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Validation of a high-performance liquid chromatographic–mass spectrometric method for the measurement of 5-chloro-2′,3′-dideoxy-3′-fluorouridine (935U83) in human plasma

John A. Dunn, Paul M. Savina*

Division of Bioanalysis and Drug Metabolism, Glaxo Wellcome Inc., 3030 Cornwallis Road, Research Triangle Park, NC 27709, USA

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Abstract

An isocratic reversed-phase LC–MS method for measuring concentrations of 5-chloro-2′,3′-dideoxy-3′-fluorouridine (935U83; I) directly and its 5′-glucuronide metabolite (5-chloro-2′,3′-dideoxy-5′-O-β-D-glucopyranuronosyl-3′-fluorouridine) indirectly in human plasma was developed, validated, and applied to a Phase I clinical study. The pyrimidine nucleoside, I, was extracted from human plasma by using anionic solid-phase extraction. The concentration of the glucuronide conjugate was determined from the difference between the molar concentration of I in a sample hydrolyzed with β-glucuronidase and the nonhydrolyzed sample. Recovery of I from human plasma averaged 90%. The bias of the assay for I ranged from −5.5 to 7.1% during the validation and from −6.0 to 1.4% during application of the assay to the Phase I single-dose escalation study. The intra- and inter-day precision was less than 8% for I and its glucuronide conjugate. The lower and upper limits of quantitation for a 50-μl sample were 4 ng/ml and 3000 ng/ml, respectively. No significant endogenous interferences were noted in human plasma obtained from drug-free volunteers nor from predose samples of HIV-infected patients.

Keywords: 5-Chloro-2′,3′-dideoxy-3′-fluorouridine; 5-Chloro-2′,3′-dideoxy-5′-O-β-D-glucopyranuronosyl-3′-fluorouridine

1. Introduction

The pyrimidine nucleoside, 935U83 (5-chloro-2′,3′-dideoxy-3′-fluorouridine; I; see Fig. 1) is a reverse transcriptase inhibitor which is active in vitro against HIV and HIV strains resistant to other nucleoside (e.g. AZT, ddI and ddC) and non-nucleoside reverse transcriptase inhibitors [1]. A Phase I single dose escalation study was undertaken to assess the safety and pharmacokinetics of I in HIV-infected individuals [2]. A specific and sensitive bioanalytical method with a high throughput was required to support this study.

Preclinical studies in monkeys [1] indicated that I was predominately excreted in the urine and less than 10% of an administered dose was biotransformed to the 5′-glucuronide conjugate (5-chloro-2′,3′-dideoxy-5′-O-β-D-glucopyranuronosyl-3′-fluorouridine; II). In humans, zidovudine (3′-azido-3′-deoxythymidine; AZT) is predominately excreted in the urine as the glucuronide metabolite with a glucuronide to parent molar ratio of 4–6:1 [3] although the preclinical studies of zidovudine in monkeys indicated a 1:1 molar urinary excretion

*Corresponding author.

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ratio. Because of the structural similarity of AZT with I, the biotransformation of I to II in humans was expected to be higher than that in monkeys and contribute significantly to the clearance of I. Therefore, the measurement of II in plasma, in addition to the measurement of parent I, was desired.

2. Experimental

2.1. Chemicals

Compound I and the internal standard (5-bromo-2′,3′-dideoxy-3′-fluorouridine; III) were synthesized in Wellcome Research Laboratories and were obtained from Compound Registration, Glaxo Wellcome (Research Triangle Park, NC, USA). The glucuronide conjugate (5-chloro-2′,3′-dideoxy-5′-O-β-D-glucopyranuronosyl-3′-fluorouridine; II) was isolated, purified, and characterized from the urine of monkeys dosed with I. The structural formulae of the compounds are shown in Fig. 1. Blank human plasma for the preparation of calibration standards and control samples was obtained from fasted drug-free volunteers. Glass distilled deionized water, used during the extraction procedure and for preparing the mobile phase, was prepared at Glaxo Wellcome. HPLC grade acetonitrile (EM Science, Gibbstown, NJ, USA), reagent grade ammonium acetate (Mallinckrodt, Paris, KY, USA), and high purity double distilled acetic acid (GFS Chemicals, Powell, OH, USA) were purchased commercially and used without further purification.

