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The potential replacement of HPLC by ^{125}I-RIA for the characterization of cyclosporin A: a bioavailability study after oral administration in healthy human subjects

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Abstract

A significant overestimation of cyclosporin A (CsA) by a radioimmunoassay using ^{125}I-labeled monoclonal antibody (^{125}I-RIA), compared to the reference HPLC method, has been reported for a limited number of samples from transplant patients. However, the extent of the discrepancy, with respect to bioavailability parameters, has not been examined for the case of the oral administration of a single dose CsA to healthy subjects where a number of factors which might be involved in this overestimation (e.g. under steady state condition and a significant accumulation of CsA metabolites) would be absent. Therefore, the objective of this study was to assess the effect of potential difference manifested by the two analytical procedures, ^{125}I-RIA and HPLC, on the bioavailability analysis of CsA. An oral CsA formulation was administered to 22 healthy male subjects and the blood samples were analysed by both ^{125}I-RIA and HPLC. Significant discrepancies in the estimated CsA concentrations by the two methods (paired t-test, $P < 0.001$) were found. The difference (bias) increased with increasing concentrations of blood CsA ($P < 0.001$). However, despite the bias in CsA estimations, the AUC and C_{max}, obtained by ^{125}I-RIA and HPLC methods showed only small differences (i.e. 2% for AUC and 7% for C_{max},). Thus, our results suggest that the bias of the ^{125}I-RIA vis-a-vis the HPLC method in the estimation of CsA blood levels may not, in practice, affect the bioavailability analysis (e.g. bioequivalence study) of CsA in a situation where a single dose CsA is orally administered to healthy subjects.

Keywords: Cyclosporin A; Bioavailability parameter; AUC; C_{max}; ^{125}I-RIA; HPLC

1. Introduction

Cyclosporin A (CsA) is a powerful immunosuppressive, orally active agent administered to prevent rejection in new organ transplants, as well as for the treatment of a variety of autoimmune diseases [1,2]. Specific and reliable methods for

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the analysis of CsA in blood have been developed to evaluate the pharmacokinetic properties of this drug. Among these, HPLC is generally accepted as the standard method despite the methodological difficulties associated with it [3]. Recently, interfacing HPLC with a mass spectrometer has further extended the HPLC method, thus permitting the microanalysis of CsA [4]. For the routine assay of CsA in blood samples, radioimmunoassay using a specific ^{125}I-labeled monoclonal antibody for CsA (^{125}I-RIA) is commonly used, because of its technical simplicity [5]. As a result, it has been reported that this method has a significantly improved throughput in the estimation of CsA level in blood samples [6]. The ^{125}I-RIA method, however, is known to overestimate CsA concentrations by 20–40% and the AUC, compared to the HPLC method for blood samples from transplant patients [6–9]. The discrepancy between the ^{125}I-RIA and HPLC methods varied depending on the studies [5,10–12], of which mechanism remains controversial. Typically, these studies have been carried out for patient TDM samples in which temporal profiles of blood CsA had not been fully covered.

Analysis of CsA is often needed for blood samples from healthy subjects, as well as for blood samples from patients, in assessing bioavailability [13] and bioequivalence [14] of CsA. Nevertheless, the discrepancy between ^{5}I-RIA and HPLC has not been examined for such cases. Thus, in the present study, a single oral dose of CsA was administered to healthy volunteers and bioavailability parameters of CsA estimated by both assay methods were then compared in order to clarify the discrepancy issue.

2. Experimental

2.1. Clinical specimens

Sandimmune Neoral® (25 and 100 mg as CsA, Norvatis Pharma AG, Basle, Switzerland) capsules were orally administered to 22 healthy human subjects in the form of a single CsA dose of 175 mg according to the protocol which was reviewed and granted by the Institutional Review Board of Seoul National University. Blood samples (8 ml) were collected in tubes containing EDTA at pre-dose, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 h after administration, and all specimens were analyzed by both the ^{125}I-RIA method [11] and HPLC.

2.2. Assays

For the determination of CsA via the ^{125}I-RIA method, the Cyclo-Trac SP® RIA kit (INCStar, Stillwater, USA) was used precisely as recommended by the manufacturer. A calibration curve was prepared using the supplied kit calibrators in the concentration range of 22–1182 ng/ml, resulting in an overall CV of standard curve samples of $< 10\%$ and percent deviations for quality control samples (i.e. accuracy) ranged from 4.2 to 6.4%.

