

Papers

The importance of “like to like” ISI calibrations with freeze dried plasmas

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Abstract

Aim—To assess reliability of like to like and cross species calibrations using two types of certified freeze dried plasma calibrants—artificially depleted of vitamin K clotting factors, and from coumarin treated patients.

Methods—Six ECAA national control laboratories provided certified values for the freeze dried plasmas in terms of the human plain international reference preparation (IRP) (BCT/441) with the manual prothrombin time technique. Eight other ECAA national laboratories determined international sensitivity index (ISI) values in full fresh plasma same species and cross species WHO calibrations against a low ISI human IRP (BCT/441) of the ECAA low ISI human thromboplastin and high ISI ECAA rabbit thromboplastin. Parallel calibrations were performed using the certified values.

Results—Calibrations on fresh plasmas of the human ECAA reference thromboplastin (stated ISI = 0.95) gave ISI of 0.957 against the human IRP and 1.66 against the rabbit IRP. The ECAA rabbit (stated ISI = 1.67) gave an identical value on the fresh plasma calibration *v* the human IRP. With freeze dried depleted plasmas certified in terms of the human IRP, the ISI of the ECAA human was 1.01, but the ECAA rabbit (stated ISI = 1.67) gave a low ISI of 1.47. The freeze dried coumarin plasmas gave an ISI of 0.943 for the ECAA human but only 1.493 for the ECAA rabbit.

Conclusions—Fresh plasmas give reliable ISI when calibrating thromboplastins in same species and cross species calibrations. Freeze dried plasmas certified in terms of a single IRP, whether artificially depleted or of coumarin plasma origin, cannot be used for calibration of dissimilar thromboplastins.

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Keywords: thromboplastin; international sensitivity index; freeze dried plasma; species specificity

Local international sensitivity index (ISI) calibration has been recommended to correct for the variable effects of coagulometers on the

international normalised ratio (INR), but the requirement for manual prothrombin time testing with an international reference preparation (IRP) in parallel with coagulometer prothrombin time testing presents great difficulties.¹⁻⁴ A new calibration step has therefore been proposed, based on freeze dried plasmas certified with IRP manual prothrombin time values, to implement the WHO/INR scheme.⁴ A report from the ECAA (in press) has shown that both freeze dried plasmas artificially depleted of vitamin K dependent clotting factors and those from coumarin treated patients gave a reasonable approximation to the conventional manual ISI calibrations at ECAA national laboratories. The WHO recommendation is for “like to like” calibrations on the basis of better precision of ISI.⁵ This is usually taken to mean employing the same species of tissue thromboplastin in the prothrombin time test. This is perhaps an oversimplification, as until recently rabbit reagents have been high ISI (less responsive) and human reagents low ISI (more responsive). Using reagents of similar responsiveness in ISI calibration is an additional aspect which cannot easily be excluded from this consideration. The adoption by WHO of a rabbit IRP with a low ISI similar to that of the human and bovine IRP, and the proposal to average the ISI of new IRP against the three current IRP, raises the possibility that calibration differences arising from different species of thromboplastins may be minimised.⁶

In an earlier ECAA study it was shown that a cross species fresh plasma calibration (that is, human *v* rabbit or rabbit *v* human) gave the equivalent results as same species calibrations using ECAA human and rabbit and relevant IRP.⁷

In the present study the effect of same species and cross species calibrations on the reliability of local ISI calibrations using both fresh plasmas and freeze dried plasmas has been compared in a multicentre calibration of the human and rabbit reference thromboplastins using freeze dried plasmas (artificially depleted and coumarin) certified in terms of the human plain IRP. The resultant ISI have been compared with the conventional ISI calibration based on fresh plasmas. The cross

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Table 1 Calibration equation slopes for fresh coumarin (n = 60) and fresh normal (n = 20) plasmas and the coefficient of variation (CV) of the slope (b). The two ECAA reference thromboplastins calibrated against the IRP BCT/441

