J. Pharm. Pharmacol. 1990, 42: 261–266
Received November 27, 1989

Detection of Poly(I):Poly(C_{12}U), Mismatched Double-stranded RNA, by Rapid Solution Hybridization: Blood Values after Intravenous Infusion

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Abstract—A rapid solution hybridization technique has been developed for estimating blood concentrations of poly(I):poly(C_{12}U), a high molecular weight bioactive double-stranded RNA. Samples were prepared by mixing 100 μL of blood with, 165 μL of 6 M guanidine thiocyanate (GuSCN) and 0·16 M EDTA pH 8·0, and freezing. Hybridizations were carried out with a [^{3}H]poly(C) probe in 3 M GuSCN for 10 min at 37°C. Ribonuclease-resistant hybrids were collected by precipitation with trichloroacetic acid and filtration. Validation studies demonstrated minor interassay variance; the assay was accurate in the range 0·1 to 10 ng poly(I):poly(C_{12}U). Thirty-one blood samples from 15 patients were collected and prepared before and immediately after an average 35 min intravenous infusion of 40–500 mg poly(I):poly(C_{12}U). Postadministration values averaged 48% (s.d. 23%) of the theoretical maximum (range 20–102%). These results confirm previous observations of rapid elimination kinetics of poly(I):poly(C_{12}U) in patients.

Poly(I):poly(C_{12}U) is a non-toxic, mismatched double-stranded RNA (dsRNA) consisting of high molecular weight single strands of polyinosinic acid annealed with high molecular weight single strands of poly(cytidylic:uridylic (12:1)) acid (Carter et al 1972, 1976). Poly(I):poly(C_{12}U) and poly(I):poly(C) with double-stranded regions in excess of 50–100 base pairs induce and activate key intracellular enzymes (Minks et al 1979; see Lengyel 1982, 1987 for reviews), and exert several biological effects in a pleiotropic manner (Carter & DeClercq 1974; Diamentstein & Blitstein 1975; Zarling et al 1981; Hubbell et al 1984; Strayer et al 1986; Lengyel 1987). In phase I and phase II clinical trials, poly(I):poly(C_{12}U) has been well tolerated without significant side-effects and with therapeutic effect (Strayer et al 1981, 1990; Brodsky et al 1985; Carter et al 1987).

Blood concentrations of the drug after intravenous infusion have not previously been determined. Conventional methods for measuring low molecular weight compounds in blood, e.g. by degrading poly(I):poly(C_{12}U) and measuring mononucleotides by HPLC, are inadequate for measuring blood values of bioactive drug because inactive constituents may also be measured. Molecular hybridization, through the base-specific hydrogen bonding properties of DNA and RNA, utilizes target specific, complementary probes to establish the presence of target sequences. Target:probe hybrids of defined complexity and mismatching may be selected for, and quantified, when hybridization is carried out under the appropriate conditions (probe excess, salt concentration, pH, temperature and time). To detect a dsRNA such as poly(I):poly(C_{12}U), the probe must complement one of the strands and must not cross-hybridize with other components present. Krueger et al (1986) have used the 'quick-blot' molecular hybridization procedure to measure high molecular weight poly(I) strands of poly(I):poly(C_{12}U) in blood plasma. This procedure involves selective immobilization of poly(I):poly(C_{12}U) from plasma followed by molecular hybridization with a DNA probe, [^{14}C]poly(dC). Relative numbers adequate for estimating the rate of poly(I):poly(C_{12}U) elimination from plasma are obtained; absolute values cannot be determined because of uncertainties and inefficiencies associated with poly(I):poly(C_{12}U) immobilization. The immobilization step could be bypassed by solution hybridization in concentrated guanidine thiocyanate (GuSCN). Dissolution of blood specimens in the chaotropic salt frees nucleic acids for molecular hybridization and inhibits nucleases (Thompson & Gillespie 1987). Because RNA:RNA hybrids are more stable than RNA:DNA hybrids (Thompson & Gillespie 1987), an RNA probe is preferred. We now describe an improved method for measuring poly(I):poly(C_{12}U) using an RNA probe, [^{3}H]poly(C), which yields blood concentrations in mg mL^{-1}.

