Evaluation of Molecular Markers for Coagulation and Fibrinolysis by Automated Chemiluminescent Enzyme Immunoassay Analyzer "HISCL-2000*i*"

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Thrombin-antithrombin complex (TAT) and plasmin-α_2 plasmin inhibitor complex (PIC) are respectively used as activation markers for coagulation and fibrinolysis for early diagnosis and treatment of thrombotic diseases. We evaluated the basic TAT and PIC measurement performance of an HISCL-2000i analyzer (Sysmex Corporation), newly inducted into our laboratory, which uses a chemiluminescent enzyme immunoassay as its measurement principle. Our study, which used specimens from patients of our hospital and healthy individuals, showed good reproducibility, assay linearity, and minimum detection sensitivity. As for the effect of coexisting substances, high concentrations of free bilirubin had a negative effect on measured TAT, but the presence of conjugated bilirubin, hemolysis and chylemia had no effect. The reference ranges used were 1.38 ng/mL for TAT and 1.07μg/mL for PIC.

The correlation of the HISCL assay with the conventional LPIA-A700 assay (Mitsubishi Chemical Medience) was good for PIC, and generally good for TAT although with discrepancy in results in some cases. So the FDP, D-dimer, soluble fibrin monomer complex, antithrombin levels, etc of the discrepant cases were investigated. The results showed that HISCL provided measured values that adequately reflected the coagulation/fibrinolysis status. HISCL-2000i requires only 17 minutes for the TAT and PIC assays, and therefore can enable rapid diagnosis. Thus, the analyzer is considered to be highly useful for clinical applications.

Key Words HISCL-2000*i*, Thrombin-Antithrombin Complex (TAT), Plasmin-α_2 Plasmin Inhibitor Complex (PIC), Activation Markers for Coagulation and Fibrinolysis

INTRODUCTION

Thrombin-antithrombin complex (TAT) and plasmin-α_2 plasmin inhibitor complex (PIC) have been used for early diagnosis and treatment of thrombotic diseases as activation markers for coagulation and fibrinolysis, respectively [1-4].

We evaluated the basic TAT and PIC measurement performance of HISCL-2000*i* (Sysmex Corporation), a fully automated immunoassay system based on chemiluminescent enzyme immunoassay (CLEIA).

1. Methods

Samples were analyzed using HISCL-2000*i* in combination with the HISCL TAT and HISCL PIC reagents. For control, samples were analyzed using the LPIA-A700 fully-automated immunoserology system (Mitsubishi Chemical Medience) in combination with LPIA-F·TAT Test II and LPIA ACE PPI II.

2. Measuring principle

A biotinylated antithrombin monoclonal antibody or biotinylated anti-plasminogen monoclonal antibody reacts specifically with thrombin or plasminogen in the sample, and binds to streptavidin-conjugated magnetic beads. Following removal of the unreacted portion of the reaction mixture, an ALP-labeled antithrombin or anti-

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α_2PI monoclonal antibody reacts specifically with antithrombin or α_2PI on the magnetic beads. After removing the unreacted portion of the reaction mixture, a luminescent substrate is added. The substrate is degraded by ALP and luminesces, and the intensity of the light is determined (***Fig. 1***). Prior to washing, the magnetic beads are gathered using a magnet and subjected to B/F separation to increase detection sensitivity.

3. Subjects

The study population comprised 128 inpatients or outpatients who presented at Kanazawa University Hospital (77 males aged 0-80 years, 51 females aged 7-97 years), as well as 180 healthy volunteers (82 males aged 22-82 years, 98 females aged 22-63 years).

4. Performance parameters

1) Reproducibility
Pooled plasma was prepared at three concentrations and analyzed once daily in 10 consecutive runs and on 5 days to obtain data for determining repeatability and daily precision, respectively.

2) Storage stability of control plasma solutions
Dedicated control plasma L and H were dissolved, dispensed to test tubes, and stored under refrigeration at 4°C or freezing at -80°C. Each tube of solution was analyzed for 28 days starting on the day of dissolution.