Centre	ECAA human v BCT/441		ECAA human v RBT/90		ECAA rabbit v BCT/441	
	b	CV(b)	b	CV(b)	b	CV(b)
1	0.9322	1.5744	0.8690	2.3747	1.5838	1.6107
2	0.9253	1.4817	0.9629	2.1974	1.5514	2.0918
3	0.9213	1.8797	0.9722	2.6272	1.4702	2.4479
4	0.9105	1.4113	0.9420	2.1631	1.6761	1.6517
5	0.9013	1.3928	0.9282	1.7992	1.5408	1.8799
6	0.9520	1.5658	0.9797	3.2801	1.6716	2.0273
7	0.8735	1.2383	0.9717	2.0957	1.5624	1.8192
8	0.9450	1.4602	0.9760	2.3628	1.7128	1.9447
Mean	0.9201	1.5005	0.9502	2.3625	1.5961	1.9342

species calibration using fresh plasmas and freeze dried plasmas has been compared by substituting the certified prothrombin time values with the human IRP in calibrations of both human and rabbit ECAA reference thromboplastins and comparing ISI with the fresh plasma calibrations according to the WHO protocol.

Method of Study

In a multicentre study, we investigated the effect of substituting freeze dried plasmas (freeze dried depleted or coumarins) and the mean normal prothrombin time (MNPT) of fresh normal plasmas to determine ISI in place of conventional WHO ISI calibration based on 60 fresh plasmas from coumarin treated patients and 20 fresh plasmas from normal healthy subjects. ISI obtained from the fresh plasma and freeze dried plasma calibrations were compared.

FREEZE DRIED PLASMAS

Artificially depleted plasmas

The set of freeze dried depleted plasmas was manufactured at the ECAA Central Facility in Manchester by artificial depletion of normal human plasma from healthy adult donors by selective adsorption of vitamin K dependent clotting factors with barium sulphate. Hepes buffer, glycine, and sucrose were added as protectives before freeze drying. Plasmas with a wide spectrum and even spread of values over the INR range of 1.5 to 4.5 when tested with the International Council for Standardisation in Haematology (ICSH) thromboplastin human plain IRP (BCT/441) were selected. Because of possible complicating effects of depletion of the non-coumarin-dependent clotting factors, factor V and fibrinogen, on the prothrombin time test, factor V and fibrinogen determinations were performed on all the artificially depleted

plasmas to ensure adequate levels. The factor V and fibrinogen content of all the artificially depleted plasmas included in the study ranged from 50% to 100% and from 150 to 400 mg/100 ml, respectively. These were tested before the multicentre certification study and samples containing less than a minimum level of 50% factor V or 150 mg/100 ml fibrinogen were excluded. The minimum quoted values are regarded as adequate for prothrombin time measurement, without having an influence on the resulting clotting time. Inter-vial variation studies, accelerated degradation, and long term stability tests were also performed on each of the depleted freeze dried plasmas. All plasmas gave a coefficient of variation of less than 3% in inter-vial studies and minimum accelerated heat degradation stability of seven days at 40°C. Long term and stability studies proved satisfactory on all samples.

Coumarin plasmas

Single donations obtained from each patient were separately freeze dried after the addition of Hepes buffer, glycine, and sucrose as protectives. These also were freeze dried at the Central Facility. Owing to the difficulty of obtaining sufficient volumes and range of plasmas from oral anticoagulant treated patients spanning the therapeutic interval of 1.5 to 4.5 INR and to avoid duplication of INR, the number of these coumarin plasmas in the study was limited to 20.

The two types of freeze dried plasma were dispensed in 0.5 ml volumes in rubber capped vials and vacuum sealed. They were reconstituted in 0.5 ml volumes with distilled water.

The 20 artificially depleted plasmas and 20 freeze dried coumarin plasmas were certified with prothrombin time values in terms of the human IRP by six ECAA national laboratories ("certifying centres").

FRESH PLASMAS

Fresh plasma were collected locally from 20 healthy normal subjects and from 60 long term patients stabilised on oral anticoagulant treatment for at least six weeks and within the INR range of 1.5 to 4.5 INR with the IRP. Testing was to be performed on eight separate days, which were not necessarily consecutive. Testing of normal plasma was to be in duplicate, but single prothrombin time tests only were to be performed on the coumarin plasmas according to the established protocol.⁸ A fresh normal plasma was to be included at the beginning and

Table 2 Calibration equation slopes for the 20 certified freeze dried depleted plasmas and MNPT entered twice, together with the coefficient of variation (CV) of the slope (b). The two ECAA reference plasma calibrations are shown