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Materials and Methods

Patients

The 15 patients in this study ranged from 20–70 years of age. There were 12 males (4 renal cell carcinoma, 6 HIV-related immunodeficiency, 2 chronic myelogenous leukaemia) and 3 females (1 renal cell carcinoma and 2 metastatic carcinoid tumors). Blood samples were also obtained from 2 normal males 25–27 years of age. The experimental protocols for the use of Ampligen in humans were approved by the USFDA and the Hahnemann University institutional review board which routinely reviews experimental protocols for safety and ethical considerations.

Sample preparation

Blood specimens were taken from patients both before and immediately after intravenous infusions of poly(I):poly (C_{12}U) (Ampligen). The drug, obtained from HEM Research, Inc. as the lyophilized salt, was reconstituted with sterile water and stored frozen at −20°C in glass vials.

Infusion times varied between 20 and 65 min. Preinfusion venous blood specimens were obtained from the same arm used for the infusion; postinfusion specimens were from the opposite arm. Blood was collected into a 1 mL tuberculin syringe and 0·1 mL aliquots were mixed into numbered tubes containing 0·165 mL of 6 M GuSCN (Fluka) with 0·16 M EDTA (Sigma) pH 7·5–8·0 (6 M GE). Specimens were stored at −80°C until assayed.

Molecular hybridization: method 1

Hybridizations were performed as follows (unless otherwise indicated): Standards were prepared by addition of varying amounts of poly(I):poly($C_{12}U$) solution (2·5 mg mL^{-1} in 0·15 M NaCl, 0·001 M $MgCl_2$ and 0·01 M phosphate pH 7·0–7·6) to fresh heparinized blood. Spiked blood (1·2 mL) was mixed with 2·0 mL 6 M GE. Thawed, prepared specimens were diluted 1:100 in 3·75 M GE. The dilutions (10 μL) were pipetted into 1·5 mL centrifuge tubes and the [^{3}H]poly(C) probe (NEN, 20·5 Ci $mmol^{-1}$, 0·05 mCi mL^{-1}) (2·5 μL) added. The tubes were vortexed and hybridized for 10 min at 37°C. Ice cold 0·3 M NaCl, 0·03 M sodium citrate with 0·1 M EDTA pH 8·0 and 15 μg mL^{-1} bovine pancreatic ribonuclease A (RNase A, Sigma) (200 μL) was added, mixed by vortexing, then incubated at 37°C for 1 h. After degradation of unhybridized probe, RNase A-resistant [^{3}H]poly(C):poly(I) hybrids were precipitated by adding ice cold 30% (w/v) trichloroacetic acid, 6% (w/v) sodium pyrophosphate (107 μL), vortexing, and standing the mixture on ice for 30 min. Precipitates were collected on Whatman GF/B circles using a twelve port Millipore vacuum manifold. Each tube was rinsed four times with 10% trichloroacetic acid, 2% sodium pyrophosphate. The filters were washed with 70% ethanol, dried under a heat lamp and counted in Econofluor (Sigma) using a Beckman LS8100 scintillation counter.

Molecular hybridization: method 2

The probe was prepared as follows: [^{3}H]poly(C) was synthesized in 10 μL containing 100 mM Tris (Sigma) pH 8·8 with 0·5 mM EDTA pH 8·0, 5 mM $MgCl_2$ (Sigma), 200 μg mL^{-1} nuclease free bovine serum albumin (Sigma), 0·1 mM cytidyl(3′-5′)cytidine (as primer, Sigma), 18 mM cytidine 5′-diphosphate (CDP, Sigma), 40 units RNase inhibitor (Promega), 0·5 mCi [5-^{3}H]+CDP (NEN) and 2·1 units *M. luteus* polynucleotide phosphorylase (Boehringer Mannheim). The mixture was incubated for 30 min at 37°C, yielding approximately 85% incorporation of CDP into poly(C). The reaction mixture was diluted to 10 mL with 50% ethanol and stored at −80°C in 0·6 mL aliquots.