3) Influences of freeze-thawing on patient samples
Patient samples (86 for TAT, 79 for PIC) were analyzed immediately after blood drawing and after freezing storage and thawing, and the measurements were compared.

4) Assay linearity
Patient samples with a high TAT or PIC value were serially diluted using the HISCL sample diluent and analyzed for TAT and PIC.

5) Minimum detection sensitivity
Standard samples containing a known concentration of TAT or PIC were serially diluted. Each diluted sample was analyzed in 10 consecutive runs, and the measurements obtained were statistically processed using the ±2SD method.

6) Influences of coexisting substances
Interference Check A Plus (Sysmex Corporation) was added to pooled plasma. The samples thus obtained were analyzed in the presence of various interfering substances in duplicate. If measured value variation exceeded 10%, each coexisting substance was judged to have influenced the assay.

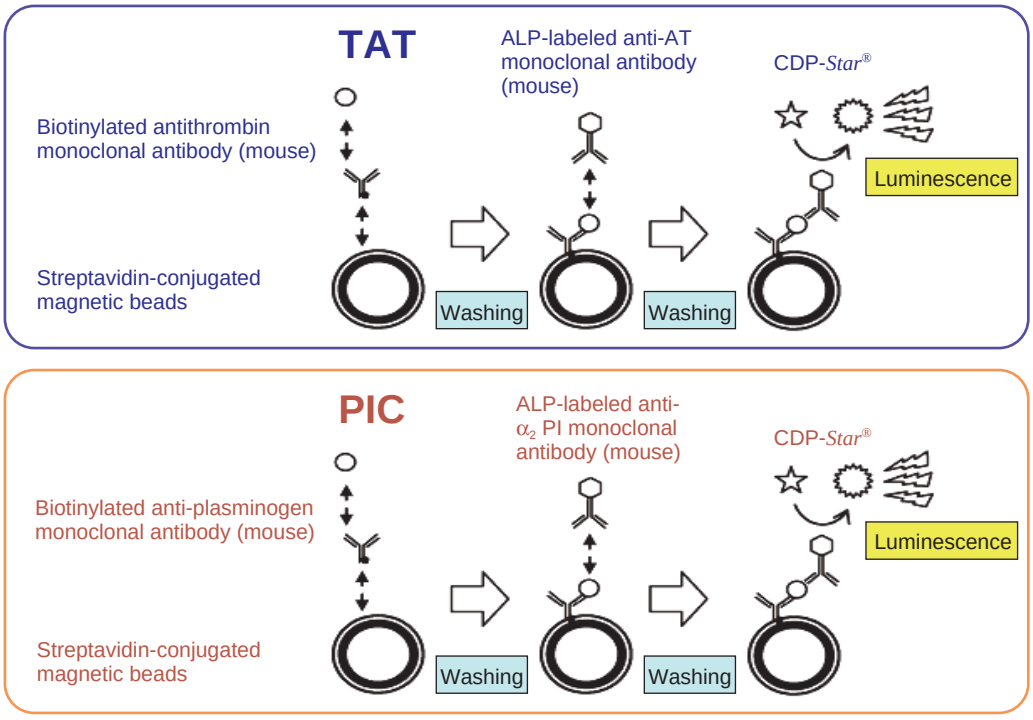


Fig. 1 *Measuring principle*

7) Reference value ranges
Reference value ranges were calculated using the parametric method in 180 healthy subjects (82 males aged 22-82 years, 98 females aged 22-63 years).

8) Correlation with conventional method
Analytical values obtained from 128 patients for TAT and 120 patients for PIC using the present assay were compared with those obtained using the conventional LPIA-A700 assay.

9) Patients with TAT value discrepancies between the present method and the conventional method

(1) A cutoff value for the TAT value correlation between the two methods was set at 4 ng/mL in accordance with the instruction manual for the HISCL TAT reagent. Higher and lower values were considered to indicate positivity and negativity, respectively. Samples from 5 patients with any discrepancy were further analyzed for other molecular markers of coagulation-fibrinolytic activity: FDP, D-dimer (DD), soluble fibrin monomer complex (SF), and antithrombin (AT).

