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Direct quantification of HIV-1 RNA by capillary electrophoresis with laser-induced fluorescence

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Abstract

HIV-1 RNA was quantitated directly by capillary electrophoresis with laser-induced fluorescence (CE–LIF). CE–LIF was used to analyze cellular RNA and various nucleotide complexes. A fluorescently labeled DNA probe (DNA/RNA complex) in conjunction with thiazole orange intercalator was determined to have optimal stability and sensitivity for RNA analysis. Based on this observation, a hybridization method using a HIV-specific fluorescently labeled probe with analysis by CE–LIF was developed. Plasma samples from a HIV-seropositive patient were lysed to obtain RNA, hybridized with the HIV-specific probe and analyzed by CE–LIF. As little as 19 fg (1710 copies per 1 ml of starting plasma) of HIV RNA can be reliably and quantitatively detected. CE–LIF appears to be an efficient and sensitive method to quantitatively analyze RNA from a variety of sources. © 1997 Elsevier Science B.V.

Keywords: Gene quantification; Human immunodeficiency virus (HIV); RNA

1. Introduction

Quantitative analysis of RNA is key to the diagnosis and monitoring of human immunodeficiency virus (HIV-1), the retrovirus which causes acquired immunodeficiency syndrome (AIDS) [1–3]. Several nucleic acid-based methods are used clinically to study this disease, including; reverse transcriptase polymerase chain reaction (RT-PCR), branched-chain signal amplification (bDNA), and nucleic acid sequence-based amplification (NASBA) [4,5]. Although these assays are fairly sensitive and reproducible, techniques which rely on amplification increase variability. Additionally, many of the newer, more potent antiretrovirals can decrease HIV-1 RNA below detection levels of current techniques. Therefore, the need for more sensitive and reliable assays is present.

Capillary electrophoresis with laser-induced fluorescence (CE–LIF) can detect molecules in the attomole range [6] and provides a nonradioactive, fast, reproducible, and sensitive method for the analysis of nucleotide fragments. Previous studies have used CE–LIF to quantify gene expression [7] and to identify HIV-1 and HTLV-I PCR products [8,9]; however, these procedures still require RT-PCR to generate DNA fragments. A report has attempted to quantify HIV-1 RNA directly from the plasma without specific HIV-1 probes [10], but plasma samples may contain non-HIV-1 viral RNA including HTLV-1 (human T-cell leukemia virus Type-1), and hepatitis A, C, D and E which would eliminate HIV-1 specificity. Additionally, contamination of plasma with leukocytes or other cells would result in the presence of nonspecific human RNA.

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This report describes a method to quantify HIV-1 RNA in plasma by using a specific, non-radioactive probe and CE–LIF. This procedure eliminates variability created by the use of amplification techniques and provides a more accurate and sensitive means of quantification.

2. Experimental

All reagents were molecular biology grade and solutions (with exception of CE buffers) were autoclaved prior to use. Glassware and disposable supplies were autoclaved prior to use.

2.1. Sample collection and RNA extraction

RNA was obtained from a HIV-seropositive patient by centrifugation of whole blood at 3000 *g* for 15 min at 4°C on a Centra GP8R (International Equipment Corporation, Needham Heights, MA, USA) refrigerated centrifuge. The plasma was separated and stored at −80°C. RNA was extracted from plasma samples using the Ultraspec II RNA isolation system (Biotecx, Houston, TX, USA) as recommended by the manufacturer. RNA was extracted from both plasma and peripheral blood lymphocytes of a HIV-seronegative normal volunteer. RNA was also obtained from *Spodoptera frugiperda* 21, grown in TC-100 serum (HyClone, Logan, UT, USA) at 27°C. RNA was resuspended in DEPC-treated water (Biotecx, Houston, TX, USA) and quantitated spectrophotometrically.

2.2. Probe synthesis

To ensure specificity, a unique gene sequence is probed. The pol region is the most genetically unique of the HIV genome and a 26-bp sequence in this region was selected (GenBank entry U62632). Uniqueness was verified by a GenBank search. A 5′-fluorescein phosphoramidite (Glenn Research, Sterling, VA, USA) (probe sequence, 5′-ACAGTATTAGAAGAYATGRRTTTGCC-3′, λ=488 nm) labeled DNA probe for this sequence was synthesized by the University of Wisconsin Biotechnology Center (Madison, WI, USA).