Standard curve specimens were generated by 1:10 dilution of 2·0 mg mL^{-1} poly(I):poly($C_{12}U$), 2·2 M GuSCN into prepared blood from an untreated normal individual, followed by serial 1·7-fold dilutions in the same prepared blood. Assays were carried out using eight place manifold pipetters in conjunction with Micronic tubes (and matcaps, Flow Laboratories, Inc.) held in 12 × 8-place carrier racks (GENETRAK Systems). Specimens were diluted 1:100 in 5 M GE. Replicate aliquots (7·5 μL) were preincubated briefly at 37°C; probe (5 μL) was added, vortexed and hybridized for 10 min at 37°C. Ice cold 0·3 M NaCl/0·03 M sodium citrate with 50 mM Tris pH 8·0, 0·1 mg mL^{-1} degraded herring testes DNA (Sigma) and 15 μg mL^{-1} RNase A (200 μL) was added, the sample vortexed, and incubated at 37°C for 1 h. Hybrids were precipitated as above and filtered on a Minifold I device through BA85 nitrocellulose (Schleicher & Schuell). The membrane was washed twice for 5 min in 10% trichloroacetic acid, rinsed for 30 s in 70% ethanol, and dried under a heat lamp. Dots were cut out then counted as above.

Data handling

Concentrations of poly(I):poly($C_{12}U$) were determined using a set of standards run in parallel with each experiment. Concentrations were converted to total blood values using estimated total blood volumes, determined according to the method of Nadler et al (1962):

Total blood volume

$$= 0{\cdot}0236 \cdot H^{0{\cdot}725} \cdot Wt^{0{\cdot}425} - 1{\cdot}229 \text{ male}$$
$$= 0{\cdot}0248 \cdot H^{0{\cdot}725} \cdot Wt^{0{\cdot}425} - 1{\cdot}229 \text{ female}$$

where H = height (cm), Wt = weight (kg).

Results and Discussion

Hybridization of [^{3}H]poly(C) to poly(I):poly($C_{12}U$) in 3 M GuSCN

Hybridization of the labelled poly(C) probe to poly(I):poly($C_{12}U$) should provide a measure of poly(I) strands over 50–100 nucleotides long (Niyogi & Thomas 1967), their equivalence with native poly(I):poly($C_{12}U$) was an assumption. While this suggested that bioactive poly(I):poly($C_{12}U$) (Minks et al 1979) was measured and that inert degradation fragments were not, this also remained an unproven assumption.

Since dissolution of biological samples in concentrated GuSCN releases nucleic acids in a hybridizable form (Thompson & Gillespie 1987), hybridization of [^{3}H]poly(C) to the poly(I) strand was attempted. Initially, poly(I):poly($C_{12}U$) was heat denatured before hybridization, but hybridization with poly(C) proceeded equally efficiently without the denaturation step (data not shown). This is probably because the poly(C) probe is better matched with poly(I) than is the poly($C_{12}U$) strand.