(2) Samples from 3 of the 5 patients with discrepant TAT values were analyzed for fibrinogen/fibrin degradation product by a Western blotting test with an anti-fibrinogen antibody to determine the coagulation and fibrinolytic status in each sample.

RESULTS

1) Reproducibility
Repeatability was determined to be CV = 1.54 - 2.53% for TAT and 1.67 - 4.57% for PIC, and 5-day daily precision was determined to be CV = 1.66 - 4.39% for TAT and 1.51 - 3.02% for PIC (***Table 1***).

2) Storage stability of control plasma solutions
Changes in dissolved control plasma over time during storage under refrigeration at 4°C and freezing at -80°C are shown in (***Fig. 2***). With an assay value falling in the daily precision range regarded as indicating no change, TAT was found to be stable under refrigeration at 4°C for 4 days and freezing at -80°C for 28 days, and PIC was found to be stable for 5 days and 14 days, respectively.

3) Influences of freeze-thawing on patient samples
A comparison of assay values obtained on the day of blood drawing and those obtained after freezing storage and thawing revealed no clinically problematic discrepancies (***Fig. 3***).

4) Assay linearity
Assay linearity was observed at concentrations up to 110 ng/mL for TAT and 38 μg/mL for PIC (***Fig. 4***).

5) Minimum detection sensitivity
Minimum detection sensitivity was determined to be 0.024 ng/mL for TAT and 0.0011 μg/mL for PIC (***Fig. 5***).

Table 1 *Reproducibility*

Repeatability

	TAT (ng/mL)			PIC (μg/m L)		
	A	B	C	A	B	C
n	10	10	10	10	10	10
mean	4.37	10.52	26.24	1.00	1.89	7.98
SD	0.07	0.27	0.44	0.03	0.03	0.37
CV(%)	1.54	2.53	1.66	2.84	1.67	4.57
MAX	4.5	11.0	27.4	1.05	1.90	8.32
MIN	4.3	10.1	25.8	0.96	1.80	7.11

Daily precision

	TAT (ng/mL)			PIC (μg/m L)		
	A	B	C	A	B	C
n	5	5	5	5	5	5
mean	4.07	10.27	26.74	1.06	1.85	8.53
SD	0.18	0.26	0.44	0.03	0.06	0.13
CV(%)	4.39	2.57	1.66	3.02	3.02	1.51
MAX	4.3	10.5	27.1	1.09	1.90	8.67
MIN	3.8	9.9	26.1	1.02	1.77	8.37