2.3. Hybridization

Sample RNA present in a concentration of 0.095 μg/ml was diluted serially with DEPC-treated water and hybridized with the DNA probe (1.0125 μg) in a buffer volume of 30 μl containing 10 m*M* Tris–HCl (pH 7.2), 1 m*M* EDTA (pH 8.0), 50 m*M* NaCl, and 1 m*M* cetyltrimethylammonium bromide (CTAB) (ACROS, Pittsburgh. PA, USA) [11]. The mixture was heated at 85°C for 10 min, and then incubated at 42°C for 4 h. The addition of CTAB to the hybridization mixture increases intramolecular crowding and decreases the time required for hybridization [11].

Following incubation, samples were digested for 30 min at 37°C with RNAase One (4.5 U) (Promega, Madison, WI, USA) in a digestion buffer of 50 μl consisting of 50 m*M* Tris–HCl (pH 7.2), 5 m*M* EDTA (pH 8.0). Samples were flash frozen at −80°C to stop enzymatic digestion.

2.4. CE–LIF analysis

Separations were performed on a P/ACE 2050 CE system (Beckman Instruments, Fullerton, CA, USA) with the temperature held constant at 20°C. Detection of hybridization samples was achieved using laser-induced fluorescence in the reversed-polarity mode (anode at the detector side) at an excitation of 488 nm and emission of 520 nm. Samples were introduced hydrodynamically by 10-s injections at 0.34 Pa across a 65 cm×100 μm coated eCAP dsDNA capillary filled with replaced linear polyacrylamide (Beckman Instruments, Fullerton, CA, USA). The capillary was conditioned with eCAP dsDNA 1000 gel buffer which contained 60 μl of LiFluor dsDNA 1000 EnhanceCE (thiazole orange) intercalator per 20 ml (Beckman Instruments, Fullerton, CA, USA). Separations were performed under constant voltage at 7.0 kV for 15–30 min. The capillary was rinsed with gel buffer for 3 min prior to each injection. The capillary was calibrated with the fluorescently labeled probe and a mixture of RNA molecular markers (Ambion, Austin, TX, USA). The five markers ranged in size from 100–500 bp. Postrun analysis of data was performed using the System Gold chromatography data system (Beckman Instruments, Fullerton, CA, USA).

3. Results and discussion

3.1. Analysis of cellular RNA

RNA was isolated from *Spodoptera frugiperda* 21, resuspended in DEPC-treated water and analyzed as described in Section 2. RNA was analyzed at 20°C without sample degradation over run time. Subsequent injections of the same sample were made after 30 and 60 min at room temperature (Fig. 1a–c). Peak areas decreased with increasing time by an average of 7.6% after 30 min and 8.2% after 60 min, indicating degradation of RNA. Samples degraded entirely after 90 min at room temperature (Data not shown). Retention times remained nearly constant over the 60-min period with C.V.s of less than 0.1% (Table 1). It appears that CE–LIF is a fast and efficient method for verification of RNA integrity.

3.2. Comparison of weight to copy number

One copy of HIV cDNA contains 9000 base pairs (bp). One pg of DNA contains 9×10^8 bp, making 1 pg equal to approximately 100 000 equivalents and 1 ag approximately equal to 0.09 copies of HIV RNA.

3.3. Determination of the injection volume

Based on literature values, the calculated injection volume is 7.1 nl when a sample is injected onto a 100-μm I.D. capillary at 0.34 Pa for 10 s [12] [13]. This injection volume was verified for our system by measuring the mass difference after injection. Twenty μl of hybridization sample was placed in a microcuvette and weighed on a Sartorius BP 210D balance (Sartorius Corporation, Edgewood NY, USA). After weighing, the microcuvette containing the sample was transferred to the auto-sampler tray and injected hydrodynamically at 0.34 Pa for 990 s (99 s×10 injections, 99 s maximum injection time). The microcuvette was then re-weighed with a mean decrease in weight after injection of 707 ng (n=3). Since the hybridization solution was very dilute, it was assumed to have the specific gravity of water (1.00 g/l), corresponding to a mean volume of 706.86 nl per 990-s injection or 7.14 nl per 10 s injection (n=3).