Centre	ECAA Human v BCT/441		ECAA rabbit v BCT/441	
	b	CV(b)	b	CV(b)
1	0.9782	3.2150	1.3439	4.5448
2	1.0049	2.8534	1.3921	4.8706
3	0.9862	3.6491	1.4274	3.3239
4	0.9614	2.8707	1.4243	3.2171
5	0.9148	3.9012	1.3349	4.2532
6	0.9996	4.5261	1.5427	4.1724
7	0.9674	3.7038	1.5141	4.3126
8	0.9377	3.7936	1.3108	2.5337
Mean	0.9688	3.5641	1.4113	3.9035

Table 3 Calibration equation slopes for the 20 certified freeze dried coumarin plasmas and MNPT entered twice, together with the coefficient of variation (CV) of the slope. The two ECAA reference plasma calibrations are shown

Centre	ECAA human v BCT/441		ECAA rabbit v BCT/441	
	b	CV(b)	b	CV(b)
1	0.8927	4.5785	1.4154	5.0621
2	0.9800	4.9158	1.4665	5.4864
3	0.9311	4.3108	1.3788	5.0858
4	0.8435	4.8377	1.4594	4.9988
5	0.8874	5.2220	1.2999	5.4560
6	0.9168	3.6175	1.5485	5.6887
7	0.9465	3.9112	1.5720	4.5868
8	0.8638	4.2829	1.3477	5.2947
Mean	0.9077	4.4596	1.4360	5.2074

end of each day's testing. Additional normal samples were to be tested on the last four days to make up the number to 20.

Prothrombin time testing of freeze dried and fresh plasmas was performed using the manual technique. Harmonisation of the manual prothrombin time technique had been attempted at a preliminary "wet workshop" held at the Central Facility.

THROMBOPLASTINS

Reference thromboplastin was ICSH human plain IRP (BCT/441). Test thromboplastins were ECAA human thromboplastin (recombinant) and ECAA rabbit thromboplastin.

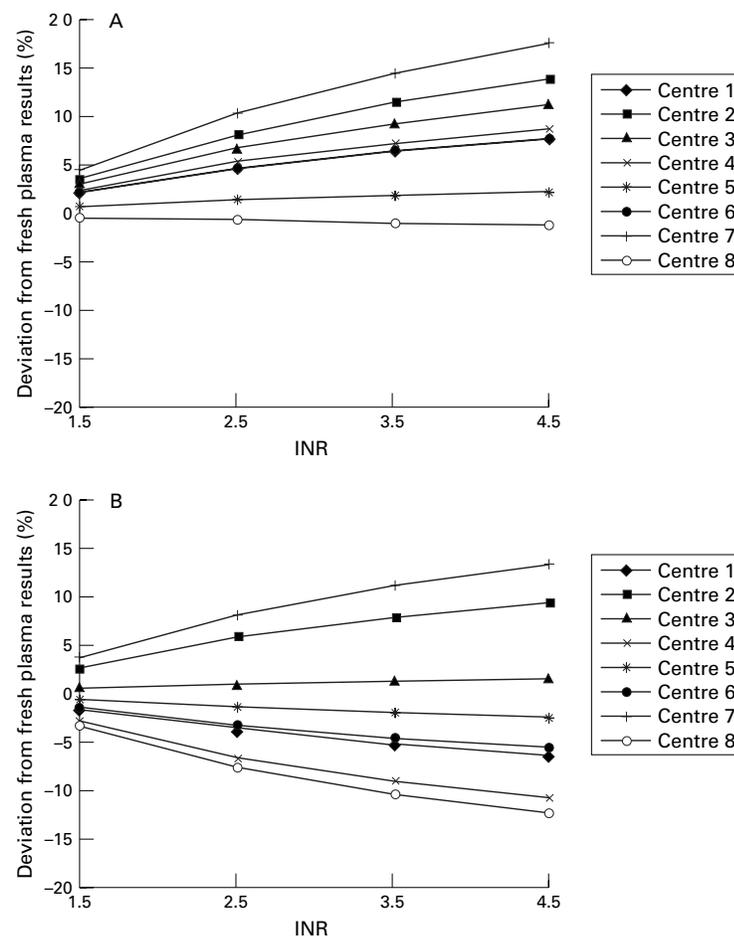


Figure 1 (A) Percentage deviation of freeze dried depleted plasma calibration INR from conventional fresh plasma calibration INR over therapeutic interval. A like to like calibration (ECAA human v BCT/441). (B) Percentage deviation of freeze dried coumarin plasma calibration INR from conventional fresh plasma calibration INR over therapeutic interval. A like to like calibration (ECAA human v BCT/441).