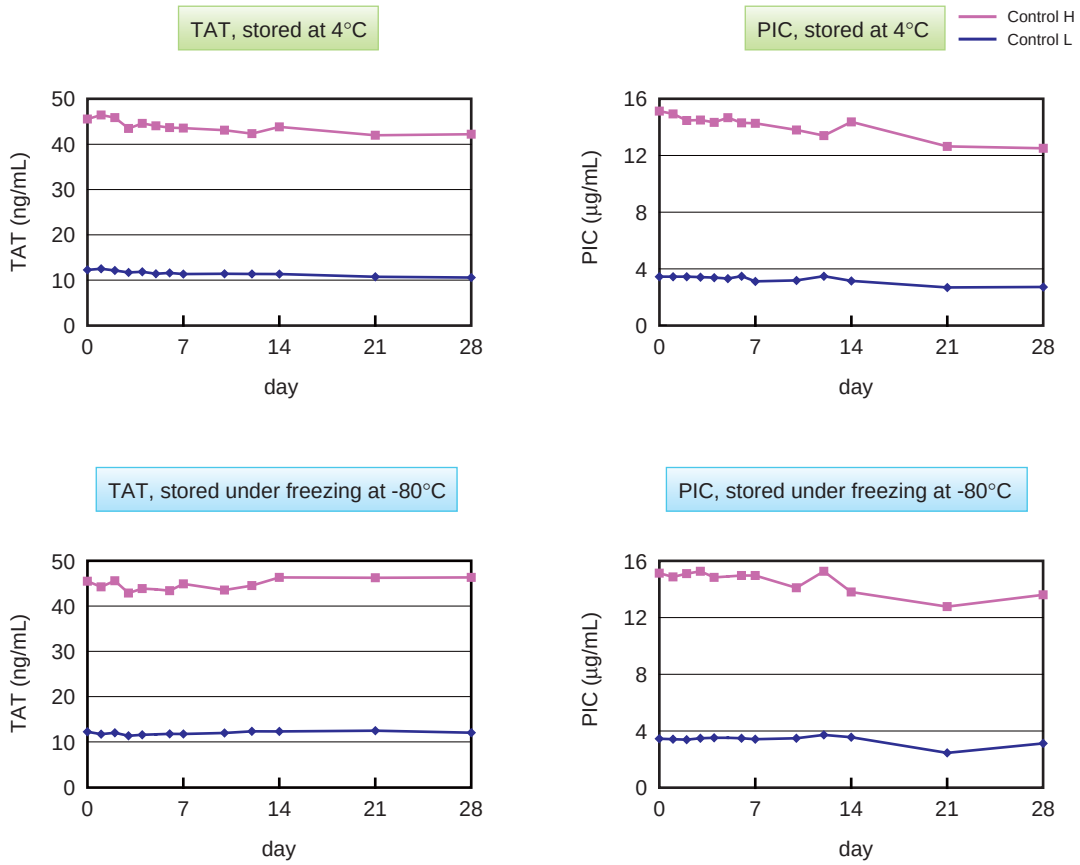


Fig. 2 *Storage stability of control plasma solutions*

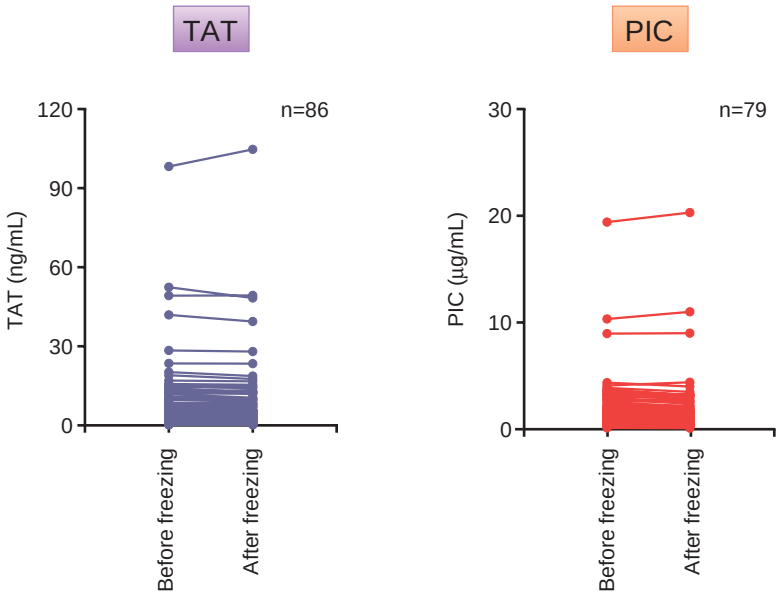


Fig. 3 *Influences of freeze-thawing on patient samples*

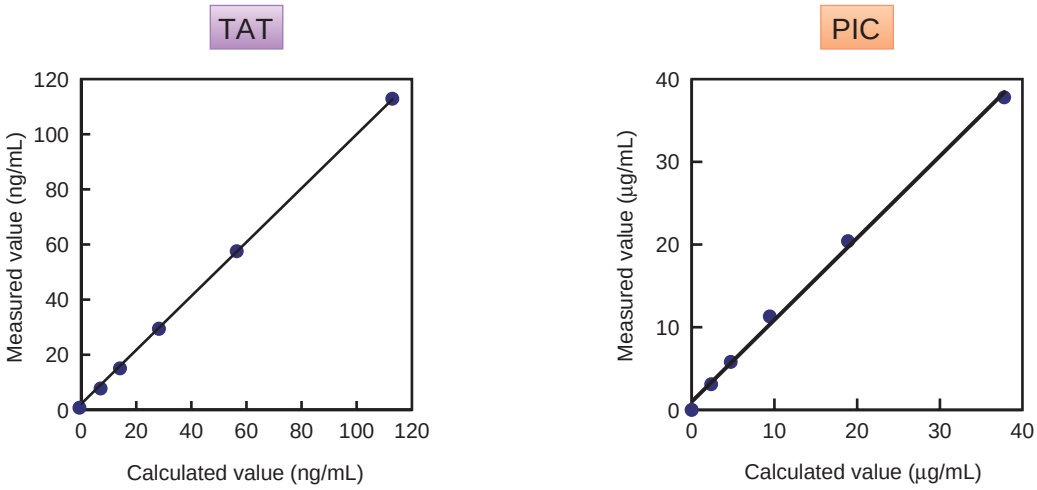


Fig. 4 *Assay linearity*

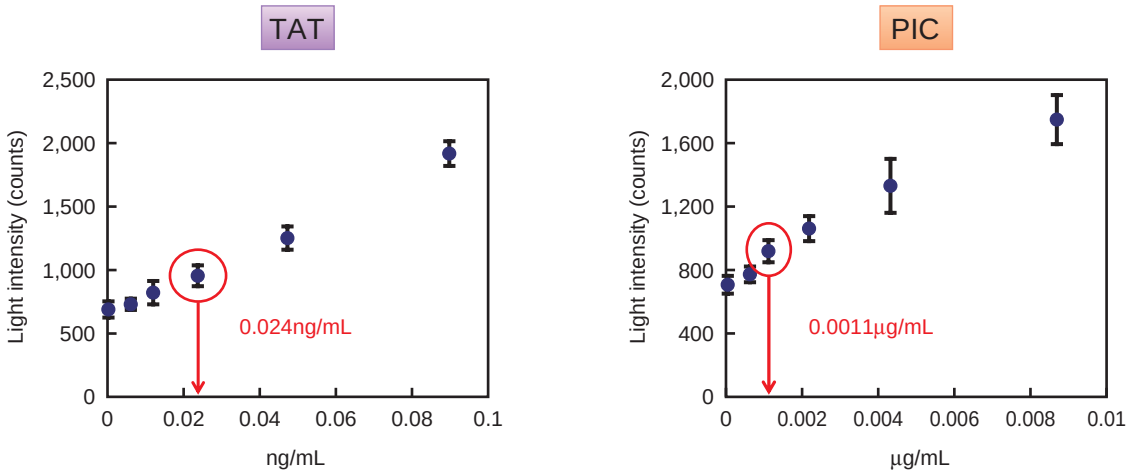


Fig. 5 *Minimum detection sensitivity*

6) Influences of coexisting substances
TAT assay was negatively influenced by free bilirubin at concentrations exceeding 15 mg/dL, whereas no influence was observed on PIC at free bilirubin concentrations up to 18.7 mg/dL. For both TAT and PIC, assay was not influenced in the presence of up to 19.7 mg/dL conjugated bilirubin, 498 mg/dL hemoglobin, and 1,440 formazin turbidity unit of chyle (***Fig. 6***).

7) Reference value ranges
Reference value ranges from 180 healthy subjects were calculated as ≤ 1.38 ng/mL for TAT and ≤ 1.07 µg/mL for PIC.

8) Correlation with conventional method
A comparison of the present method and the conventional method revealed a regression equation of $y = 0.823x + 2.138$ with a correlation coefficient of $r = 0.941$ for TAT; although a general correlation was found, discrepant values were obtained from some patients. For PIC, a good correlation was found, with a regression equation of $y = 1.009x + 0.014$ with a correlation coefficient of $r = 0.986$ (***Fig. 7***).