For the determination of CsA via HPLC, the analysis was carried out essentially according to the method described by Salm et al [12]. Briefly, blood samples were cleaned using a solid phase extraction column prior to chromatographic separation on a μ-Bondapak C18 column (3.9×300 mm I.D., 10 μm particle sizes, Waters, Milford, USA). The mobile phase was a mixture of acetonitrile-methanol-deionized water (55:15:30, v/v/v). The elution profile was determined by UV absorption at 210 nm, and the retention times of CsA and the internal standard (cyclosporin D) were 17 and 22 min, respectively. Both the standard samples of CsA and the internal standard (Galena, Czech Republic) were in excess of 98% purity. Calibration curves were constructed by plotting the ratio of the peak heights of CsA to cyclosporin D against the concentrations of CsA added, and were linear over the concentration range of 40–1000 ng/ml. The precision and accuracy of the HPLC assay were determined by analyzing quality control samples (250, 500 and 1000 ng/ml). The overall coefficient of variation (i.e. both inter- and intra-day) for the quality control samples was consistently $< 10\%$. The percent deviation for the quality control samples (i.e. accuracy) was in the range of 2.2–6.8%.

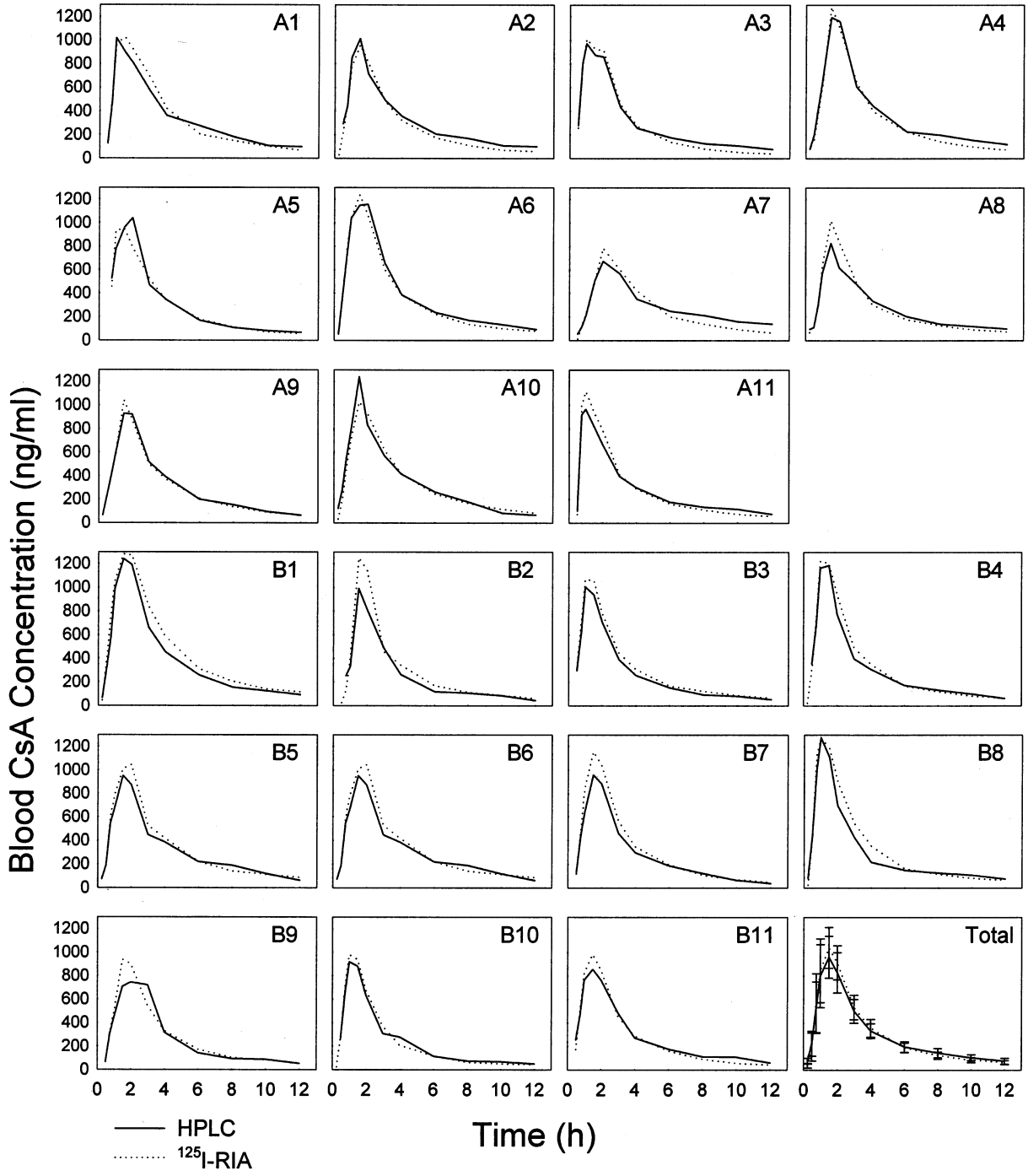

Fig. 1. Temporal profiles of CsA concentrations, as determined by the ^{125}I-RIA (●) and HPLC (○) methods, after a single oral administration of Sandimmune Neoral® to 22 male subjects at a CsA dose level of 125 mg.