Ammonium acetate buffer (50 m*M*, pH 5.5) was used to prepare analytical stock solutions, reconstitute β-glucuronidase (Type X-A from E-Coli; Sigma, St. Louis, MO, USA), and to dilute human plasma samples not undergoing hydrolysis prior to solid-phase extraction. The β-glucuronidase enzyme solution was prepared by adding 5–5.5 ml of the ammonium acetate buffer to a vial containing approximately 3.5 mg of the β-glucuronidase preparation (enzyme activity was approximately 6000–6500 units/ml of solution depending on the initial assay activity of the solid powder). A solution of ammonium acetate (7.8 m*M*, pH ~6.74) served as the reconstitution solvent and as the injection vehicle for HPLC. The HPLC mobile phase was 15% acetonitrile in a 9.2 m*M* ammonium acetate buffer.

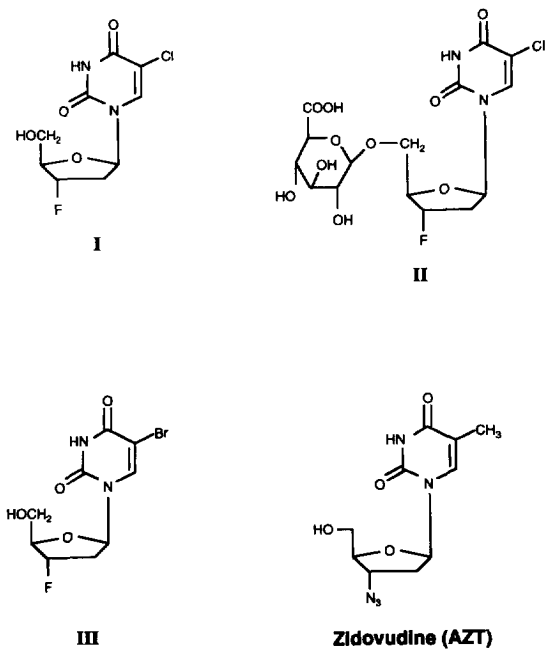

Fig. 1. Chemical structures of 935U83 (I), glucuronide conjugate (II), internal standard (III), and zidovudine (AZT).

2.2. Instrumentation and chromatographic conditions

The HPLC system used in the validation and application of the method consisted of a Model 625 multisolvent delivery system, a Model 715 or 717 WISP auto-injector (Waters Associates, Milford, MA, USA), and a Model API III biomolecular mass analyzer (PE Sciex, Thornhill, Canada). An Inertsil ODS2 5 μm, 50×4.6 mm reversed-phase column (MetaChem, Torance, CA, USA) was used for the analysis with a 3 mm guard column (MetaChem). Sample solid-phase extraction was performed using anion-exchange extraction cartridges (QMA Sep-Pak Plus, Waters Associates) and a MilliLab Workstation (Waters Associates). The mass spectrometer was operated in the negative ion mode. Mass calibration was assessed before each run and mass assignment was within ±0.3 a.m.u. of 263 (^{35}Cl isotope) and 265 (^{37}Cl isotope) for I, and of 307 (^{79}Br isotope) and 309 (^{81}Br isotope) for III.

2.3. Preparation of analytical standards, calibration standards, and control samples

Two analytical stock solutions (approximately 100 μg/ml) of I were prepared independently in water–acetonitrile (5:95) from separate weighings. An analytical stock solution (approximately 100 μg/ml) of III was prepared in water–acetonitrile (5:95). A stock solution of II (approximately 100 μg/ml) was prepared in methanol. Portions of the I and the II stock solutions were diluted with 50 m*M* ammonium acetate (pH 5.5) buffer to prepare the analytical spiking solutions that were used to spike plasma for preparation of the calibration standards and controls. A portion of the internal standard (III) stock solution was diluted with 50 m*M* ammonium acetate (pH 5.5) buffer to a concentration of 140 ng/ml. All solutions were stored in a 2°C refrigerator when not in use.

Calibration standards (4–3100 ng/ml) and quality control samples (6–2500 ng/ml) of I were prepared in pooled blank human plasma from the independently prepared analytical standard of I. Quality control (QC) samples of II were prepared in pooled blank human plasma from the analytical standard solutions at approximate concentrations of 13–4330 ng/ml.

