

Performance Analysis of a Rapid HPLC Determination with the Solvent Demixing Extraction of HIV Antiproteases and Efavirenz in Plasma

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Abstract

A rapid and simple high-performance liquid chromatographic method without internal standardization is evaluated for the drug-level monitoring of most marketed antiproteases and efavirenz. Following plasma deproteinization with acetonitrile, the analytes are extracted into the solvent while it is demixed by the addition of a saturating amount of neutral salt. The organic supernatant is diluted by half with water up to the polarity of the mobile phase before being injected. The isocratic mobile phase is unbuffered water–acetonitrile (52:48), and the stationary phase is LiChrospher 100 RP-8 (5 μm). Analytes are eluted between 4 min (amprenavir and indinavir) and 20 min (nelfinavir). A spreadsheet program including analysis of variance (ANOVA) and regression is used for both the overall validation of milligrams-per-liter determinations and the performance evaluation of analytical steps from chromatographic raw data. Extraction shows acceptable 5% repeatability and nearly 100% recovery, although it is somewhat concentration-dependent. The calibration function is better fitted by bilogarithmic than arithmetical regression, and the ANOVA of raw data is found quite predictive of the quality of the final determinations.

Introduction

The present consensus on the therapy of human immunodeficiency virus (HIV) infection acknowledges as first-line treatment a three-drugs regimen combining a protease inhibitor and two inhibitors of the retrovirus reverse transcriptase (1–4).

There is growing interest in using drug-level monitoring to optimize antiretroviral therapy (5–13). Drugs can be assayed in plasma with a number of high-performance liquid chromatographic (HPLC) methods recently published for HIV protease inhibitors (14–17), the nonnucleoside reverse transcriptase inhibitor efavirenz (EVZ) (18), or all together (19). Most involve

either the liquid–liquid or solid-phase extraction (SPE) of biological samples, which are two techniques that are somewhat lengthy and have questionable between-operator robustness when manually processed. Concerning data processing, calibrators are most often a set of concentrations in geometrical progression (serial dilutions), the concentration–peak height or peak-area relationship is commonly fitted by linear regression, and the best fit is sought by using or not weighting factors of $1/\text{concentration}$ or $1/\text{concentration}^2$. Finally, the performance evaluation of methods generally is limited to the overall (mg/L) retrieval of control sample values, which is the pragmatic approach required by current quality control guidelines. In this study, we describe an HPLC method for the determination of the transcriptase inhibitor EVZ together with any one of most marketed antiproteases [nelfinavir (NFV) and its M8 metabolite (NF8), zidovudine (ZDV), zalcitabine (ZCZ), didanosine (DDI), and amprenavir (APV)] in their proposed therapeutic ranges (20) (Figure 1). Distinctive features of this work include the analysis of performance upon raw data in addition to overall milligrams-per-liter validation and in particular thorough evaluation of the technique of extraction by solvent demixing already proposed (21). Required computations were made with an already described

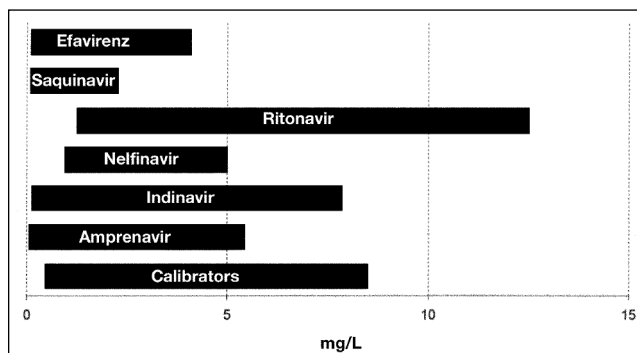


Figure 1. Concentration range of calibration in the method presented (lowest bar) as compared with the proposed therapeutic ranges of drugs under study (20).

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spreadsheet program (22) that was extended to work out calibrations, as well.

Experimental

Chemicals

Methanol and acetonitrile were HPLC-grade reagents from Carlo Erba (Val de Reuil, France) and Merck-Clevenot (Nogent-sur-Marne, France), respectively.

APV was from GlaxoWellcome (Stevenage, U.K.), IDV from MSD (Paris, France), RTV from Abbott Laboratories (Abbott Park, IL), and NFV mesylate and SQV from Roche Products (Welwyn Garden City, U.K.).

EVZ solution was obtained by dissolving the content of a capsule of Sustiva (batch RI 692) in methanol.

NF8 was provided as a methanolic solution at 500 mg/L by Dr. M. Legrand (Pharmacology, Pitie-Salpetriere Hospital, Paris, France) (23) for the Cophar quality control.