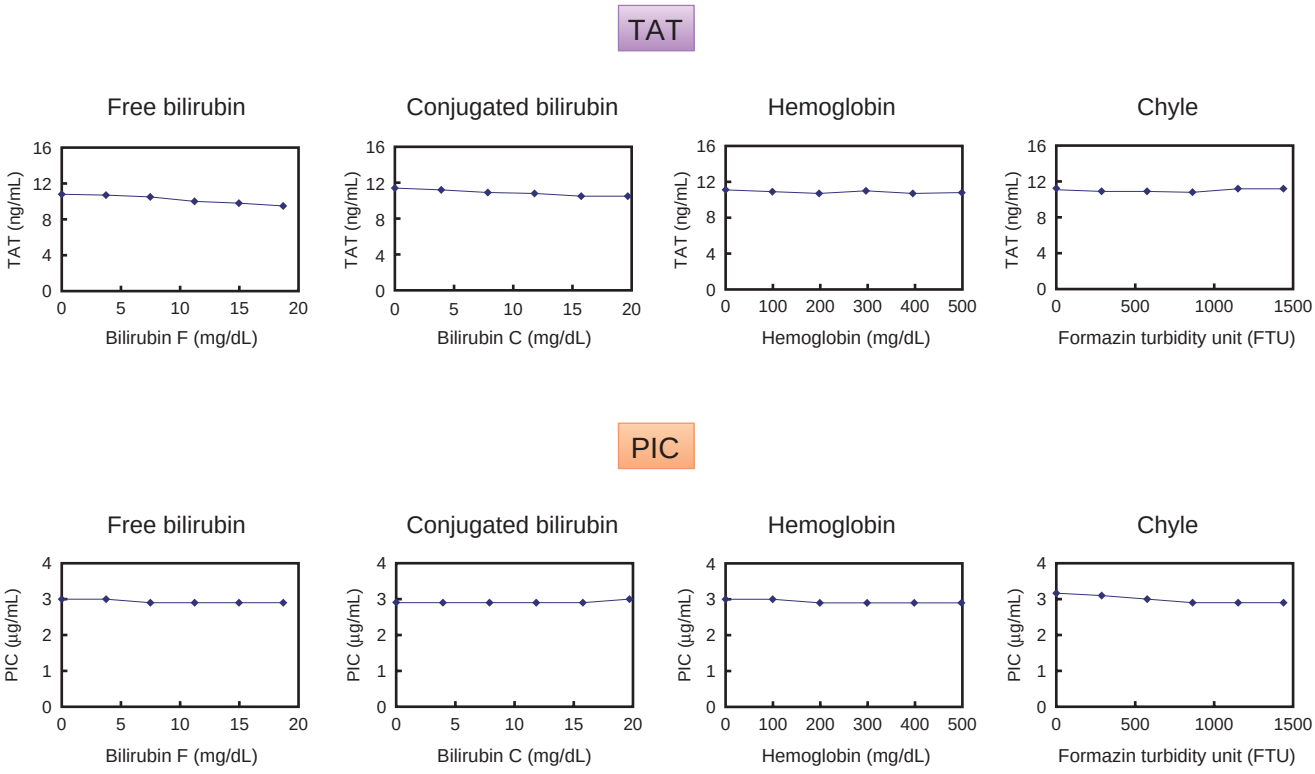


Fig. 6 *Influences of coexisting substances*

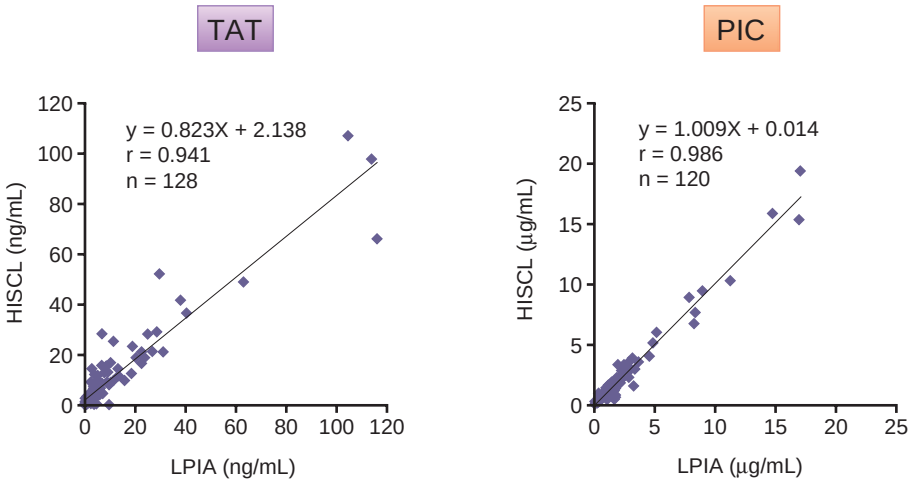
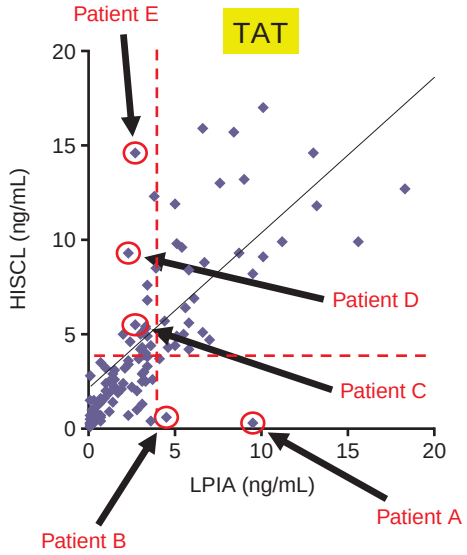


Fig. 7 *Correlation with conventional method*

9) Patients with TAT value discrepancies between the present method and the conventional method

(1) The measured values of molecular markers for coagulation-fibrinolysis in the 5 deviating patients (A-E) are shown in (***Fig. 8***). FDP, DD, and SF were detected at normal levels in patients A and B (lower TAT values obtained with the present method than with the conventional method), whereas in patients C, D, and E (higher TAT values obtained with the present method than with the conventional method), FDP, DD, and SF were detected at abnormal levels.

(2) Western blotting of samples from patients A, D, and E with discrepant values detected no band suggestive of coagulation and fibrinolytic activity in patient A, whereas in samples from patients D and E, bands suggestive of DD and D fractions appeared (***Fig. 9***).



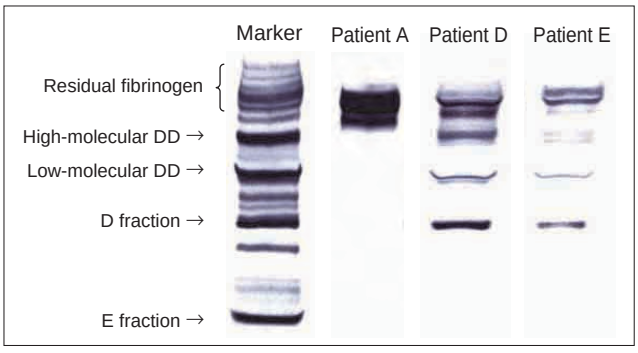
Patients A-E

Other coagulation and fibrinolytic system markers

	TAT (ng/mL)		FDP (μg/mL)	DD (μg/mL)	SF (μg/mL)	AT (%)
	HISCL	LPIA				
Patient A	0.3	9.5	1.5		2.2	113
Patient B	0.6	4.5	1.6	0.7	4.8	95