2.4. Sample preparation and analytical procedure

Calibration standards, plasma and QC samples (50–100 μl) were spiked with 14 ng of III and either 10 m*M* ammonium acetate buffer (100–150 μl), for I analysis, or β-glucuronidase enzyme solution (100–150 μl), for II analysis, then vortexed. The tubes containing the enzyme solution were incubated for approximately 2 h in a water bath at 37°C. The buffered human plasma was added to the Sep-Pak QMA anionic-exchange cartridges, preconditioned with 2 ml of acetonitrile and 3 ml of water. Following an air purge (0.2 min), I and III were eluted from the column with two 0.75 ml portions of acetonitrile. The eluates were dried under nitrogen with heating (40–45°C), and the residue was reconstituted with injection vehicle (200–400 μl). A portion of the reconstituted sample (10–100 μl) was injected onto the analytical column and eluted at 1 ml/min with a run time of 5 min. Calibration standards and QC samples (duplicates at each concentration) were interspersed throughout the LC–MS run. The concentration of II in a sample was determined from the difference between the molar concentration of I in a hydrolyzed and non-hydrolyzed sample.

2.5. Data analysis and calculations

Data were acquired using Routine Aquisition and Display (RAD) software supplied with the PE Sciex biomolecular mass analyzer. Integration of I and III peak areas and calculation of I to III peak-area ratios were performed using MacQuan software. Regression and data analyses were performed by using SAS (version 6.06; Cary, NC, USA).

2.6. Assay validation

2.6.1. Calibration model and lower limit of quantitation

Calibration standards (range=4–3100 ng/ml, $n=4$ at each of nine concentrations) were extracted and assayed. A linear model was fit to the concentration/peak-area ratio data using unweighted and weighted ($1/c$, $1/c^2$) least-squares regression. Linear least-squares regression was also performed following natural logarithm transformation of the data. For each regression analysis, plots of the Studentized residuals were used to show empirically how well the model described the data and to demonstrate that the variance of the residuals was stable over the range of the concentrations in the calibration curve.

2.6.2. Stability

The stability of I in analytical standard solutions, stored at 2–3°C, was examined by using a calibration curve constructed with freshly prepared standards and controls. The possible hydrolysis of II to form I was tested by processing the analytical standard solutions through the method and looking for the presence of any I. The QMA cartridges completely retained II.

The stability of processed samples prior to injection was checked during the course of a normal 24-h analytical run. Human plasma was spiked in duplicate at three concentrations and extracted as described. Following reconstitution of the samples the solutions at each concentration were combined, then divided into eight injector vials and injected over a 25-h period. The concentrations were de-

termined from the calibration curve and visually inspected for trends. Time zero was arbitrarily assigned to the time of the first injection for each concentration.

The stability of I in human plasma stored at −40°C was investigated. Human plasma was spiked with I at 100, 300, 1515 and 10 000 ng/ml. These stability control samples were assayed on the day of preparation and the remainder of each control was divided into multiple tubes and stored at −40°C. The controls were removed and assayed periodically.

The stability of I in human plasma was examined over seven freeze–thaw cycles. Control samples were prepared in blank human plasma at 10, 100 and 450 ng/ml, analyzed immediately after preparation, then stored in a −20°C freezer for analysis on seven different occasions.

Samples (n=16) for heat inactivation assessment were prepared by spiking blank human plasma with I to obtain a final concentration of 582 ng/ml. Eight tubes were placed into a −20°C freezer. The remaining eight tubes were incubated at 58°C in a shaking water bath (65 rpm) for 5 h then placed into a −20°C freezer. On a second occasion, an assessment was made by spiking tubes of human plasma (n=6) at concentrations of 25.3, 253 and 1010 ng/ml. Three tubes at each concentration were frozen and the remaining three were treated as above.

2.6.3. Recovery

Samples (n=5) for internal standard recovery were prepared by spiking 100 μl of blank human plasma with 16.2 ng of III and then extracting the samples according to the method as described above in Section 2.4. After elution from the QMA cartridge, 15.5 ng of I were added before evaporation under nitrogen to serve as the internal standard for III assessment. Samples (n=5) for the determination of I recovery were prepared by spiking 100 μl of human plasma with 15.5 ng of I and then extracting the samples as above. After elution from the QMA cartridge, 16.2 ng of III was added before evaporation under nitrogen to serve as the internal standard for I assessment. Samples (n=5) that served as the basis for comparison for the calculation of recovery for each compound were prepared by extracting 100 μl of blank human plasma and adding 16.2 ng of III and 15.5 ng of I to the QMA eluant which was dried under nitrogen and analyzed as described.