Drug-free human plasma was obtained from Etablissement de Transfusion Sanguine (Montpellier, France). Bovine albumin (Fraktion V, Boehringer, Mannheim, Germany) was dissolved in water (25 g/L) to make a blood-free matrix intended for extraction studies.

Equipment

The chromatographic apparatus included an SP8800 pump, a UV1000 spectrophotometer, and an AS100 autosampler [all from Thermostechnique Products (Les Ulis, France)] coupled to a D2000 integrator from Merck (Darmstadt, Germany). The column was a LiChroCART 250-4 from Merck filled with LiChrospher 100 RP-8 (5 μ m).

Chromatography

The isocratic mobile phase was a 48:52 mixture of acetonitrile and water (v/v) used at a flow rate of 1.5 mL/min at room temperature. Fifty microliters of sample was injected with the autosampler adjustable volume loop injector. Peak heights were measured at 240 nm. Every chromatographic run was duplicated in order to estimate measurement imprecision.

Sample extraction by acetonitrile demixing

Samples were extracted according to the solvent demixing procedure already published for anionic and nonelectrolyte drugs (21,22,24–27). Briefly, 1 mL acetonitrile was mixed with 1 mL plasma sample or calibrator in a glass tube by forceful pipetting under vortex stirring in order to avoid the formation of protein clusters. A saturating amount (~ 0.5 g) of solid potassium chloride was added, and the tube was vigorously vortex mixed and then centrifuged (5 min, 1500 g, 5°C). Of the approximately 0.7-mL demixed acetonitrile supernatant, 0.50 mL was diluted with an equal volume of water in an autosampler vial for injection. The volume injected (50 μ L) thus contained the amount of analytes extracted from approximately 0.5/0.7 times 50/1000 times 1 mL, 0.036 mL, or 36 μ L of plasma. This calculation assumes that all analytes are concentrated in the 0.7 mL of acetonitrile following solvent demixing (as will be further discussed in “Discussion” section).

Preparation of titrated samples for both raw data analysis of variance and validation

A separate titrated stock solution of every compound was made in methanol at a common concentration of 200 mg/L except for NF8, which was available as a 500-mg/L solution. In order to express all of the results in milligrams per liter of pure substances, an NFV mesylate weighted amount was augmented by the correcting factor of 1.16 (ratio of NFV mesylate to NFV molecular weights).

Because preliminary chromatograms had shown IDV and APV retention times to be indistinguishable in our conditions, two separate validation sessions were needed.

Session A samples included (in the order of retention times) APV, NF8, RTV, EVZ, SQV, and NFV. Two titrated working solutions were extemporally prepared at concentrations of 100 and 10 mg/L for every analyte. In order to make the higher-level working solution (AHi), 0.500 mL of each of the five stock solutions and 0.200 mL of the Cophar NF8 solution were added in a glass tube, the solvent evaporated to dryness under a flow of nitrogen at room temperature, and the residue redissolved in 1.0 mL of methanol–water (50:50). The lower-level solution (ALo) was obtained by diluting solution AHi ten times in methanol–water.

Session B samples included IDV, RTV, EVZ, SQV, and NFV. Two working solutions (BHi and BLo, which were 100 and 10 mg/L, respectively, of every analyte) were prepared the same way.

Each session included two sets of titrated samples: one designed for statistical analysis [analysis of variance (ANOVA) samples] and the other intended for calibration and results validation. Ten ANOVA samples were prepared by incorporating adequate volumes of the working solutions to plasma and albumin–water in order to obtain 5 levels of every analyte in both of the matrices: 0.5 to 1.0 mg/L (from the lower-level solution) and 2.0 to 4.0 to 8.0 mg/L (from the higher-level solution). Two 1-mL aliquots of every ANOVA sample were racked up as a set of 10 duplicated (i.e., 20 total) assay samples.

In each session, a set of five calibration samples was also prepared the same way in plasma from the same working solutions.

Data processing

Experimental data from the two sessions presented were used in two ways.

Peak heights (raw millivolts data from integrator) of ANOVA samples were processed in order to estimate the main instrumental characteristics of the technique: limit of detection (LOD), extraction and detection precision, and detection linearity.

LOD was estimated as the analyte concentration generating a peak height of three times the chromatogram baseline noise around the corresponding retention time following the injection of an extract of blank plasma. Baseline noise (in millivolts) was graphically estimated as the width of the chromatographic track during the 30 s preceding the start of a peak.