Patient C	5.5	2.7	42.5	14.9	19.7	69
Patient D	9.3	2.3	59.8	27.4	16.7	133
Patient E	14.6	2.7	12.1	6.9	8.5	
	Reference Interval		< 5.0	< 1.0	< 7.0	70~130

Fig. 8 *Patients with TAT value discrepancies between HISCL and LPIA - 1*

Western blotting test (primary antibody:antifibrinogen antibody)



	TAT (ng/mL)		FDP (μg/mL)	DD (μg/mL)	SF (μg/mL)
	HISCL	LPIA			
Patient A	0.3	9.5	1.5		2.2
Patient D	9.3	2.3	59.8	27.4	16.7
Patient E	14.6	2.7	12.1	6.9	8.5

Fig. 9 *Patients with TAT value discrepancies between HISCL and LPIA - 2*

DISCUSSION

The repeatability CV was calculated to be 1.54 - 2.53% for TAT, and 1.67 - 4.57% for PIC. The daily precision was calculated to be 1.66 - 4.39% for TAT and 1.51 - 3.02% for PIC, which were found to be nearly equivalent to those obtained using HISCL-2000*i* at three other facilities [5-7]. Regarding the repeatability of the fully automated clinical laboratory test system STACIA (Mitsubishi Chemical Medience), a successor to comparator LPIA-A700 in the present study, Kazama et al. determined the repeatability CV to be 3.24 - 5.74% for TAT and 0.31 - 1.20% for PIC. The daily precision CV values of 11.47 - 11.99% for TAT and 0.68 - 1.77% for PIC. Similar values of basic performance parameters for STACIA were reported from other facilities [9-13].