Hybridizations between poly(I):poly($C_{12}U$) and 2 ng of [^{3}H]poly(C) probe were performed at pH 7·0 or 8·0 in 3 M GuSCN, and the products of hybridization were exposed to RNase A in NaCl/Na citrate buffer at pH 7·0 or 8·0 (Table 1). Improved hybridization signals were obtained when the

Table 1. Optimization of poly(I):poly($C_{12}U$) assay conditions: pH during hybridization and RNase treatment.

	pH during:		Counts min^{-1}:		
	Hybridization	RNase treatment	poly(I):poly($C_{12}U$) plus	minus	Signal:Noise
1·2 ng	8	8	1812	162	10·2
	7	8	1566	218	6·2
	8	7	886	262	2·4
	7	7	451	252	0·8
0·6 ng	8	8	1012	173	4·8
	7	8	893	156	4·7
	8	7	460	221	1·1
	7	7	291	190	0·5

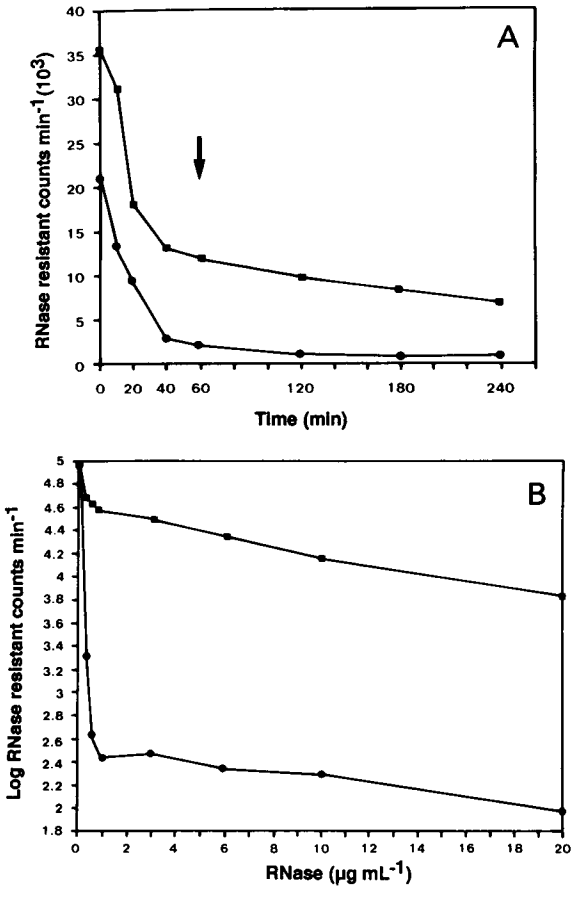

FIG. 1. Determination of RNase A conditions. (A) RNase kinetics: 300 μL of 6 ng μL^{-1} poly(I):poly($C_{12}U$) in 3·75 M GE was added to a tube containing 75 μL of 0·73 ng μL^{-1} [^{3}H]poly(C); a 10-fold dilution in 3·75 M GE was assayed similarly. The hybridization was at 37°C for 18 h. 30 mL of 0·6 μg mL^{-1} RNase A in 0·3 M NaCl 0·03 M sodium citrate with 0·1 M EDTA pH 8·0 were added, triplicate 1 mL aliquots were placed in 1·5 mL tubes containing 0·5 mL ice cold 30% TCA, 6% $NaPP_i$. Triplicate aliquots were similarly taken after appropriate times, and precipitates were collected as in Method 1. Arrow indicates time chosen for standard operating procedure. ■——■ 1·0 ng; ●——● 0·1 ng poly(I):poly($C_{12}U$). (B) RNase concentration and hybrid stability: Triplicate 10 μL aliquots of poly(I):poly($C_{12}U$) in 3·75 M GE (60 ng) or 3·75 M GE alone were hybridized for 2 h at 37°C. 1 mL of the appropriate RNase A solution was added, and the samples processed as in Method 1. ■——■ 60 ng poly(I):poly($C_{12}U$); ●——● no poly(I):poly($C_{12}U$).

