasmas were not significant, with EDTA-anticoagulated plasmas being the most stable. In contrast, after 6 months of storage at −70°C the RNA levels in both serum and the heparinized plasmas had a significant mean decrease of >0.300 log units.

While the vast majority of samples are tested within 1 week of collection, research samples are often held for longer periods of time and batch tested at later dates. For this type of delayed testing, plasma RNA stability over extended periods of time is a critical issue. Two conditions must be considered when designing viral load testing protocols for these situations. First, RNA levels may be slightly reduced with a cycle of freeze-thaw. Second, storage over time also generally results in a loss of viral RNA. Though both components may independently result in insignificant changes in detectable HIV-1 RNA copies, the combination of both factors could result in significant reductions in RNA levels. This was clearly demonstrated with heparinized plasma samples where significant decreases in RNA levels resulted from a combination of losses from both a cycle of freeze-thaw and storage over time.

Under optimal conditions specimens for plasma HIV-1 viral load testing should be collected as EDTA-anticoagulated whole blood, processed within several hours of collection, and the cell-free plasma stored at −70°C until testing. Although it is always advantageous to process specimens as soon as possible, this is not always practical when specimens are collected from outpatient sites and transported to reference centers for testing. Our results clearly indicate that for up to 30 h post-collection, EDTA-anticoagulated plasma or whole blood are the most suitable and stable matrices for viral load testing. A comparable alternative is to collect whole blood in EDTA-PPTs and centrifuge as soon as possible. The level of HIV-1 RNA in EDTA-anticoagulated plasma is stable for at least 6 months when properly processed and stored at −70°C. This is extremely important for assessing the results of clinical trial studies where viral load measurements are often performed in batches several months after specimen collection.

It is critical that blood collection, processing, and storage methods which provide the highest degree of HIV-1 RNA stability be standardized and employed by all sites responsible for the collection, transportation, and testing of such samples. This standardization will ensure not only the accuracy of the test results but allow the comparison of data, especially when specimens are collected and transported from sites which are a part of multicenter clinical trial studies. The correlation between our results and similar studies using both bDNA and RT-PCR testing indicates that the optimal blood collection, processing, and storage parameters determined in our studies should apply in general to all viral load testing, independent of test methodology.

ACKNOWLEDGMENTS

This study was funded in part by the Jane and Dayton Brown and Dayton Brown Jr. Virology Laboratory. NASBA test kits were kindly provided by Organon Teknika Corporation, and VACUTAINER blood collection tubes were provided by Becton Dickinson and Company.

We thank Kristine Witt for editorial review and Andrew Stead for statistical analysis. We sincerely thank the volunteer patients and nurses from the North Shore University Hospital Center for AIDS Research and Treatment.

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