Storage stability of control plasma solutions

Control plasma solution data stability during refrigerating storage was found for 4 days for TAT and 5 days for PIC. While the dedicated control plasma supplies are freeze-dried preparations claimed to be stable at 4°C for 5 days following dissolution with 1 mL of purified water [14,15], the present study found a slight reduction starting on the 1st day of storage even under refrigeration. The sampling volume for HISCL-2000*i* was very small at 10 µL, and was less than 200 µL even with dead volume taken into account. For these reasons, dissolution, dispensing, and -80°C freezing are expected to allow TAT and PIC to yield stable data for 28 and 14 days respectively, and to lead to cost reductions.

TAT, a complex formed by thrombin resulting from coagulation system activation and inhibitor AT, serves as a coagulation activation marker [2,3]. In DIC, TAT is not specific but important because a normal TAT value rules out activated coagulation at the time of blood drawing [2]. The present study showed that acceptable values were obtained from plasma even after freeze-thawing, demonstrating feasibility of analyzing frozen samples in cases where off-hours service is not available or testing is outsourced.

Acceptable values of assay linearity and minimum detection sensitivity were confirmed as reported by many authors [5-7]. As for influences of coexisting substances, TAT assay was negatively influenced by high concentrations of free bilirubin, with no influence observed of conjugated bilirubin, hemolysis, and chyle. Negative influences of free bilirubin in TAT assay using HISCL-2000*i* were reported from other facilities [6,7], and similar findings were reported from TAT assay using STACIA [9-11,13]; the reason remains unknown.

Although the TAT value correlation between the present method and the conventional LPIA-A700 assay was generally good, discrepancies were found in some patients. As shown in (***Fig. 8***), patients A and B tested negative with HISCL-2000*i* and positive with LPIA-A700; however, FDP, DD and SF measurements remained within the respective reference value ranges. Patients C, D, and E tested positive with HISCL-2000*i* and negative with LPIA-A700; however, higher measurements were obtained for FDP, DD and SF. Western blotting with antifibrinogen antibody of samples from patients A, D, and E detected no band in the sample from patient A, whereas in the samples from patients D and E, bands appeared at positions for DD and D fractions, suggesting increased coagulation and fibrinolysis. Judging from these results, the HISCL TAT reagent was considered to better reflect the coagulation activity state in the body than other activation markers for coagulation and fibrinolysis. The observed discrepancies are attributable to a possible nonspecific reaction that occurred at the time of measurement using LPIA-A700. The LPIA-F·TAT Test II kit is based on the 2-step sandwich technique by a time-resolved fluorescent enzyme immunoassay with Europium (Eu) latex. Since the Eu latex reagent in LPIA-F·TAT Test II is based on a Eu latex sensitized with anti-human ATIII polyclonal antibody, a nonspecific reaction mediated by latex particles or the polyclonal antibody [16,17] might influence the assay.

CONCLUSION

The present study confirmed that the fully automated immunoassay system HISCL-2000*i* possesses good analytical performance for detection of TAT and PIC. Furthermore, HISCL-2000*i* requires only 17 minutes for TAT and PIC assays, which is about half the 30-minute time requirement for the conventional method. With these features, HISCL-2000*i* is highly useful in actual clinical settings.

An abstract of this article was presented at the 12th meeting of the Japanese Society for Laboratory Hematology and the 43rd meeting of the Japan Society for Clinical Laboratory Automation. This study was conducted after approval by the medical ethics committee of Kanazawa University (examination No. 922).

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