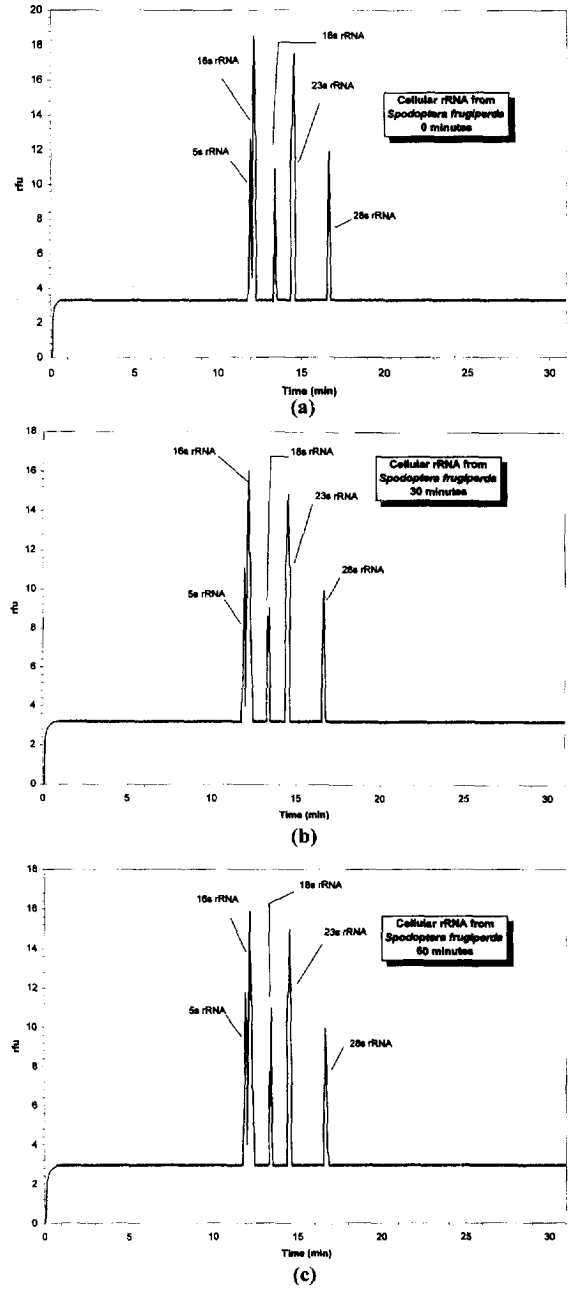

Fig. 1. Electropherogram of cellular RNA (1.856 μg/μl) obtained from *Spodoptera frugiperda* culture. (a) Injection at time 0. (b) Second injection of same sample after 30 min at room temperature. (c) Third injection of same sample after 60 min at room temperature.

Table 1
Stability of cellular RNA at room temperature

Peak injection	Peak area					Migration time				
	5S	16S	18S	23S	28S	5S	16S	18S	23S	28S
Time 0	1523	2891	586	2717	921	11.96	12.17	13.45	14.58	16.68
Time 30 min	1277	2605	650	2368	1002	11.94	12.21	13.44	14.54	16.64
Time 60 min	1371	2603	598	2183	911	11.94	12.20	13.45	14.55	16.66
Mean	1390.33	2699.67	611.33	2422.67	944.67	11.95	12.19	13.45	14.56	16.66
S.D.	124.13	165.70	34.02	271.1	49.9	0.012	0.021	0.006	0.021	0.02
C.V.%	8.9	6.1	5.5	11	5	0.1	0.1	0.04	0.1	0.1
% change over 30 min	−17	−10	+10	−13	−8					
% change over 60 min	−10	−10	+2	−20	−1					

3.4. Capillary electrophoresis analysis with various nucleotide complexes

Thiazole orange present in the buffer intercalates into every one of two DNA bp and every one of 10 RNA bp [14]. Although the intercalation parameters of thiazole orange into a DNA/RNA complex is unknown, it is assumed to be between 10 and 50%, providing a 10–50% enhancement in sensitivity over the RNA/RNA complex. The addition of a fluorescein label to the probe provides a double detection system over intercalator alone, also enhancing sensitivity. Various nucleotide complexes were analyzed to determine detection limits and reproducibility with this system and to determine the optimal probe composition.

3.4.1. Analysis of RNA/RNA complexes

A solution of RNA fragments was used to determine the detection limits of the unlabeled RNA/RNA complexes. RNA standards present in a concentration of 0.5 mg/ml (0.1 mg/ml per fragment) initially, were diluted 1:10 and 1:100 with DEPC-treated water and injected for 2.5–15.0 s. A linear relationship between the peak area and concentration was demonstrated from 11 to 107 pg (r^2=0.9924, y=69.072+112.45x). The intra-day and inter-day migration time precision was 1.1 and 0.16% (n=6), respectively, the intra-day peak area precision was 0.9% (n=6) and the inter-day peak area precision was 1.0%. Minimal detectable concentration was 500 fg/nl (signal-to-noise ratio 3:1).