ECAA human freeze dried reference thromboplastin (human recombinant (low ISI) thromboplastin donated by Ortho DS, Raritan, New Jersey, USA) was calibrated previously against the ICSH human plain IRP in a multi-centre exercise.⁷ ECAA rabbit thromboplastin (high ISI) was prepared at the ECAA central facility.

Certification of freeze dried plasmas

The certification of prothrombin times of the freeze dried plasmas in terms of the human plain IRP by the manual prothrombin time technique was performed by six of the 14 ECAA national centres ("certifying centres"). Each of the plasmas was tested in quadruplicate by each of the "certifying centres." The overall mean prothrombin time of each freeze dried plasma from all six centres was regarded as its certified value.

Thromboplastin calibrations with certified freeze dried plasmas

The evaluation of the certified freeze dried plasma calibrations was performed on the results from the remaining eight ECAA national centres only ("test centres"). The eight centres tested the 20 freeze dried depleted plasmas and the 20 freeze dried coumarin plasmas with the ECAA human and ECAA rabbit reference thromboplastins. Four replicate tests were to be performed on each plasma with each thromboplastin.

Thromboplastin calibrations with fresh plasmas

The eight test centres undertook parallel conventional WHO plasma calibrations on the human ECAA reference thromboplastin versus the human plain IRP, and the ECAA rabbit reference thromboplastin versus the human plain IRP (cross species calibration). Twenty fresh plasmas from normal subjects were collected locally, as were 60 plasmas from long term stabilised patients on coumarin treatment.

STATISTICAL ANALYSIS

Conventional fresh plasma ISI thromboplastin calibrations

All data from the centres were analysed at the central facility. The ISI with the fresh plasma calibrations was determined by the eight ECAA test centres. These had not been involved in the certification of the freeze dried plasmas. The procedures adopted in previous collaborative studies for establishing the ISI of thromboplastin IRP were used.⁷⁻¹⁰ Prothrombin times were plotted on a double logarithmic scale. IRP results on the vertical axis and test thromboplastin prothrombin time on the horizontal. All prothrombin time results were included except those which departed from the orthogonal regression line by two or more standard deviations and those outside the 1.5–4.5 INR range with the IRP. The ISI of the ECAA reagents was calculated against the slope of the human plain IRP (ISI = 1.04). Within laboratory precision of the calibration line was estimated from the coefficient of variation (CV) of the slope (b) of the orthogonal regression line (CV(b) = 100 × SD(b)/b).