Because each ANOVA sample in both matrices was extracted twice and each of the two extracts was chromatographed twice, each session generated 2 matrices multiplied by 5 concentrations, 2 extracts times, and 2 measures, thus equaling to 40 peak height data. Napierian logarithmic transforms of both peak heights and concentrations were submitted to standard ANOVA for a design of three crossed factors (sessions were random with 1 or 2 levels,

concentrations were fixed with 5 levels, and sample matrix fixed with 2 levels) together with one nested factor (the extract, enclosing spectrophotometric measurement variance as residual variance). The third crossed factor was included for checking the feasibility of substituting a synthetic matrix such as albumin-water for human plasma in calibrators.

The design also enabled estimation of the slope of the bilogarithmic linear regression line, its deviation from the value of 1 expected from the hypothesis of analytical linearity, and overall lack of fit of the regression straight line (in particular second-order curvature).

For this purpose variance components (mean squares) were submitted to F significance tests and slope deviation from 1 to a *t*-test. Roots of mean squares were directly read as arithmetical relative standard deviations (RSDs) or coefficients of variation (see the Discussion section).

ANOVA samples were also used as control samples for assay validation. Milligrams-per-liter values were calculated in each session with the associated calibration curve and submitted to current quality control assessment [accuracy, precision, and lower limit of quantitation (LOQ)] (see the Discussion section). Calculations were made through both arithmetical and logarithmic regressions. First, the five-point calibration line was fitted by the unweighted linear regression of calibrator peak heights (Y_{cal}) versus calibrator concentrations (C_{cal}), providing the calibration parameters *a* and *b* according to:

$$Y_{cal} = a + b \cdot C_{cal} \quad \text{Eq. 1}$$

Control levels (X_{ctr}) were thereafter calculated from ANOVA sample peak heights (Y_{ctr}) with the reciprocal function:

$$X_{ctr} = (Y_{ctr} - a)/b \quad \text{Eq. 2}$$

Second, in parallel logarithmic computation the calibration line was fitted by bilogarithmic linear regression as:

$$\ln(Y_{cal}) = a_{ln} + b_{ln} \cdot \ln(C_{cal}) \quad \text{Eq. 3}$$

Control levels were then calculated as antilogarithms of the reciprocal function:

$$X_{ctr} = \exp[(\ln(Y_{ctr}) - a_{ln})/b_{ln}] \quad \text{Eq. 4}$$

In both cases, estimated levels (X_{ctr}) were compared to expected concentrations (C_{ctr}) as determination percent relative deviates (*d*):

$$d = 100(X_{ctr} - C_{ctr})/C_{ctr} \quad \text{Eq. 5}$$

Each set of determination deviates (obtained from either arithmetical or logarithmic computations) was submitted to standard statistics: the mean deviate (which is the instrumental average bias) and its standard deviation (which is the overall imprecision of the assay). In addition, the distribution of determination deviating among the analytes and concentrations was graphically

evaluated on scatter plots.

Extraction recovery from plasma was roughly estimated by the ratio of peak heights from separate pairs of spiked samples, one of extracted plasma and the other of unextracted acetonitrile for which the volume spiked was 0.7 times that of plasma (the approximate volumetric extraction recovery of acetonitrile demixing). Comparative recovery from plasma and albumin-water was calculated the same way from all ANOVA samples in either matrix.

Results

Figure 2 shows a chromatogram. Estimations of the LODs of the seven analytes are listed in Table I together with retention times.

The milligrams-per-liter level estimates of ANOVA samples analyzed in Table II were those calculated using the bilogarithmic regression of calibrators (as justified in the Discussion section). The factor analysis part included (as percent RSDs) imprecision estimates of HPLC measurements (between-duplicated mea-