RNA/RNA complexes can be reliably quantitated by CE–LIF, however, the linear range (1 100 000–10 700 000 HIV equivalents) and the minimum detectable quantity (50 000 HIV equivalents) are not as sensitive as current HIV assays with detection limits of (350–10 000 HIV equivalents) [15] (Table 2).

3.4.2. Analysis of DNA/DNA complexes

The fluorescently labeled probe present initially in a concentration of 2.025 mg/ml was diluted 1:1000 to 1:100 000 000 with DEPC-treated water and injected for 2.5–15 s. The concentration of the fluorescently labeled DNA fragment when diluted 1:1000 and injected for 10 s is 14 pg/7.1 nl. A linear relationship between the peak area and concentration was demonstrated from 0.072 to 21.46 pg (r^2=0.99, y=516.88+18.01x). The intra-day and inter-day migration time precision was 0.18% (n=9) and 0.22% (n=6), respectively, the intra-day peak area precision was 7.3% (n=6) and the inter-day peak

Table 2
Comparison of detection limits and reproducibility of various nucleotide complexes

Nucleotide complex	Minimum detectable quantity		Precision	
	Weight	HIV equivalents	Migration time C.V.%	Peak area C.V.%
RNA/RNA	500 fg	50 000	0.16–1.1	0.9–1.0
DNA/DNA-fluorescein	36 ag	4	0.18–0.22	7.3–11
DNA/RNA-fluorescein	190 ag	21	0.18–0.22	5.6–7.3

area precision was 11% (n=9). The minimal detectable level was 36 ag (signal-to-noise ratio=3:1).

Fluorescently labeling the probe increased the sensitivity of the assay by an order of magnitude from femtograms to attograms and extended the lower limit of the linear range from 11 pg to 72 fg which is approximately equal to a lower detection limit of 7200 HIV equivalents (Fig. 2, Table 2). Although the peak area precision was less precise at the lower detection levels (7.3–11% compared to about 1%) it compares favorably to other published methods including bDNA with a limit of detection of 10 000 eq/ml and a precision of 11–42% and RT–PCR with a detection limit of 350 copies/ml and a precision of 13–93% [15]. The decreased precision may represent the effect of dilution on the fluorescently labeled probe. However, with a minimum detectable level of 36 ag, which is approximately 4 equivalents of HIV, further evaluation of linearity in the lower range is warranted.

3.5. Analysis of hybridization samples

The hydridization product consists of an RNA/DNA complex 26 bp in length. Hybridizations were carried out on a sample obtained from a seropositive HIV patient and a seronegative normal volunteer. RNA (0.095 μg/μl) was diluted 10^{-1} to 10^{-12}, hybridized and analyzed as previously described. A dilution of 10^{-7} (19 fg which is equal to 1710 copies of HIV RNA) was determined to be optimal and

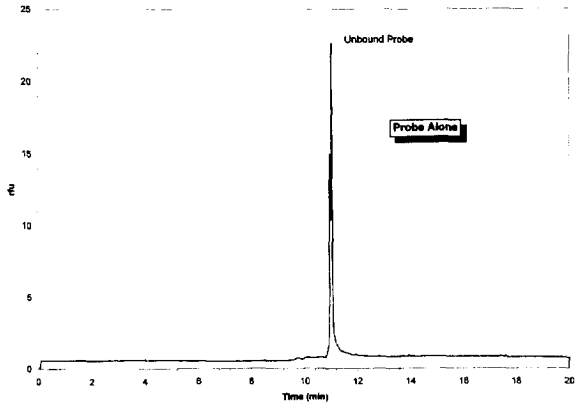

Fig. 2. Electropherogram analysis of fluorescently labeled HIV-specific probe alone diluted in DEPC treated water to a concentration of 72 fg/7.1 nl, which elutes at 11 min.

subsequent hybridizations were performed in triplicate on three different occasions and analyzed by CE–LIF (Fig. 3a–c). Hybridization products were injected hydrodynamically without further sample preparation and quantitated by comparing the peak area obtained to the standard curve. The intra-day and inter-day migration time precision was 0.18 and 0.22% (n=9), respectively, the intra-day peak area precision was 7.3% (n=9), and the inter-day peak area precision was 5.6% (n=9), and represents both the hybridization and capillary electrophoresis portions of the assay.

This is consistent with our detection limits, with DNA/DNA complexes having the lowest detection limits (36 ag) DNA/RNA complexes (1.9 fg) RNA/RNA (500 fg) (Table 2).