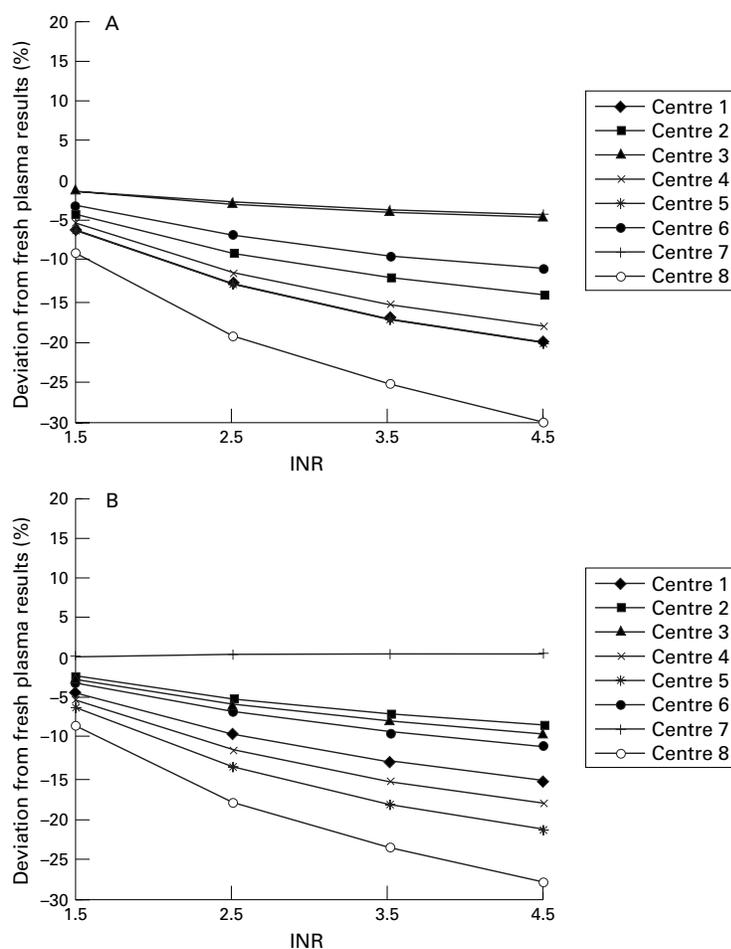


Figure 2 (A) Percentage deviation of freeze dried depleted plasma calibration INR from conventional fresh plasma calibration INR over therapeutic interval. A cross species calibration (ECAA rabbit v BCT/441). (B) Percentage deviation of freeze dried coumarin plasma calibration INR from conventional fresh plasma calibration INR over therapeutic interval. A cross species calibration (ECAA rabbit v BCT/441).

Modified thromboplastin calibrations with freeze dried plasmas

Freeze dried plasma calibrations were determined at each of the eight test centres by placing the certified BCT/441 values for the log prothrombin time of the 20 freeze dried plasmas on the vertical axis and the locally determined log prothrombin time results for the 20 freeze dried plasmas with the ECAA human reagent on the horizontal axis. The mean MNPT for the reference preparation obtained by the six "certifying centres" was also inserted twice on the vertical axis and the locally determined MNPT entered twice on the horizontal axis.

Assessment of INR obtained using local system ISI derived from certified freeze dried plasma calibrations

The reliability of INR derived from a local ISI using either artificially depleted or freeze dried coumarin plasma calibrations was assessed for the test centres. Conventional WHO type fresh plasma calibrations had previously been performed at each of the eight test centres. A local fresh plasma ISI (ISI_F) and a local fresh normal MNPT (M_F) has thus been estimated for the ECAA thromboplastins. These locally esti-

mated parameters have been used to calculate INR (INR_F) for prothrombin times of 20, 30, 40, ..., 70 seconds. INR have also been calculated for these prothrombin times using the locally determined freeze dried plasma ISI (ISI_L) and the MNPT (M_F) of the 20 fresh normal plasmas.

Assuming that the hypothetical prothrombin times of 20 to 70 seconds are locally determined results using the ECAA thromboplastins, INR can be determined for these prothrombin times using the locally determined fresh plasma calibration parameters (INR_F) and the freeze dried plasma calibration parameters (INR_L). Percentage deviation of the freeze dried plasma calibration scheme INR from the fresh plasma calibration scheme INR has been calculated as:

$$d_i = 100 [(INR_{L,i} - INR_{F,i}) / INR_{F,i}] \quad (1)$$

where $INR_{L,i} = (i/M_F)^{ISI_L}$ and $INR_{F,i} = (i/M_F)^{ISI_F}$ and where i represents prothrombin time; thus $i = 20, 30, 40, \dots, 70$.

Results

All centres involved in the certification and testing of freeze dried plasmas returned a complete set of results. Table 1 gives the fresh plasma calibration slopes for the eight test centres for the ECAA thromboplastins calibrated against the human IRP. The ECAA human calibrations have a mean slope of 0.9201, which gives an ISI of 0.9569. The ECAA rabbit calibrations have a mean slope of 1.5961 and an ISI of 1.6599 for the cross species calibration. These ISI values show close agreement with the mean values of the 14 ECAA national laboratories.⁷ Table 1 also shows good agreement in the cross species calibration against the rabbit plain IRP of the ECAA human on fresh plasmas (mean slope = 0.9502, ISI = 0.988).

Table 2 shows the freeze dried depleted plasma calibration slopes for the ECAA reference reagents against the human plain IRP BCT/441. The mean slopes for the ECAA human and rabbit calibrations are 0.9688 (ISI = 1.01) and 1.4113 (ISI = 1.47), respectively. These results give an average deviation of 5.3% for the human route and 11.6% for the cross species calibrations.