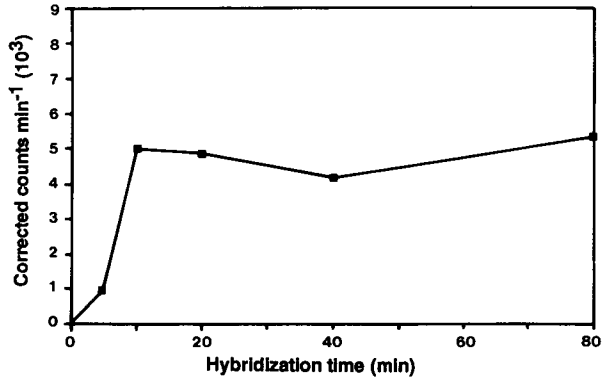

FIG. 2. Hybridization kinetics. Poly(I):poly($C_{12}U$) in 3·75 M GE was diluted 60-fold with 3·75 M GE. Triplicate 10 μL aliquots (1 ng) were hybridized for the indicated time, then 1 mL of 0·6 μg mL^{-1} RNase A was added; processing proceeded as in Method 1. A sample with no poly(I):poly($C_{12}U$) was subtracted to correct for assay noise.

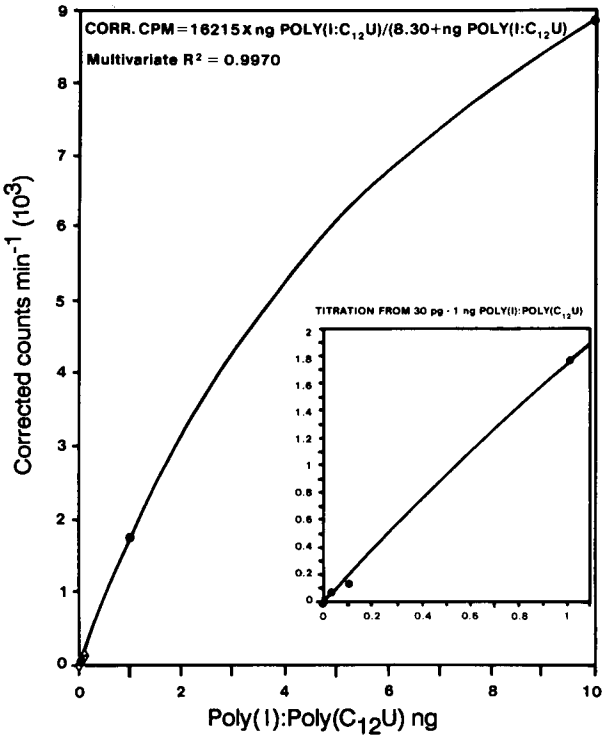

FIG. 3. Titration of poly(I):poly($C_{12}U$) in 3·75 M GE. Poly(I):poly($C_{12}U$)/3·75 M GE (6 ng μL^{-1}) was diluted with 3·75 M GE to create a series of titration samples. Assays were performed as described in Method 1, except that the target was heat denatured at 75°C for 5 min before a 2 h hybridization; 1 mL of 0·6 μg mL^{-1} RNase A was then added. Inset: expanded view of 0, 0·03, 0·1, and 1 ng. The curves were generated using a multivariate regression program in RS1.

hybridization was carried out at pH 8·0. Furthermore, when RNase degradation of unhybridized probe was conducted at pH 8·0, signals improved 2·6–7·3-fold, while noise levels dropped 40–60%. Increased probe availability probably resulted from the decreased helical nature of poly(C) at pH 8·0 compared with pH 7·0 (Gianonni & Rich 1964). Subsequent experiments were conducted at pH 8·0.

[^{3}H]poly(C):poly(I) hybrids were not completely resistant to RNase degradation. Exposure of hybridization products to RNase A resulted in a rapid degradation of unhybridized [^{3}H]poly(C) during the first 20–30 min followed by a considerably slower destruction of the [^{3}H]poly(C):poly(I) hybrid (Fig. 1A). The extent of hybrid destruction was accentuated at higher RNase concentrations (Fig. 1B). The nuclease condition chosen for all subsequent experiments was 200 μL of NaCl/Na citrate with 0·1 M EDTA pH 8·0, 15 μg mL^{-1} RNase A (final RNase concentration of 14 μg mL^{-1}) incubated at 37°C for 1 h. Hybridizations with poly(I):poly($C_{12}U$) excess (60 ng) yielded signal:noise ratios around 80. Increasing salt concentration and lowering temperature during RNase treatment increased noise without proportional increases in signal (data not shown). Extended RNase incubations reduced noise, signal and the signal:noise ratio.