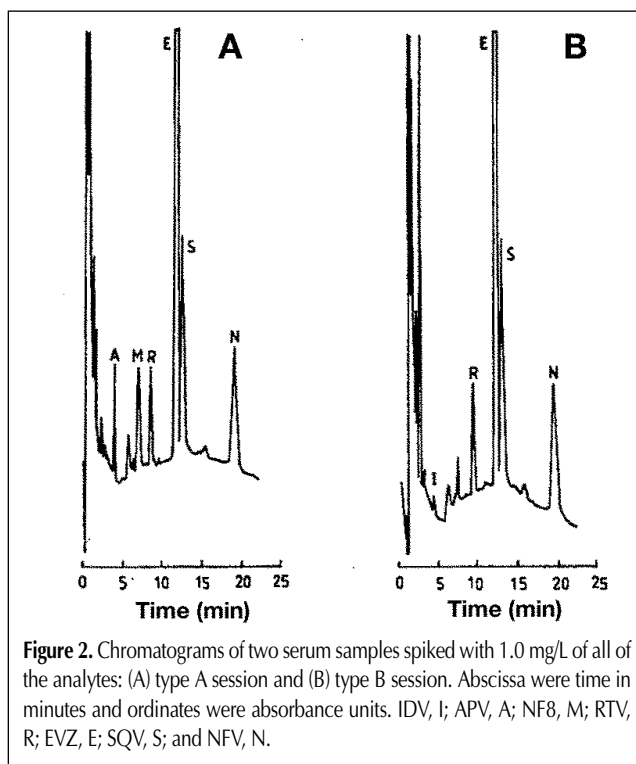


Figure 2. Chromatograms of two serum samples spiked with 1.0 mg/L of all of the analytes: (A) type A session and (B) type B session. Abscissa were time in minutes and ordinates were absorbance units. IDV, I; APV, A; NF8, M; RTV, R; EVZ, E; SQV, S; and NFV, N.

Table I. Retention Times and Estimated LODs of Analytes in Order of Increasing Retention Times

	IDV	APV	NF8	RTV	EVZ	SQV	NFV
RT* in min	4.5	5.15	8.3	10.0	13.25	14.15	21.3
(range)	(4.4–4.6)	(5.1–5.2)	(8.2–8.4)	(9.9–10.1)	(13.2–13.3)	(14.1–14.2)	(20.9–21.6)
LOD in μ /L	175	20	20	20	1	10	20
* RT, retention time.							

tures) and extraction (extraction component of average measurements variance of duplicated extracts). Also included were the mean percent relative differences of determinations between sessions (session 2 minus session 1) and between matrices (plasma minus albumin–water).

The regression analysis part included slopes of the bilogarithmic regression line of estimated versus expected milligrams-per-liter values in both matrices, which was then compared with the theoretical slope of 1 that was expected from strict accuracy. Also included was the overall percent lack of fit from the common regression line as well as the relative contribution of its main nonrandom components: second- and third-order

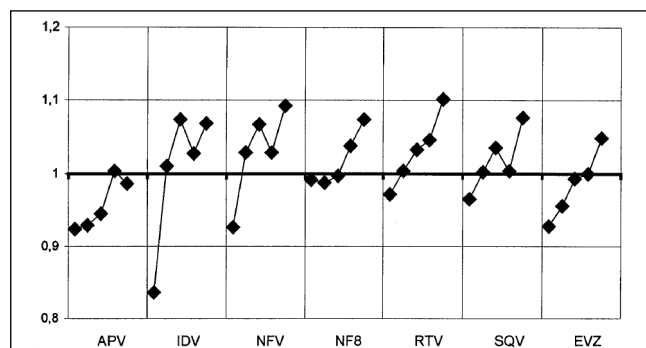


Figure 3. Concentration-dependent recovery of analytes from plasma extracts relative to albumin–water extracts. Ordinate was the ratio of the mean peak height from plasma extracts versus the mean peak height from water extracts at every tested concentration of all seven analytes. Concentrations of every analyte were equidistant (log scale) from 0.5, 1.0, 2.0, 4.0, to 8.0 mg/L (left to right, respectively).

common curvatures and deviations from parallelism in either matrix.

Extraction recovery from plasma was found to be not lower than 90% (not reported). Figure 3 shows the concentration-dependent recovery ratio from plasma relative to albumin–water.

Discussion

Figure 1 shows that the range of calibration points was in accordance with the proposed therapeutic ranges of most drugs under study. The retention time interference of APV and IDV was not detrimental because these equivalent drugs were not used together. However, the detection of IDV at the 240-nm wavelength was not optimal. When needed, IDV was better calibrated and assayed at 210–220 nm.

Analysis of milligrams-per-liter data

Items in Table II are informative about method global precision (factor analysis) and accuracy (regression analysis). Imprecision was currently expressed as %RSD or coefficient of variation. In the ANOVA design presented in this study, calculations were made with logarithmic transforms of entered data. The root of the mean square (i.e., standard deviation) of any ANOVA logarithmic component, if small enough (< 20%), was quite a good estimate (90% to 100%) of the corresponding arithmetical RSD. Thus, the RSD of the chromatographic determination was directly read as the root of the mean square of the “between-duplicated measures” component. Extraction RSD had to be calculated further from the “between-extracts” component mean square.