2.6.4. Specificity

Human plasma from fasted drug-free volunteers (four male, three female) and predose samples from the Phase I study patients (n=11) were analyzed by the method. The chromatograms were visually inspected for chromatographic peaks from endogenous substances which would interfere significantly with the integration of the peaks for I or III.

2.6.5. Accuracy and precision

Spiked control samples (n=5) at each of five concentrations (6.31, 12.6, 93.4, 934 and 2802 ng/ml) of I were assayed on two occasions to determine the intra-day accuracy and precision of I. The concentrations of I were calculated by using a weighted ($1/c^2$) linear regression model and these concentrations were then compared to the nominal concentrations. The calculated values at each concentration were averaged and the percentage bias was calculated to estimate accuracy and the CV was calculated as a measure of precision.

The inter-day accuracy and precision of the method was assessed from control samples analyzed in conjunction with clinical assay runs from a single dose escalating Phase I study. Estimates of the variances were made using ANOVA (PROC NESTED in SAS which corrects for unbalanced data). Estimates of the total variance, the intra-day and the inter-day precision were made.

3. Results

3.1. Calibration model and lower limit of quantitation

A plot (Fig. 2) of the Studentized residuals for the least-squares linear regression analysis (n=4 at nine concentrations) indicated that the variance associated with the response (peak area ratio) was homogenous when a $1/c^2$ weighting function was used. The limits of quantitation for a 50 μl sample were 4 and 3100 ng/ml. Samples with concentrations of I above 3100 ng/ml were diluted into the range with drug-free human plasma because the Studentized residuals

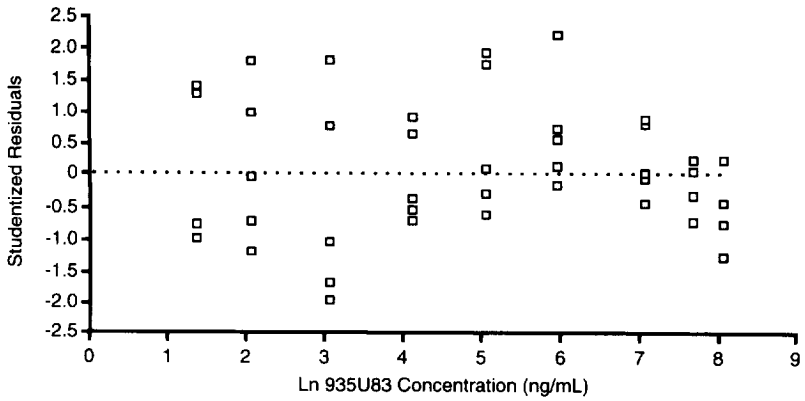

Fig. 2. Typical studentized residual plot for the $1/c^2$ weighted I calibration curve regression during validation ($n=4$ at nine concentrations; two observations hidden).

suggested that the model significantly overestimates the actual concentration. These results were verified during application of the method to the Phase I clinical study [2].

3.2. Stability

Analytical standard solutions of I prepared in acetonitrile, diluted with 50 m*M* ammonium acetate buffer (pH 5.5) and stored refrigerated at 2–3°C were stable for at least 580 days (concentrations within 8% of nominal with no detectable trend). No detectable hydrolysis of II to I was observed for analytical standard solutions prepared in 50 m*M* ammonium acetate (pH 5.5) buffer over the same period of time. No evidence of I instability was observed in the processed samples over a 25-h period.

Compounds I and II were stable in human plasma when stored frozen at −40°C for at least 15 months. The daily assayed concentrations were within 10% of the nominal value throughout the assessment period.

Compound I was stable through at least six freeze–thaw cycles. The averaged assayed concentration measured after each freeze–thaw cycle was within 8% of the nominal concentration with no visual evidence of any trend, indicating that plasma could be frozen and thawed at least six times with no appreciable change in the concentration of I.

The process of heat inactivation of HIV had no statistically significant ($p<0.01$) effect on the determination of I concentrations (%bias for unheated samples ranged from −4.7 to 1.6). No conversion of II to I was observed during the inactivation process.

