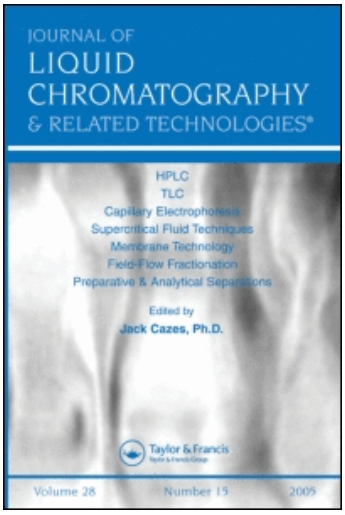


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# High Performance Liquid Chromatographic analysis of the Biological Response Modifier Synthetic Double-Stranded RNA (dsRNA, Ampligen)

Michael G. Rosenblum[a]; Lawrence Cheung[a]
[a] Department of Clinical, Immunology and Biological Therapy The University of Texas M. D. Anderson Hospital and Tumor Institute, Texas

**To cite this Article** Rosenblum, Michael G. and Cheung, Lawrence(1986) 'High Performance Liquid Chromatographic analysis of the Biological Response Modifier Synthetic Double-Stranded RNA (dsRNA, Ampligen)', Journal of Liquid Chromatography & Related Technologies, 9: 13, 2869 — 2877
**To link to this Article: DOI:** 10.1080/01483918608074156
**URL:** http://dx.doi.org/10.1080/01483918608074156

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(13), 2869-2877 (1986)

# HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE BIOLOGICAL RESPONSE MODIFIER SYNTHETIC DOUBLE-STRANDED RNA (dsRNA, AMPLIGEN)

Michael G. Rosenblum and Lawrence Cheung
*Department of Clinical Immunology and Biological Therapy*
*The University of Texas*
*M. D. Anderson Hospital and Tumor Institute*
*6723 Bertner Avenue*
*Houston, Texas 77030*



ABSTRACT

A high performance liquid chromatographic method was developed for analysis of the antitumor agent synthetic, double-stranded RNA (dsRNA, Ampligen) in plasma. In this procedure the agent was extracted from plasma by ion-exchange chromatography and then degraded to its primary components, inosine and cytidine, by treatment with nuclease and alkaline phosphatase. Inosine and cytidine derived from degradation of ampligen were quantified by reversed phase HPLC. Standard curves for quantitation of inosine derived from ampligen were generated by addition of various amounts of ampligen to plasma. Utilizing this method, extraction of drug from the plasma was approximately 70-80%. Standard curves were linear over the concentration range of 0.25 to 100 ug/ml plasma. There were no peaks from plasma which interfere with quantitation of inosine. The approximate lower limit of detection of drug by this method was 0.25 ug/ml plasma. Interassay and intra-assay mean variability of standards was 9.6 and 3.2% respectively. Analysis of plasma samples obtained from one patient after infusion of ampligen (640 mg/$m^2$) show that this method is sensitive enough to monitor plasma for clinical pharmacology studies of ampligen.



 0148-3919/86/0913-2869$3.50/0

## INTRODUCTION

The high molecular weight biological response modifier synthetic, mismatched double-stranded RNA (dsRNA, Ampligen) is a biologically active but less toxic congener of the interferon inducer, polyinosine: polycytidylic acid (poly I:C) (1,2,3). Ampligen has recently received considerable attention because of its immunomodulatory and direct antiproliferative activity against a variety of human tumors both *in vitro* and *in vivo* (4,5,6,7). Although the mechanism of antiproliferative activity is not known, part of the effect of Ampligen may be due to induction of endogenous interferon production or release since treatment of cells with neutralizing antibodies to interferon can partially block the antiproliferative effects of Ampligen. In *in vitro* preclinical studies, Ampligen shows impressive synergistic antiproliferative activity when combined with human recombinant interferons against various human tumor cell lines (8,9,10).

To assist in the pharmacological analysis of Ampligen phase I clinical trials, we developed a sensitive high performance liquid chromatographic method for quantitation of this agent in plasma obtained from patients treated with Ampligen.



## MATERIALS AND METHODS

### Materials

All reagents and solvents for HPLC were reagent grade or higher. Alkaline phosphatase (TYPE III-R from *E. coli*) as well as the inosine and cytidine standards were purchased from Sigma Chemical Company. Nuclease $P_1$ (from Penicillium citrinium) was purchased from Boehringer Mannheim Biochemicals.