When analyzing milligrams-per-liter results, the regression straight line of slope 1.0 is the identity line, which is of strict equality between measured and nominal concentrations. Global inaccuracy can be evaluated from deviations of the regression slope around 1 and from the variance of the lack of fit component of ANOVA. We found it convenient to express the amount of lack of fit also as the root of the variance estimate of the lack of fit component, which is to be read as a percent discrepancy similar to the former ones.

Calibration being somewhat better with bilogarithmic than with arithmetic regression

There are two conditions in which bilogarithmic linear regression is theoretically more appropriate than arithmetical regression: (a) when arithmetical abscissa are in geometric progression (logs provide equal intervals) and (b) when residual standard deviations are proportional to ordinate values [in other words when residual error RSD is constant (logs then restore homoscedasticity; i.e., constant variance)]. The first condition almost always exists for calibrators in manual assay methods, because of the common practice of serial dilution. The second is usual for

Table II. ANOVA of Milligrams-per-Liter Determinations Calculated with Bilogarithmic Linear Regression*

	APV	IDV	NFV	NF8	RTV	SQV	EVZ
Analysis of regression							
Slope in plasma	1.037	1.095	1.031	1.022	1.014	1.011	1.012
Slope in albumin–water	1.005	1.020	1.002	0.989	0.969	0.982	0.969
Lack of fit (%RSD)	0.41	10.96	16.69	0.41	3.58	2.63	1.47
Second-order curvature (%RSD)	1.03	2.18	7.03	0.42	3.14	6.07	4.01
Third-order sigmoidicity (%RSD)	0.17	10.52	10.34	0.94	0.18	4.21	1.40
Unparallelism (%RSD)	9.84	23.09	12.88	10.15	19.52	12.78	19.01
Opposite curvature (%RSD)	0.02	15.98	7.62	4.04	1.40	0.99	0.50
Factor analysis							
Between measures (%RSD)	0.85	3.93	1.04	2.30	2.49	2.34	0.48
Between extracts (%RSD)	6.43	7.87	6.03	8.96	6.42	5.99	5.68
Extraction component (%RSD)	4.51	4.82	4.20	6.12	4.19	3.90	4.01
Between sessions (2–1) [†]	–	–	–9.76	–	–3.48	7.87	–5.19
Between matrices (plasma–water) [†]	–2.10	4.87	7.44	4.84	7.51	9.56	2.77
IA [‡] concentration matrix (%RSD)	5.51	15.00	9.11	5.50	9.92	7.60	9.65
IA concentration session (%RSD)	–	–	6.18	–	3.48	1.59	3.94
IA matrix session (%RSD)	–	–	18.74	–	6.90	22.70	5.40
IA triple (%RSD)	–	–	13.37	–	4.10	6.17	3.94

* The slope value expected from strictly accurate determinations was 1.

[†] % difference.

[‡] IA, interaction between factors.

high values, though residual error tends to become arithmetically constant as measured values come down toward the LOQ. From an empirical standpoint, bilogarithmic regression can provide a better fit in case of small departures from arithmetical proportionality, such as a nonzero intercept (in which case the "curva-

ture" component in the bilogarithmic regression is expected to be significant) or actual arithmetical curvature resulting from, for example, adsorptive extraction losses higher for lower concentrations.

Our results confirmed logarithms to work better. Table III presents, in the same format as Table II, the comparison of the statistical analysis estimates obtained from arithmetical and logarithmic calculations using the ratio of the former to the second. Whenever a deviation or an RSD was concerned, a ratio close to 1 indicated that both calculations were equivalent, a ratio less than one indicated that the arithmetical model was better, and a ratio larger than one indicated that the logarithmic model was better. Clearly, although many variance components were not affected by the regression model, the "lack of fit" component most often was larger (i.e., fitting was poorer) after arithmetical regression, though IDV was little affected and NFV was possibly better.

Quality was predictable directly from ANOVA of raw data

When designing an assay method, it may be useful to get suitable information about the quality level reached at every step without having to work out the whole assay procedure. One factor of quality is extrinsic to methods, the accuracy of calibrators, which can be assessed only through interlaboratory checking. The others are intrinsic (repeatability and precision, of course), but also intrinsic bias differently affecting individual calibration levels (for example, better extraction recovery from higher than from lower concentrations). Raw chromatographic data already include this information. We thus tested whether and how calibration computations changed raw data ANOVA estimates.

Table IV allows for the comparison of ANOVAs of milligrams-per-liter determinations (following logarithmic fitting selected as better) and raw data. In a similar way as in Table III, each component value was the ratio of the final milligrams-per-liter determination ANOVA component to the raw data ANOVA same component. Again, 1 denoted equality, more than 1 denoted the final estimate being enlarged (i.e., worsened by calibration calculations as long as imprecision and lack of fit were concerned), and less than 1 denoted its being diminished (i.e., bettered).