3.3. Recovery

The recovery from human plasma of I was 90% (range=88–93%) and of III was 87% (range=84–91%).

3.4. Specificity

Representative selected ion chromatograms of plasma obtained from drug-free volunteers and from patients in the Phase I clinical study are presented in Fig. 3. No endogenous peaks interfered significantly with I or III integration in either plasma from drug-free volunteers or at any of the predose samples in patients receiving escalating doses of I.

3.5. Accuracy and precision

The intra-day accuracy (expressed as %bias) and precision for the analysis of I are presented in Table 1. The intra-day bias ranged from −4.1 to 7.1% on day 1 and −5.5 to 3.0% on day 2 of the validation. The precision ranged from 2.8 to 13% on day 1 and from 2.1 to 5.4% on day 2.

The inter-day accuracy and precision for I and II, calculated from the analysis of controls included with the Phase I study samples, are presented in Table 2 and Table 3, respectively

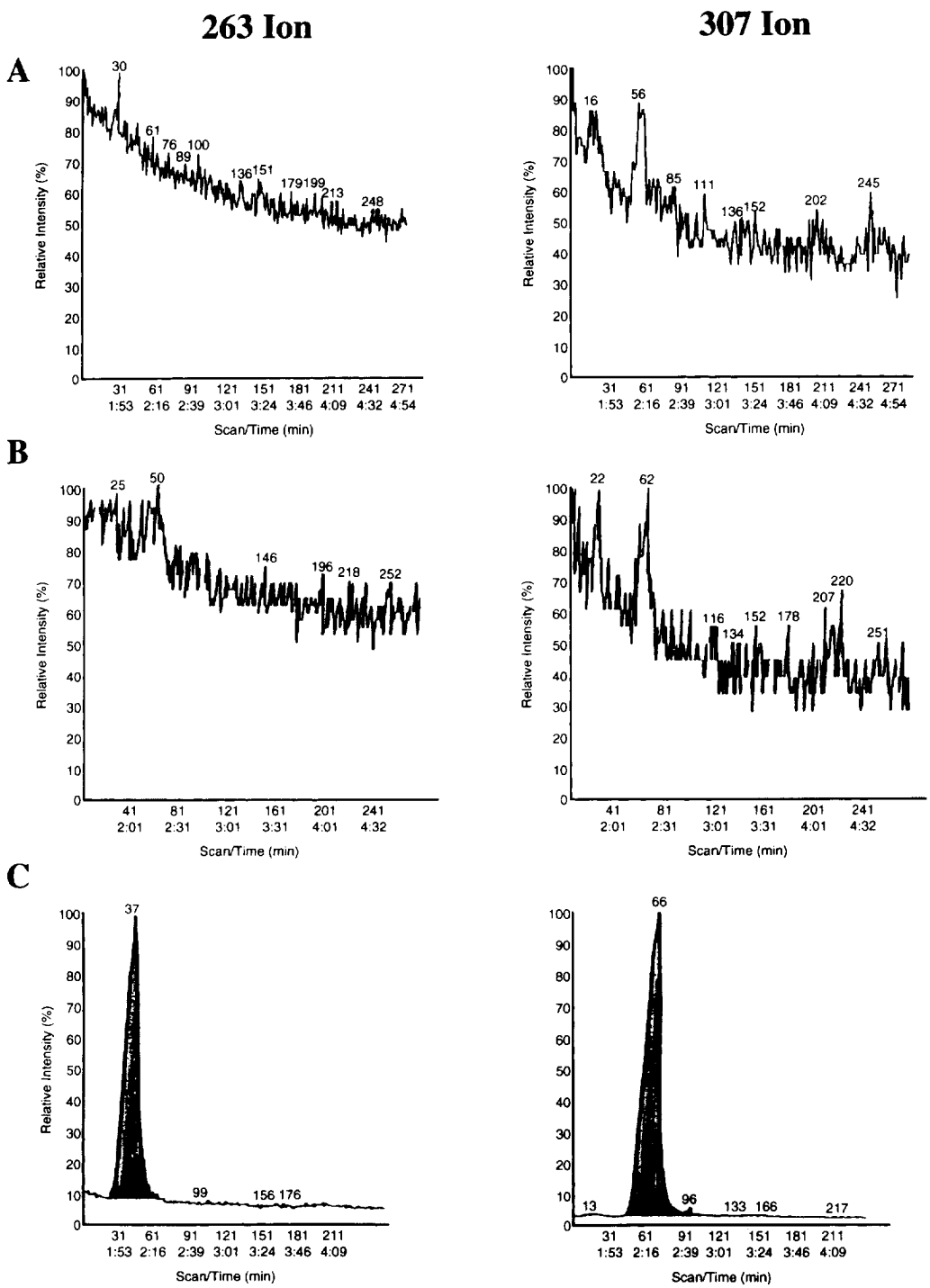

Fig. 3. Selected ion chromatograms of human plasma: (A) from a drug-free volunteer, (B) from a predose sample of an HIV-infected patient, and (C) spiked with I (25 ng/ml) and III (140 ng/ml). The 263 ion corresponds with I and the 307 ion corresponds with the internal standard III.

Table 1
Accuracy and precision for the analysis of compound I in human plasma by LC–MS

Validation run	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Precision (% R.S.D.)	Bias (%)
Day 1 ($n=5$)	6.31	6.59	13.0	4.4
	12.6	12.1	3.9	−4
	93.4	100.0	3.5	7.1
	934	954	2.8	2.2
	2802	2688	3.6	−4.1
Day 2 ($n=4$)	6.31	6.07	3.0	−3.9
	12.6	12.3	4.5	−2.6
	93.4	96.2	5.4	3
	934	919.0	3.5	−1.6
	2802	2649	2.1	−5.5

Table 2
Accuracy and precision for the measurement of compound I, by LC–MS, in control samples used in a Phase I dose escalation study

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	n[a]	Precision (% R.S.D.)		Bias (%)
			intra-day	inter-day	
6.18	5.94	23	7.6	0.0	−3.9
12.4	11.6	18	6.7	1.7	−5.8
59.3	56.6	9	2.4	2.7	−4.6
119	120	18	6.2	2.2	1.4
296	285	9	2.6	3.2	−3.8
593	598	19	4.9	0.0	0.8
988	995	3	7.6	0.0	0.7
1235	1179	6	2.3	2.0	−4.6
1976	1858	4	2.5	1.8	−6.0
2498	2420	18	3.7	1.8	−3.1

[a] Number of independent runs. Not all concentrations were used in each analytical run.

Table 3
Accuracy and precision for the measurement of compound II, by LC–MS, in control samples used in a Phase I dose escalation study