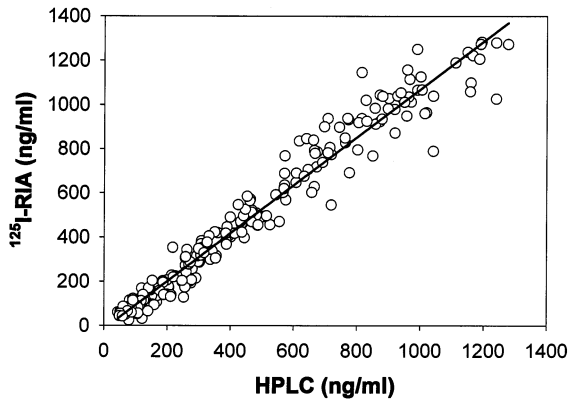

Fig. 2. Comparison of CsA concentrations measured by the ^{125}I-RIA and HPLC methods for 239 whole blood samples from 22 healthy volunteers following a single oral administration of Sandimmune Neoral® at a CsA dose level of 125 mg. Regression analysis revealed that ^{125}I-RIA = 1.085 × HPLC − 16.34 ng/ml, $r = 0.9818$, $S_{y/x} = 70.20$, $S_b = 0.01363$ and $S_a = 7.386$.

Table 1
Bioavailability parameters calculated based on CsA levels estimated by the ^{125}I-RIA and HPLC methods[a]

Assay method	AUC (ng × hr/ml)	C_{max} (ng/ml)	t_{max} (h)
^{125}I-RIA	3630	1076	1.445
HPLC	3555	1005	1.481
^{125}I-RIA/HPLC (%)	102.1%	107.1%**	97.54%

[a] Each value represents the mean for 22 subjects. A paired t-test was used for statistical comparison for each bioavailability parameter.

** $P < 0.001$ between the two assay methods.

2.3. Data analysis

The noncompartmental pharmacokinetic characteristics of CsA for each subject were calculated by using Winnonlin™ program (Scientific Consulting, NC, USA). The area under the blood CsA concentration-time curve (AUC) was calculated using blood concentration data for up to 12 h by the linear trapezoidal rule since, in most subjects, the 24 h concentration of CsA was below the detection limit. The maximum whole blood concentration, C_{max}, and the time of its occurrence, t_{max}, were compiled from concentration-time data.

The correlation between blood CsA concentrations obtained by the ^{125}I-RIA (y) and HPLC (x) was determined by linear regression analysis. The standard deviation for y on x ($S_{y/x}$) and the standard errors for the slope (S_b) and intercept (S_a) of the regression line are included in the analysis. A two-sided t-test [15] was performed for the slope and intercept to assess discrepancies between the assay methods. Blood concentrations, AUC, C_{max} and t_{max} were statistically compared by the paired t-test for differences between the assay methods. A P value of < 0.05 was considered significant.

3. Results and discussion

CsA was readily detected in collected blood samples by both ^{125}I-RIA and HPLC methods. Fig. 1 shows temporal blood CsA concentration profiles for each subject and the means of all subjects ($n = 22$). Nearly superimposable profiles were obtained for all subjects regardless of the assay methods. This is consistent with the result of the linear regression analysis of blood CsA concentrations from the two assay methods (Fig. 2), in which a good agreement was obtained between the assay methods (^{125}I-RIA = 1.085 × HPLC − 16.34 ng/ml, $r = 0.9818$, $n = 239$; $S_{y/x} = 70.20$, $S_b = 0.01363$, $S_a = 7.386$).

However, the slope (1.085) and intercept (− 16.34 ng/ml) of the regression line (Fig. 2) were significantly different from unity and zero respectively (t-test, $P < 0.05$, when analyzed by the method of Loo et al [16]), indicating overestimation of blood CsA by the ^{125}I-RIA compared to the HPLC. Bland and Altman plot [15] of the differences in CsA estimates between the two methods against the average CsA concentrations (Fig. 3) revealed a positive slope of 0.1006 for the regression line, which was statistically different from zero ($P < 0.001$). It appears to indicate that

