Comparative Metabolite Profiling and Identification of a GalNAc-Conjugated siRNA, siRNA01, in Plasma Prepared with Various Anticoagulants, Serum, and *In Vivo* Plasma Using LC-UV-HRMS

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Introduction

Following subcutaneous administration, GalNAc-conjugated siRNA enters the blood circulatory system, where it can be metabolized by nucleases. The metabolic stability of the siRNA in the blood is crucial for the effectiveness of siRNA-based drugs. Therefore, a reliable *in vitro* incubation system that can predict the *in vivo* metabolism of siRNA in blood is critical, especially for screening lead compounds with high metabolic stability. However, systematic research on this issue has not yet been reported in detail.

Objective

The main objective was to identify a suitable *in vitro* incubation system that could predict the metabolism of siRNA01 by nucleases in *in vivo* blood.

Method

In this study, a comparative analysis of the metabolite profiles of siRNA01, a GalNAc-conjugated siRNA available on the market, was conducted in plasma prepared with various anticoagulants and in serum. A well-established ion-pairing LC-UV-HRMS method was used for the analysis. These results were then compared with *in vivo* rat plasma metabolite profiles.

Results

Metabolite profiling of siRNA01 in rat plasma, prepared with EDTA and sodium heparin anticoagulants, and in serum, revealed that the predominant metabolite of the antisense strand (AS) in both sodium heparin-anticoagulated plasma and serum is 3' n-1, with relative abundances of 17.48% and 26.78%, respectively (Table 1). A noteworthy result was that this major metabolite was not detected in EDTA-anticoagulated rat plasma (Figure 1). This may be due to the chelation of EDTA with divalent metal ions, such as Ca^{2+} and Mg^{2+}, thereby inhibiting nuclease activity. No relevant metabolites of the sense strand (SS) were detected in these systems, indicating that the SS is more stable than the AS in plasma or serum. Metabolite profiling of siRNA01 in *in vitro* monkey and human plasma, prepared with both EDTA and sodium heparin anticoagulants, as well as in serum, yields results consistent with those observed in the rat matrices (Data not shown).

In the rat plasma administered with siRNA01, the main metabolite of the AS was 3‘ n-1 (14.62%, Table 1). These results are similar to those of the *in vitro* metabolic profiles of sodium heparin-anticoagulated rat plasma and serum (Table 1). Additionally, minor SS-related metabolites, including those with a loss of one GalNAc (3.43%), two GalNAcs (0.71%), and three GalNAcs (1.05%), were detected in *in vivo* rat plasma, which were not detected in the *in vitro* plasma and serum samples.

Besides, the metabolite profiles of siRNA01 in freshly prepared rat plasma anticoagulated with sodium heparin and serum were similar to those of their corresponding cryopreserved matrices (Figure 1).

Table 1. Comparative metabolite profiling and identification of siRNA01 in *in vitro* rat plasma with sodium heparin, serum, and in vivo rat plasma

Code	Metabolic Changes	Relative Abundance (MS Peak Area, % Total) *In vitro*		*In vivo*
		Plasma	Serum	Plasma (AUC)
M1	AS 3' n-9	0.53	0.05	ND
M2	AS 3' n-7	0.36	0.49	0.23
M3	AS 3' n-5	0.50	0.48	ND
M4	AS 3' n-1	17.48	26.78	14.62
AS	Full-length AS	81.13	72.20	85.15
SS	Full-length SS	100.00	100.00	94.81
M5	SS-GalNAc	ND	ND	3.43
M6	SS-2GalNAc	ND	ND	0.71
M7	SS-3GalNAc	ND	ND	1.05

ND: not detected; $\%\text{Total} = \frac{\text{Peak Area of a Related Component}}{\text{Peak Area of Total Related Component}} \times 100$

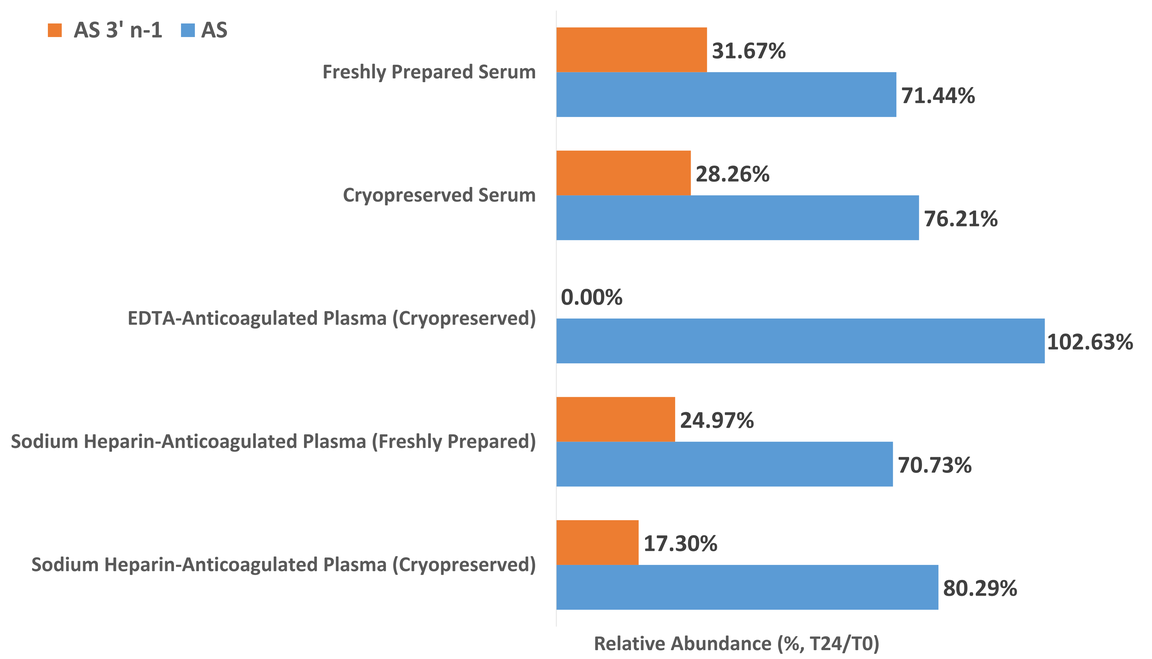

Figure 1. Comparative analysis of AS and its main metabolite AS 3' n-1 in fresh and cryopreserved rat plasma (with various anticoagulants) and serum.

Conclusion

- Both sodium heparin-anticoagulated plasma and serum can be utilized for the metabolic study of siRNA and other oligonucleotides. These two models can predict the metabolite profiles of oligonucleotides in blood *in vivo*, and help to evaluate whether the related metabolites can be metabolized by the nucleases in the bloodstream.
- EDTA-anticoagulated plasma may not be suitable for the metabolic study of siRNA1 and other oligonucleotides, possibly due to EDTA's reported inhibition of nuclease activity by the chelation of divalent metal ions.
- Commercially cryopreserved matrices are acceptable for siRNA01 metabolic studies, obviating the need for fresh preparation.

References

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