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	n[a]	Precision (% CV)		%Bias (%)
			intra-day	inter-day	
8.57	8.56	24	7.8	2.7	−0.1
15.6	16.5	17	6.2	5.3	5.6
33.3	32.8	8	2.6	2.6	−1.5
66	67	17	5.4	7.2	1.0
329	327	8	4.1	1.1	−0.5
660	659	17	5.0	0.0	−0.1
919	917	8	3.8	4.0	−0.2
1818	1810	7	3.0	2.0	−0.4
2647	2648	11	4.1	0.7	0.0

[a] Number of independent runs. Not all concentrations were used in each analytical run.

4. Discussion

A method for the measurement of I and II in human plasma has been described. The validation experiments have shown that the assay has very good accuracy and precision. The assay was shown to be robust over a number of analytical runs during the application to the analysis of clinical samples. No endogenous interferences were noted. Because the treatment of HIV-infection usually requires the use of many agents, the specificity of mass spectrometry provides an added advantage during the analysis of clinical samples. The confirmation of stability for I and II following HIV heat-inactivation procedures minimizes the health risk to laboratory personnel.

Because the HPLC runtime is short more than 100 samples could be analyzed per day with the limitation being the capacity of most auto-injectors. The rate-limiting step for the analyst is the labor involved in sample preparation which can be ameliorated by using semi-automatic solid-phase extraction procedures (described here) or a fully automatic robotic adaptation.

The excellent sensitivity of the method provides enough data to describe the terminal elimination phase in the plasma of patients receiving the smaller doses of I. In addition, smaller volumes of blood may be taken in which the volume of blood taken for the study is of special concern in this patient population.

5. Conclusions

A sensitive and specific LC–MS method for the direct determination of I and the indirect determination of II in human plasma was developed and validated. Validation experiments have shown that the assay has very good precision and accuracy over a wide concentration range, and that no interferences caused by endogenous substances were observed. The short runtime (<5 min) and integrated sample analysis software allowed for a high throughput of samples.

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