### Methods

To 3 ml of plasma in disposable glass test tubes (16 x 100 mm), 1 ml of 0.9% saline and various concentrations of double-stranded RNA were added. The samples were mixed rapidly, allowed to stand

for 5 minutes on ice, and then applied to small (5 ml) polypropylene columns (Isolab, Inc.) containing 1 ml of DE-52 ion exchange resin (Whatman, Inc.) previously equilibrated in a buffer containing 20 mM Hepes and 90 mM KCl (Buffer G.) and washed with 1 ml of 0.1M KCl (pH 6.5) prior to sample loading. After the samples were loaded, the columns were washed three times with 3 ml of 0.1 M KCl and then sequentially with 1 ml each of 0.2M and 0.4M KCl. The samples were finally eluted with 0.8M KCl directly into 16 x 100 mm disposable glass test tubes. A 200 ul aliquot of the 0.8M KCl extract was added to a 1.5 ml plastic micro-centrifuge tube (Fisher Scientific, Co.), the samples were heated at 95°C for 2 minutes and then cooled rapidly in an ice bath. Then, 4 ul of 20 mM $ZnSO_4$, 10 ul of alkaline phosphatase (1 mg/ml), and 40 ul of nuclease $P_1$ (1 mg/ml in 30 mM NaOAc pH 5.3) were added, the samples were mixed rapidly and incubated for 1 hr. at 37°C. A 30 ul aliquot of 0.5M Tris, pH 7.5, was added and the samples were further incubated for 1 hr. at 37°C, cooled and chromatographed as described below.



High Performance Liquid Chromatography

The HPLC system consisted of a Water's model 721 system controller, an automatic sample injector (WISP Model 710B), two M6000A pumps, a variable-wavelength U.V. detector (Model 481) and a Waters Model 730 data module. Chromatographic separations were performed using a Waters Bondapack C-18 column (10 micron particle size, 3.9 mm x 30 cm). The mobile phase was 0.5M NaOAc pH 6.5 with a constant flow rate of 2 ml/min. The column effluent was monitored for UV absorbance at 260 nm.

## RESULTS AND DISCUSSION

The development of this HPLC procedure was based upon the total enzymatic degradation of Ampligen to its major primary components namely cytidine and inosine. As shown in Figure 1, cytidine and inosine were well-separated in this chromatographic system eluting at 4.4 and at 13.4 minutes respectively. Degradation of Ampligen to its cytidine and inosine components by enzymatic digestion and

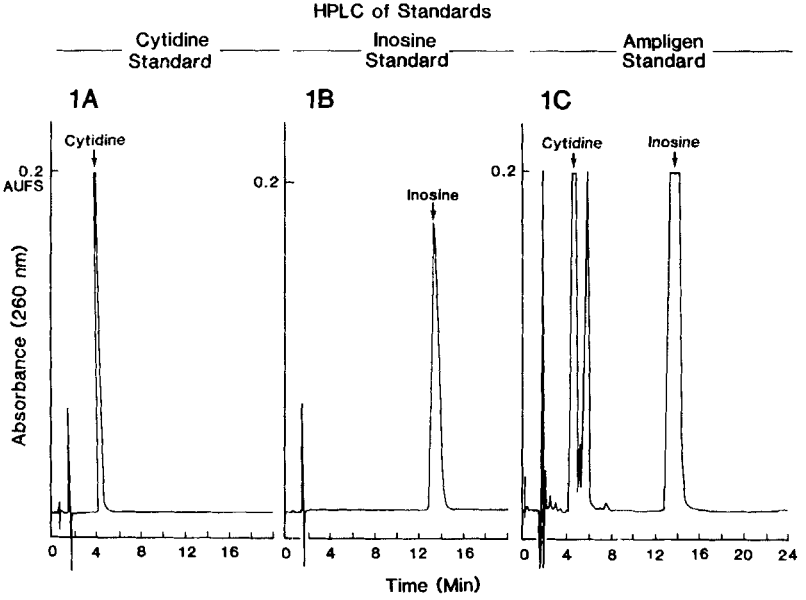


FIGURE 1.

A. Chromatogram of cytidine standard which represents 0.5 μg of injected material. Retention time for cytidine was 4.4 minutes at a flow rate of 2 ml/min.

B. Chromatogram of inosine standard which represents 0.5 μg of injected material. Retention time for elution of inosine was approximately 13.4 minutes after injection.

C. Chromatogram of Ampligen after enzymatic degradation (see methods) to its inosine and cytidine components. This chromatogram represents 100 μg of Ampligen.

subsequent chromatographic separation of the component showed the expected generation of the cytidine and inosine major components.