Table 2. Poly(I):poly(C_{12}U) Hybridization: dilutions in saline and blood.

		Sample/diluent[a]		
		[1][b] Saline	[2][b] Whole blood	[3][b] Whole blood
Dilution	ng per assay	3·75 M GE	3·75 M GE	Blood/3·75 M GE
1:1	30·0	16111 (120)	18048 (169)	14466 (11)
1:2	20·0	15497 (116)	14365 (135)	10699 (8)
1:5	10·0	14295 (107)	12707 (119)	8767 (6)
1:9	6·0	9826 (73)	10843 (101)	7245 (5)
1:20	3·0	6788 (50)	6101 (57)	3244 (2)
1:60	1·0	2983 (21)	2711 (25)	2364 (1)
1:100	0·6	1839 (13)	1858 (17)	1747 (0·5)
1:200	0·3	1348 (9)	466 (3)	1373 (0·1)
Background	0	133	106	1195

[a] Undiluted prepared samples: 6 ng μL^{-1} poly(I):poly(C_{12}U), 3·75 M GE.
[b] Counts min^{-1} (signal:noise).

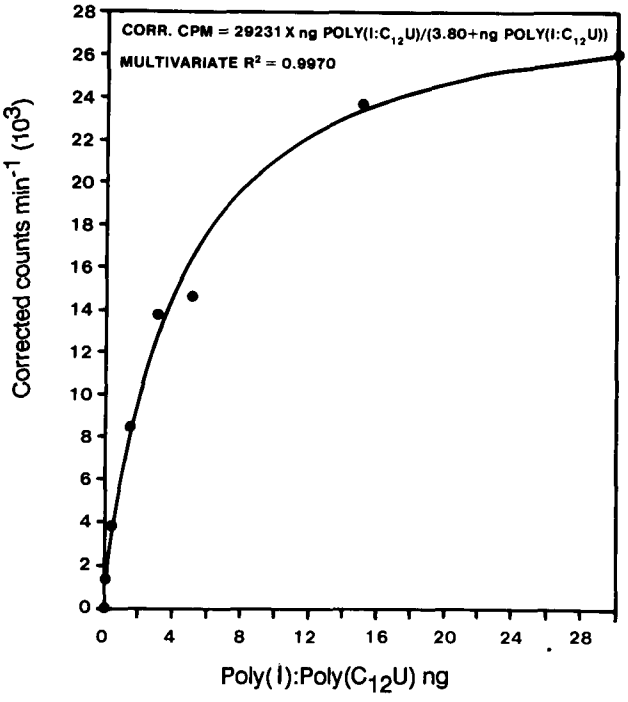

FIG. 4. Poly(I):poly(C_{12}U) standard curve in blood/3·75 M GE. By combining known amounts of poly(I):poly(C_{12}U) with blood, then adding concentrated GE solution, several independent standards were constructed (0, 0·05, 0·15, 0·3, 0·5, 1·5 and 3·0 ng). Hybridizations carried out as described in Method 1. The curve was generated using a multivariate regression program in RS1.

As expected for complementary RNA homopolymers, hybridization kinetics were rapid. Hybridization of 1 ng of poly(I):poly(C_{12}U) to 2 ng of [^{3}H]poly(C) was essentially complete within 10 min (Fig. 2). Using a constant 2 ng of probe to hybridize increasing amounts of poly(I):poly(C_{12}U) (0·03–10 ng), the relationship between amount of poly(I):poly(C_{12}U) and hybridization signal fit a saturation curve of general form $Y=(AX)/(B+X)$ (multivariate $r^2=0{\cdot}9970$, $P<0{\cdot}001$, Fig. 3). Thus, even though the [^{3}H]poly(C) probe was not employed in weight excess, poly(I):Poly(C_{12}U) could be accurately measured up to 10 ng (Figs 3, 4).