Clearly, most ANOVA components were not affected (ratio of ~ 1) by calibration calculations, particularly the within-session imprecision of measurement and extraction duplicates. Moreover, lack of fit was diminished in four cases, as was between-sessions variation in all cases involved. This was not unexpected, because calibrators and controls tend to vary in parallel ways and cancel

Table III. Compared ANOVA Components of Milligrams-per-Liter Determinations through Arithmetical Versus Bilogarithmic Calibration*

	APV	IDV	NFV	NF8	RTV	SQV	EVZ
Analysis of regression							
Slope in plasma	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Slope in albumin-water	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lack of fit (%RSD for all terms)	7.7	1.0	0.8	44.0	4.0	3.1	13.2
Second-order curvature	2.8	1.0	1.0	41.5	4.3	1.0	4.7
Third-order sigmoidicity	5.9	1.0	0.6	5.5	20.1	1.0	3.5
Unparallelism	1.0	1.0	1.5	1.0	1.0	1.0	1.1
Opposite curvature	10.2	1.0	0.8	0.9	0.5	0.9	0.9
Factor analysis							
Between measures (%RSD)	1.0	1.0	0.9	1.1	1.1	1.0	1.0
Between extracts (%RSD)	1.0	1.0	0.9	1.1	1.0	1.0	1.1
Extraction component	1.0	1.0	0.9	1.1	1.0	1.0	1.1
Between sessions (2-1)	-	-	0.5	-	1.0	-2.2	1.0
Between matrices (plasma-water)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
IA concentration matrix (%RSD)	1.0	1.0	1.2	1.0	1.0	1.0	1.1
IA concentration session (%RSD)	-	-	1.2	-	1.1	1.5	0.8
IA matrix session (%RSD)	-	-	0.3	-	1.1	1.0	1.0
IA triple (%RSD)	-	-	0.8	-	1.0	1.0	1.0

* Every value is the ratio of an ANOVA component following arithmetical calibration to the same component following bilogarithmic calibration: 1 denotes equivalence, > 1 denotes variance enlargement by arithmetical computation, and < 1 denotes variance enlargement by logs.

Table IV. Compared ANOVA Components of Milligrams-per-Liter Determinations Versus Measurement Raw Data*

	APV	IDV	NFV	NF8	RTV	SQV	EVZ
Analysis of regression							
Slope in plasma	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Slope in albumin-water	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lack of fit (%RSD for all terms)	0.3	1.0	1.0	0.3	1.0	0.3	0.3
Second-order curvature	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Third-order sigmoidicity	1.0	1.0	1.0	1.0	1.1	1.0	1.1
Unparallelism	1.0	1.0	0.6	1.0	1.0	1.0	1.0
Opposite curvature	1.0	1.0	1.0	1.0	1.0	1.1	0.9
Factor analysis							
Between measures (%RSD)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Between extracts (%RSD)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Extraction component	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Between sessions (2-1)	-	-	0.7	-	0.4	-0.5	0.6
Between matrices (plasma-water)	1.0	1.0	1.2	1.1	1.1	2.2	1.6
IA concentration matrix (%RSD)	1.0	1.0	0.7	1.0	1.0	1.0	1.0
IA concentration session (%RSD)	-	-	1.2	-	1.0	0.7	1.1
IA matrix session (%RSD)	-	-	4.0	-	1.0	1.0	1.0
IA triple (%RSD)	-	-	1.1	-	1.0	1.0	1.0

* Every component is expressed as the ratio of the former to the second: 1 denotes equivalence, > 1 denotes component being enlarged by sessional calibration, and < 1 denotes component being reduced by calibration.

out. Thus, checking quality at intermediate steps when working a method out by merely analyzing raw data, as we have long been used to doing, appears to be safe and time saving until the final check of the fully developed method.

The solvent-demixing extraction

Extraction repeatability, which the ANOVA design in this study allows to assess separately, was approximately 5% RSD (Table II).

Figure 3 shows the ratio of recovery from plasma over albumin–water to be regularly dependent on the analyte concentration in the range studied. Recovery from plasma was lower at the low concentration of analyte and higher at higher concentration than recovery from albumin–water. Because other tests had suggested plasma recovery not to be less than 90%, such a pattern would confirm the assumption that recovery from albumin–water was more or less complete, and extraction from plasma underwent adsorptive losses that are well-known to lessen as concentration goes up within the adsorption saturable range. In this study, the only possible cause for adsorption was in precipitated plasma proteins. This may be a drawback of this study's technique or more likely of the liquid–liquid extraction of cationic substances. Whether SPE is free of such adsorption effects or not, this has not yet been assessed to our knowledge.