Standard curves for quantitation of ampligen in plasma were generated by the method of addition of various amounts of drug to control human plasma followed by extraction, enzymatic degradation and HPLC analysis of the resulting products. The concentration of ampligen in plasma sample was based upon the generation of inosine

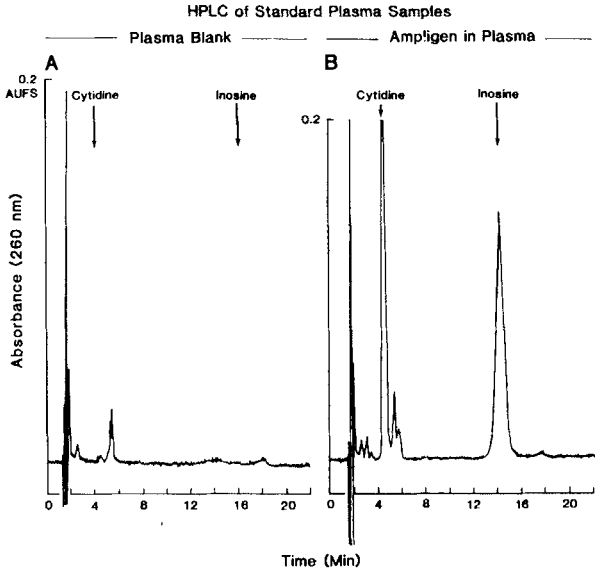


FIGURE 2.

A. Chromatogram of enzyme-treated plasma without addition of Ampligen (plasma blank).

B. Chromatogram of plasma with 10 μg Ampligen added/ml of plasma, treated enzymatically to promote ampligen degradation and chromatographed as described in method section.

after enzymatic degration. Peak areas representing inosine were calculated and peak areas were plotted versus the amount of Ampligen added. Standard curves focusing on inosine generated were linear over the range of 0.25 to 100 ug Ampligen/ml plasma (standard curves not shown). As shown in Figure 2, there were no peaks which interfere with quantitation of inosine in plasma. Utilizing this method, extraction of ampligen from plasma was approximately 70-80% efficient. Interassay variability of standards (50 ug/ml) was 10.5, 14.7, 8.4, 11.6, 6.3, 10.7, and 5% (mean 9.6%) and the variability of intra-assay standards was 2.0, 1.0, 9.5, 2.0, 3.0 , and 1.5% (mean 3.2%). The lower limit of detection (LLD) of Ampligen was approximately 0.25 ug per ml of plasma.

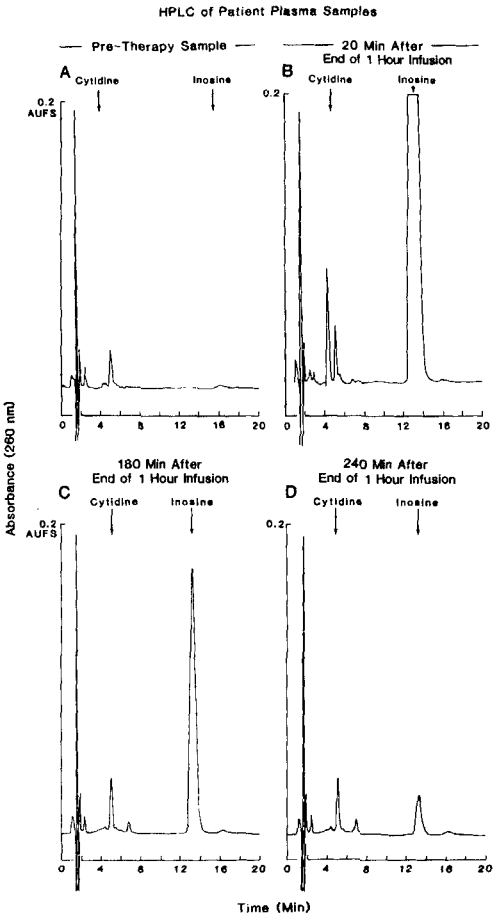


FIGURE 3:

3A - Plasma sample from a patient prior to administration of Ampligen.

3B - Plasma sample from a patient 20 min after end of a 1 hr infusion of Ampligen (640 mg/$m^2$, 1280 mg total). Peaks represent 27.69 ug/ml of Ampligen based upon generated inosine.

3C - Plasma sample from A patient 180 min after end of a 1 hr infusion of ampligen (640 mg/$m^2$, 1280 mg total). Peaks represent 9.24 µg/ml of Ampligen based upon generated inosine.

3D - Plasma sample from a patient 250 min after end of a 1 hr infusion of Ampligen (640 mg/$m^2$, 1280 mg total). Peaks represent 2.46 µg/ml of ampligen based upon generated inosine.