Hybridization of [^{3}H]poly(C) to poly(I):poly(C_{12}U) in blood

Blood containing poly(I):poly(C_{12}U) was prepared as described. Prepared blood containing poly(I):poly(C_{12}U) that was diluted in 3·75 M GE (Table 2, col 2) showed comparable hybridization signals and noise levels to poly(I):poly(C_{12}U) in saline/3·75 M GE (Table 2, col 1). However, when poly(I):poly(C_{12}U) in prepared blood was diluted with blood/3·75 M GE (Table 2, col 3), signals were reduced and noise levels were significantly elevated. Therefore, prepared blood specimens were diluted 100-fold in 3·75 M GE before assaying. The 100-fold dilution permitted peak poly(I):poly(C_{12}U) blood values to fall within the linear range of the standard curve. Standard curves were generated by spiking blood from a normal individual with known amounts of poly(I):poly(C_{12}U) (Fig. 4). Blood from another normal individual spiked in the same way yielded the same standard curve (data not shown).

Assay validation samples were made separately and assayed in parallel with standard curves in successive assays (Table 3). Overall, the observed values in this assay were 94·1% (s.d. 12·6%) of the theoretical input. Average coefficients of variation (CV) for validation sample replicates on a particular day (8·5%) were approximately equal to the average interassay CV (8·8%), indicating acceptable day-to-day precision. Normalized interassay variance was negligible.

Blood values of poly(I):poly(C_{12}U) in patients

Thirty-one clinical specimens were collected before and immediately after 20–65 min infusions of poly(I):poly(C_{12}U). Samples were coded and analysed by the GuSCN hybridization procedure. Postadministration poly(I):poly(C_{12}U) values following an average 35 min intravenous infusion were 48·1% ± 22·6% (mean ± s.d.) of the theoretical maximum and ranged between 19·8% and 102·1% (Table 4). Significant differences in postadministration values from the theoretical maximum reflected the rapid elimination of poly(I):poly(C_{12}U) from blood during the infusion (Krueger et al 1986). When poly(I):poly(C_{12}U) was administered in doses of 200–500 mg, the assay accurately detected concentrations of as low as 0·004 mg

Table 3. Accuracy and precision of hybridizations in blood.

Input (ng)	% input (CV)[a] I	II	III	IV	Inter-assay mean	Inter-assay CV	Inter-assay variance[b]
11·25	91·2 (11·2)	103·5 (6·1)	92·1 (5·7)	93·0 (2·8)	95·0	6·1	0·0393
3·75	n.d.	85·0 (6·4)	94·5 (11·1)	79·4 (4·9)	86·3	8·8	0·0253
2·25	97·4 (17·7)	n.d.	[c]	95·2 (2·2)	96·3	1·6	0·0005
1·125	110·9 (11·4)	87·4 (5·3)	106·6 (13·3)	n.d.	101·6	12·3	0·0174
0·75	91·7 (11·1)	94·7 (3·0)	n.d.	104·0 (4·5)	96·8	6·6	0·0032
0·225	88·4 (18·3)	56·9 (7·3)	113·3 (12·9)	102·0 (6·5)	90·2	27·1	0·0149

[a] Per cent of input = (Observed × Input^{-1} × 100); CV = (sample s.d. mean^{-1} × 100); hybridization Method 2 was utilized, I to IV were four successive assay dates; validation samples prepared as described in Results and Discussion; n.d. = not done.
[b] Normalized interassay variance = (sample s.d.)^2 × (interassay mean)$^{-1}$.
[c] This value was eliminated due to extreme disparity in the replicates.