In this approach, cellular RNA is hybridized with a HIV-1-specific probe. A complex is formed if HIV-1 RNA is present and unbound RNA is digested with RNAase One. Samples are then analyzed by CE–LIF. Two peaks elute if HIV-1 RNA is present; the first, eluting at approximately 11 min is the DNA/DNA unbound probe complex. The second, the DNA/RNA complex, elutes at approximately 12 min. Although the complexes are both 26mers, the DNA/RNA complex has a different secondary structure and slightly higher molecular mass and a subsequently longer retention time.

Any double-stranded nucleotide complex, as well as the fluorescently labeled probe, will be detected with this approach. All samples are digested under identical conditions, and when no complex formation (normal volunteer) occurs there is excess RNA present for digestion. The relatively greater quantity of RNA (when compared to the HIV-positive sample) may be incompletely digested and nonspecifically interact with the fluorescently labeled probe, providing a cluttered response. Additionally, the negative control contains no RNA, and probe is the only substrate for RNAase One. A larger degree of digestion occurs, leaving a smaller peak area in comparison to samples containing RNA.

4. Conclusions

We have used CE–LIF to analyze cellular RNA, and a variety of nucleotide complexes. The double

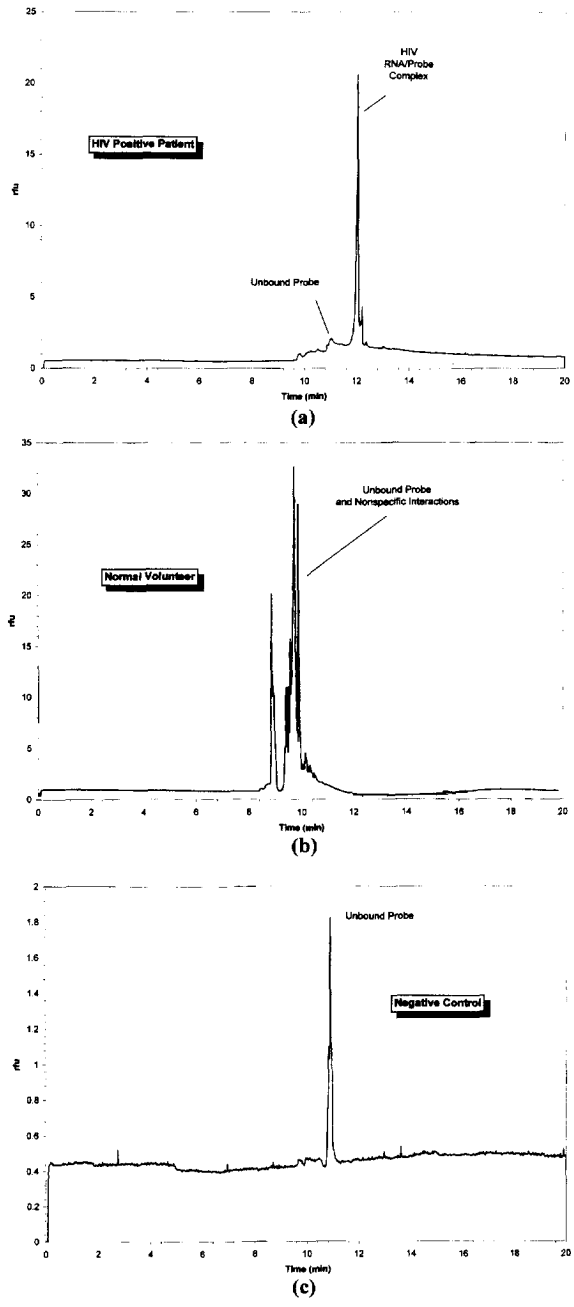

Fig. 3. Electropherogram analysis of hybridization products. RNA samples obtained from a HIV-seropositive patient and a seronegative volunteer were hybridized with a HIV-specific probe and analyzed as described in Section 2. (a) HIV RNA/Probe complex elutes at 12 min, indicating the presence of HIV RNA in the patient's serum. (b) Seronegative volunteer. (c) Negative control containing all reaction components except RNA.

detection system is linear from 0.072–21.46 μg, the migration time precision is less than 1% and the peak area precision ranges from 1–11% depending on the concentration and the nucleic acid analyzed. The minimal detectable level is 36 ag, which corresponds with 4 equivalents of HIV. With the hybridization strategy, we are able to reliably and quantitatively detect as little as 19 fg (1710 copies per 1 ml of starting plasma) of HIV RNA. This approach appears to be an efficient and sensitive method to quantitatively analyze RNA from a variety of sources and warrants further evaluation and comparison to established HIV assay techniques.

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