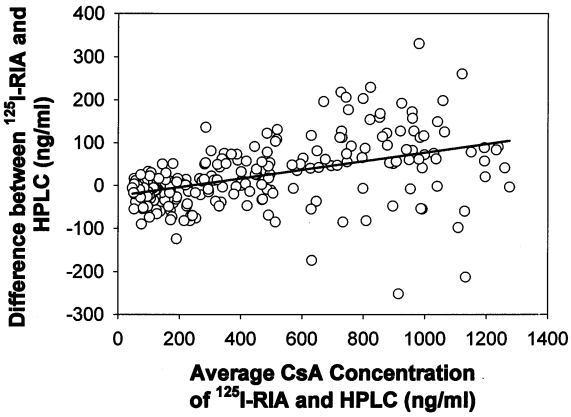

Fig. 3. Bland and Altman plot of the concentration difference between the ^{125}I-RIA and HPLC methods versus average concentration measured by the ^{125}I-RIA and HPLC methods. Linear regression revealed a correlation of $r = 0.4659$ ($P < 0.001$), slope = 0.1006, $S_{y/x} = 67.00$, $S_b = 0.0124$ and $S_a = 6.948$.

extent of overestimation of the ^{125}I-RIA increases as a function of blood CsA levels. Similar degree of overestimation by the ^{125}I-RIA [17] or a good agreement of ^{125}I-RIA and HPLC methods [16] has already been reported for spiked whole blood samples from normal volunteers.

It is contrary to cases for blood samples from transplant patients, in which the ^{125}I-RIA by the Cyclo-TracSP® RIA kit used to reveal a much larger overestimation of CsA compared with the HPLC. The overestimation by the ^{125}I-RIA reached 2.62- [16] or 1.73-fold [18] for renal transplant patients, 1.73-fold for cardiac transplant patients [16] and 1.4–1.7-fold for various transplant patients [19]. Thus, the overestimation seems to be common to all the blood samples from the transplant patients, and does not seem to be associated with types of the organ transplantation. The cross-reactivity of the ^{125}I-RIA to metabolites [5,6,10] is often suspected as a possible mechanism of the overestimation, since significantly higher blood concentration of major CsA metabolites, AM1 and AM9, was observed for transplant patients. For example, comparable blood levels of AM1 and AM9 to that of CsA following multiple oral administration have been reported for renal transplant patients [20], and even higher blood concentration of these metabolites relative to CsA levels has been reported for kidney-, liver-, or heart-transplant patients [21,22]. As the result, AUC ratios of metabolites to CsA in renal transplant patients who received multiple doses of CsA were higher (1.03 for AM1 and 0.5 for AM9, [22]) than those in healthy subjects who had received a single CsA dose (0.69 for AM1 and 0.41 for AM9, respectively [23]).

Consistent with this hypothesis, a bias of up to 20–40% has been reported for steady state blood CsA levels and AUC in kidney-, liver- or heart-transplant patients who had received multiple oral CsA doses [7–9]. However, further studies are called for before a firm conclusion can be made, because the cross-reactivity of the ^{125}I-RIA to CsA metabolites (AM1 and AM9) is controversial depending on the reports [24,25]. For example, Tredger et al [24] reported 15 and 5% cross-reactivity of IncstarRIA (the same method as used the present study) for AM1 and AM9, respectively, while Murthy et al [26] reported only 1 and 5% cross-reactivity, respectively. Thus, other factors including a patient blood matrix, for example, might also be involved in the bias between the normal blood and patient blood [16].

Despite of the overestimation of the ^{125}I-RIA method, the bias between the two methods in estimating bioavailability parameters was minimal (Table 1). No significant differences between the methods were observed for AUC and t_{max}. Significant ($P < 0.001$) difference was observed for C_{max}, but the difference was only 7%. It appears to indicate that such a subtle overestimation by the ^{125}I-RIA in healthy subjects (Figs. 2 and 3), contrary to the cases for transplant patients [16,18,19], does not actually affect the estimation of bioavailability parameters of CsA, especially in a single dose study.

4. Conclusion

A CsA concentration-dependent overestimation of CsA by the ^{125}I-RIA method was observed for blood samples from healthy subjects who received single oral dose of CsA (Figs. 2 and 3). However, the overestimation was minimal compared to the cases for transplant patients [16,18,19]. As a re-

sult, minimal bias between the ^{125}I-RIA and HPLC methods was observed in estimating bioavailbility parameters of CsA from healthy subjects (Table 1), which is contrary to the substantial (up to 40%) bias for TDM samples from transplant patients [7–9]. Therefore, the ^{125}I-RIA method appears to be an alternative assay method to the HPLC method in estimating bioavailability of CsA so long as CsA is administered to healthy subjects in a single dose. However, as indicated by Steimer [27], the assay methods of CsA, including the ^{125}I-RIA, need to be validated more carefully before the HPLC is replaced, in order to avoid substantial erratic CsA dosing to transplant patients.

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