Table 4. Poly(I):poly($C_{12}U$) postadministration values in patients[a].

Sample no. patient	Diagnosis	n	Dose (mg)	IT (min)	PV (mg)	PV × Dose^{-1} (%)	CV (%)	Inter-assay variance
14 a	RCC	1	300	30	181	60	4·5	
30 a	RCC	1	300	25	168	56	6·0	
16 b	HIV	1	200	30	110	55	7·0	
34 b	HIV	1	200	25	99	49	3·5	
59 b	HIV	1	200	25	66	33	5·3	
71 b	HIV	1	200	30	116	58	6·6	
18 c	CML	4	300	25	152	51	8·0	0·090
38 c	CML	1	300	30	111	37	1·3	
61 c	CML	4	300	35	83	28	10·4	0·010
20 d	RCC	1	250	30	63	25	0·5	
36 d	RCC	4	250	30	113	45	4·3	0·085
22 e	RCC	4	300	35	101	34	5·4	0·028
46 e	RCC	4	500	55	116	23	5·9	0·002
50 e	RCC	4	500	65	112	22	9·0	0·005
74 e	RCC	4	500	35	433	87	10·5	0·107
24 f	MCT	4	300	35	176	59	4·3	0·245
52 f	MCT	4	300	40	153	51	9·3	0·350
26 g	RCC	4	250	50	81	32	5·1	0·032
54 g	RCC	4	250	45	67	27	24·5	0·007
28 h	MCT	4	300	35	125	42	14·7	0·026
32 i	CML	1	300	40	181	60	10·5	
40 j	HIV	4	200	30	53	27	4·9	0·030
42 k	HIV	4	200	30	42	21	14·4	0·048
44 l	RCC	4	40	25	18	44	21·2	0·044
65 l	RCC	1	40	20	15	37	4·5	
48 m	HIV	4	400	35	374	94	9·1	0·048
57 m	HIV	1	400	20	385	96	13·6	
69 m	HIV	4	400	20	408	102	7·3	0·185
67 n	HIV	4	500	55	99	20	8·4	0·001
80 n	HIV	4	500	50	301	60	8·7	0·053
76 o	HIV	1	200	25	113	56	1·8	

[a] Samples were numbered chronologically as received. Abbreviations: a–o refer to the 15 patients studied (females: d, f, and h); RCC: renal cell carcinoma; HIV: HIV-related immunodeficiency; CML: chronic myelogenous leukaemia; and MCT: malignant carcinoid tumor. PV: postadministration value, the mean of 'n' triplicate analyses of the sample over a 3 week period; IT: infusion time; CV = (sample s.d. × mean^{-1} × 100); Normalized interassay variance = (sample s.d.)^2 × (interassay mean)$^{-1}$.

mL^{-1} 1–2 h after the infusion. However, after 2 days no poly(I):poly($C_{12}U$) could be detected above background (< 0·003 mg mL^{-1}, data not shown). The mode of elimination of this dsRNA is under investigation.

Nineteen of the thirty-one clinical samples were diluted 1:100 in 5 M GE and stored at −80°C. Analysis of these samples after 11 months of storage, utilising Method 2, yielded results within 11·4% ± 8·8% of those presented in Table 4 (data not shown). Utilisation of Method 2 with a more highly labelled probe and less sample dilution could provide accurate results with an order of magnitude more sensitivity. This rapid assay yields accurate blood value results for poly(I):poly($C_{12}U$) and may be adapted to measuring other RNAs in clinical specimens.

Acknowledgements
This work was supported in part by Grant CA29545 from the NIH and a grant from the Advanced Technology Center of Southeastern Pennsylvania. The authors are grateful to Susan Novak and Evelyn Ross for drawing blood samples,

John Gray and Peter Wong at DuPont (Wilmington, Delaware) for insights on the curve fitting, Edward Pequignot for advice on statistics, and Bob Solomon for many valuable discussions.

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