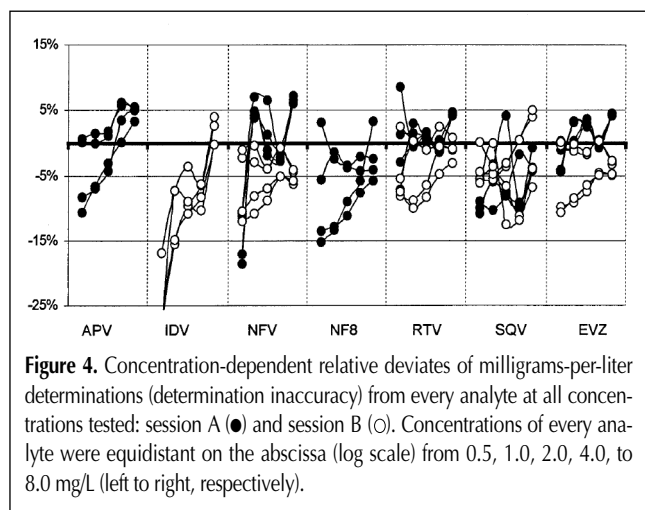


Figure 4. Concentration-dependent relative deviates of milligrams-per-liter determinations (determination inaccuracy) from every analyte at all concentrations tested: session A (●) and session B (○). Concentrations of every analyte were equidistant on the abscissa (log scale) from 0.5, 1.0, 2.0, 4.0, to 8.0 mg/L (left to right, respectively).

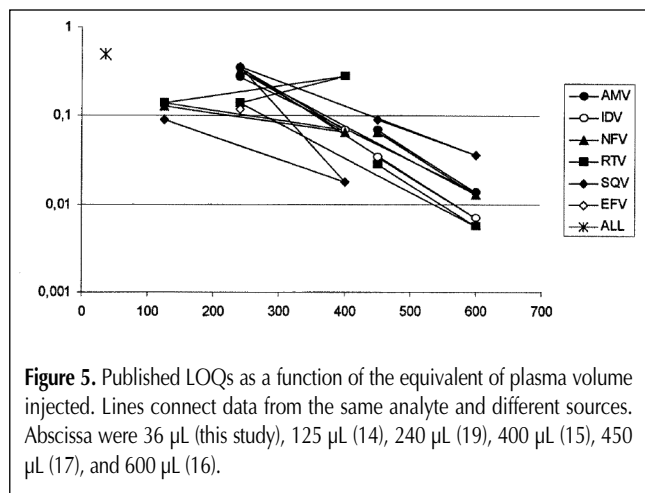


Figure 5. Published LOQs as a function of the equivalent of plasma volume injected. Lines connect data from the same analyte and different sources. Abscissa were 36 μ L (this study), 125 μ L (14), 240 μ L (19), 400 μ L (15), 450 μ L (17), and 600 μ L (16).

Lower LOQ

LOQ is defined by the Food and Drug Administration as the concentration at which the analyte peak (response) is still identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% (28). Concentrations were found lower than 20% down to 0.5 mg/L, which was thus the LOQ validated in this study for all analytes except NFV.

Figure 4 shows that the LOQ was limited by concentration-related inaccuracy more than imprecision.

We found that LOQs published in literature were inversely correlated with the equivalent of the plasma volume that was injected. Plasma equivalent injected volume can be calculated as the injection volume times the volume reduction ratio (plasma sample volume over final volume of injected extract or SPE eluate). Figure 5 shows this correlation and also that our results located within its extrapolation. This indicates that this study's extraction technique, however simple and rapid, shows sensitivity comparable with more sophisticated ones and can compete at the expense of two more steps under validation—exsiccating the supernatant aliquot and redissolving it into a smaller volume.