Table 3 shows the corresponding results for the freeze dried coumarin plasma calibrations. The mean slopes for the ECAA human calibrations were 0.9077 (ISI = 0.9433) and for the ECAA rabbit thromboplastin, 1.4360 (ISI = 1.493). The average deviations from the fresh plasma calibrations were 1.35% and 10.0%, respectively, for the two routes.

INR assessment of freeze dried plasma calibrations

To assess the like to like species and cross species freeze dried plasma calibrations INR were calculated for prothrombin times of 20 to 70 seconds, corresponding approximately to a therapeutic interval of 1.5 to 4.5 INR, using the locally determined fresh plasma ISI and fresh plasma MNPT. INR were also calculated for the same prothrombin time using local freeze dried plasma calibration ISI and fresh

plasma MNPT. The resultant INR were compared with those obtained from the fresh plasma calibrations and deviations assessed (see equation (1)).

Figure 1 (A and B) shows the percentage deviations in term of INR for the like to like species calibrations (ECAA human *v* BCT/441). Results for the eight test centres are shown. In the majority of cases, deviation from the fresh plasma ISI results increase for greater INR, with results within 10% of the fresh plasma ISI. In all but one case (centre 8) the depleted plasma calibration ISI overestimated the equivalent fresh plasma results. Average deviation for all centres over the therapeutic interval of 1.5 to 4.5 INR was approximately 6% for the freeze dried depleted plasmas and 5% for the freeze dried coumarin plasmas. In fig 2 (A and B), the percentage deviations in term of INR for the cross species calibrations (ECAA rabbit *v* BCT/441) are shown. As with the like to like calibrations, deviations increase over the therapeutic interval. Average deviation over the therapeutic interval was, however, somewhat greater, at approximately 14% for the two types of freeze dried plasma.

Discussion

This report provides new evidence of the requirement to use the "like to like" principle in ISI calibration when freeze dried plasma calibrants are certified in terms of the thromboplastin IRP. The two types of freeze dried plasma calibrants studied, artificially depleted and coumarin, give a reasonable approximation to the ISI results of conventional fresh plasma calibrations in a multicentre exercise using the like to like principle, when the low ISI ECAA human reagent is calibrated against the low ISI human IRP. When, however, the high ISI ECAA thromboplastin of rabbit origin is calibrated against the low ISI human IRP using both freeze dried artificially depleted and freeze dried coumarin plasmas, the results are different from fresh plasma calibrations, although similar to each other. Substantially lower ISI results of 1.47 and 1.49 respectively were obtained as opposed to the 1.66 with the fresh plasma calibrations.

There is close agreement in ISI from the fresh plasma calibrations with the IRP, both of like to like and cross species (human and rabbit) versus the ECAA reagents of human and rabbit origin.⁷ This appears to be a satisfactory vindication of the recommendation to average the ISI of the new WHO rabbit IRP in terms of the three previous IRP of rabbit, human, and bovine origin.⁶

The precision of the calibration measured by the coefficient of variation of the slope is less than in the previous ECAA study of the minimum numbers required for a reliable calibration.¹¹ The latter calibration was, however, based on a slightly larger test sample, consisting of 20 abnormal plasmas and seven freeze dried normal samples. A MNPT was used (twice) to represent the normal value in this study, thus reducing the number of samples to 22. Perhaps more important, however, is that in the present calibration of the

ECAA rabbit, a higher ISI reagent is being used than in the study of the minimum requirements. It is likely that higher ISI reagents may require larger numbers of calibrant plasmas to establish a reliable ISI.

The results suggest that certified prothrombin time values for freeze dried plasmas cannot be used for ISI calibration of dissimilar thromboplastins. Accordingly the solution is that certified freeze dried plasma ISI should be provided for any set of freeze dried plasma calibrants used for local ISI determination in terms of the relevant IRP, human, rabbit, or bovine. The type of reagents for which a set of freeze dried plasma ISI calibrants are to be used must be specified in each instance. Certified values with freeze dried plasmas (either depleted or coumarin) with a low ISI human plain IRP cannot be used for calibrating a higher ISI rabbit reagent unless some correction factor is used.

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