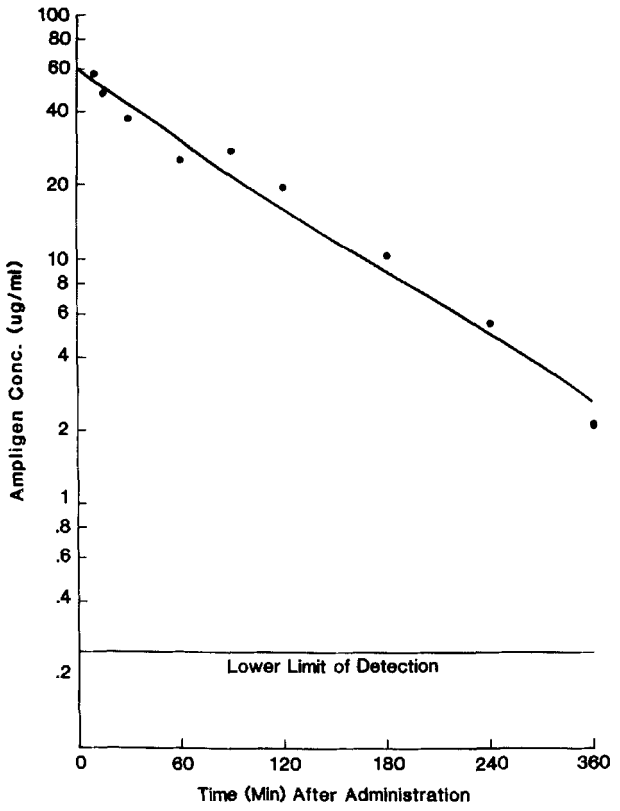


FIGURE 4:

Plasma disappearance curve from a cancer patient treated with Ampligen (100 $\mu g/m^2$). Plasma samples were withdrawn at various times after the end (time=0) of a 1 hr drug infusion.

Plasma samples were obtained from a patient prior to therapy 20 minutes, 180, and 240 minutes after the end of a 1 hr Ampligen infusion. As shown in Figure 3A the pretherapy patient sample contained no peaks which interfere with Ampligen quantitation. In addition, both inosine and cytidine were generated by enzymatic degradation (Fig. 3B). Analysis of this patient sample containing Ampligen prior to in vitro enzymatic degradation showed no endogen ous free inosine or cytidine which would be indicative of in vivo metabolism of Ampligen.

Application of this analytical method to plasma samples obtained from a patient at various times after administration of Ampligen (dose - 640 mg/m2) as a 1 hr infusion are shown in Figure 4. At this dose, the HPLC method allowed quantitation of ampligen concentrations in plasma for up to 6 hrs. after the end of drug infusion thus demonstrating the utility of this method for clinical pharmacology studies. However, although this method for quantitation of Ampligen in plasma appears to be sensitive, accurate and reproducible, it does not account for possible *in vivo* metabolism of the drug since smaller molecular-weight components of Ampligen should behave in this analytical method identically to smaller amounts of parent drug. Attempts to develop a method for sensitive quantitation of ampligen based upon gel permeation HPLC and molecular weight analysis have been unsuccessful. However, for a complete understanding of the clinical pharmacology of Ampligen, a method for analysis of possible lower molecular weight metabolites of this agent may be essential to complement the current assay.



REFERENCES

1. Carter, W.A., Pitha, P.M., Marshall, L.W., Tazawa, I.L., Tazawa, S., Ts'o, P.O.P. J. Molec. Biol., 70 , 567, 1972.

2. Carter W.A., Strayer, D.R., Gillespie, D.H., Brodsky, I, Greene, J.J., Ts'o, P.O.P. Augmenting Agents in Cancer Therapy, edited by E.M. Hersh, Raven Press, New York, 1981, 177.

3. Zarling, J.M., Schlais, J., Eskra, L., Greene, J.J., Ts'o, P.O.P, and Carter, W.A., J. Immuno. 124, 1852, 1980.

4. Ts'o, P.O.P., Alderfer, J.L., Levy, J., Marshall, L.W., O'Malley, J., Horoszewicz, J., and Carter, W.A., Molec. Pharm., 12, 299, 1976.

5. Lin, S.L., Greene J.J., and Ts'o, P.O.P., Nature, 297, 417, 1982.

6. Hubbell, H.R., Liu, R-S., and Maxwell, B.L., Cancer Res., 44, 3252, 1984.

7. Hubbell, H. R., Carter, W.A., and Strayer, D.R., Proceedings of AACR, Vol. 25 (#928), May 9-12, 1984, Toronto, Ontario, Canada.

8. Rosenblum, M.R. and Gutterman, J., Proceedings of the AACR, Vol. 26 (#1105), May 22-25, 1985, Houston, Texas.

9. Hubbell, H.R., Carter, W.A., Williams, R.D. and Strayer, D.R., Proceedings of AACR, Vol. 25 (#1418), May 9-12, 1984, Toronto, Ontario, Canada.

10. Hubbell, H. R., Proceedings of AACR, Vol. 26 (#1091), May 22-24, 1985, Houston, Texas.