Acknowledgments

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References

1. C.D. Holtzer and M. Roland. The use of combination antiretroviral therapy in HIV-infected patients. *Ann. Pharmacother.* **33**: 198–209 (1999).
2. R. Samuel and B. Suh. Antiretroviral therapy 2000. *Arch. Pharm. Res.* **23**: 425–37 (2000).
3. C.C. Carpenter, D.A. Cooper, M.A. Fischl, J.M. Gatell, B.G. Gazzard, S.M. Hammer, M.S. Hirsch, D.M. Jacobsen, D.A. Katzenstein, J.S. Montaner, D.D. Richman, M.S. Saag, M. Schechter, R.T. Schooley, M.A. Thompson, S. Vella, P.G. Yeni, and P.A. Volberding. Antiretroviral therapy in adults: updated recommendations of the International AIDS Society—USA Panel. *JAMA* **283**: 381–90 (2000).
4. C.C. Carpenter, M.A. Fischl, S.M. Hammer, M.S. Hirsch, D.M. Jacobsen, D.A. Katzenstein, J.S. Montaner, D.D. Richman, M.S. Saag, R.T. Schooley, M.A. Thompson, S. Vella, P.G. Yeni, and P.A. Volberding. Antiretroviral therapy for HIV infection in 1998: updated recommendations of the International AIDS Society—USA Panel. *JAMA* **280**: 78–86 (1998).
5. T.N. Kakuda, L.M. Page, P.L. Anderson, K. Henry, T.W. Schacker, F.S. Rhame, E.P. Acosta, R.C. Brundage, and C.V. Fletcher. Pharmacological basis for concentration-controlled therapy with zidovudine, lamivudine, and indinavir. *Antimicrob. Agents. Chemother.* **45**: 236–42 (2001).
6. E.P. Acosta, K. Henry, L. Baken, L.M. Page, and C.V. Fletcher. Indinavir concentrations and antiviral effect. *Pharmacotherapy* **19**: 708–12 (1999).
7. G. Gatti, A. Di Biagio, R. Casazza, C. De Pascalis, M. Bassetti, M. Cruciani, S. Vella, and D. Bassetti. The relationship between ritonavir plasma levels and side effects: implications for therapeutic drug monitoring. *AIDS* **13**: 2083–89 (1999).
8. C. Marzolini, A. Telenti, L.A. Decosterd, G. Greub, J. Biollaz, and T. Buclin. Efavirenz plasma levels can predict treatment failure and

- central nervous system side effects in HIV-1-infected patients. *AIDS* **15**: 71–75 (2001).
9. C. Merry, D. Back, and M. Barry. Abstract 104. Presented at the 7th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, January 30–February 2, 2000.
 10. P. Clevenberg, J. Duran, and R. Garraffo. Abstract 260B. Presented at the 8th Conference on Retroviruses and Opportunistic Infections, Chicago, IL, February 4–8, 2001.
 11. C.V. Fletcher, T. Fento, and C. Powell. Abstract 259. Presented at the 8th Conference on Retroviruses and Opportunistic Infections. Chicago, IL, February 4–8, 2001.
 12. J. Duran, P. Clevenberg, R. Garraffo, P. Halfon, S. Icard, P. Del Giudice, N. Montagne, J.M. Schapiro, and P. Dellamonica. Importance of protease inhibitor plasma levels in HIV-infected patients treated with genotypic-guided therapy: pharmacological data from the Viradapt study. *AIDS* **14**: 1333–39 (2000).
 13. P. Langmann, M. Zilly, B. Weissbrich, C. Schlor, T. Vath, E. Richter, and H. Klinker. Therapeutic drug monitoring of saquinavir in patients during protease inhibitor therapy with saquinavir alone or in combination with ritonavir or nelfinavir. *Eur. J. Med. Res.* **5**: 59–82 (2000).
 14. M. Bouley, C. Briere, C. Padoin, O. Petitjean, and M. Tod. Sensitive and rapid method for the simultaneous quantification of the HIV-protease inhibitors indinavir, nelfinavir, ritonavir, and saquinavir in human plasma by reversed-phase liquid chromatography. *Ther. Drug. Monit.* **23**: 56–60 (2001).
 15. M. Legrand, G. Aymard, V. Calvez, R. Tubiana, M. Cadart, and B. Diquet. Méthode de dosage simultané de quatre antiprotéases par chromatographie liquide haute performance dans le plasma humain. *J. Pharm. Clin.* **19**: 61–68 (2000).
 16. J.M. Poirier, N. Radembino, P. Robidou, and P. Jaillon. Simultaneous determination of the five HIV-protease inhibitors: amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir in human plasma by solid-phase extraction and column liquid chromatography. *Ther. Drug. Monit.* **22**: 465–73 (2000).
 17. R.P.G. van Heeswijk, R.M.W. Hoetelmans, R. Harms, P.L. Meenhorst, J.W. Mulder, J.M. Lange, and J.H. Beijnen. Simultaneous quantitative determination of the HIV protease inhibitors amprenavir, indinavir, nelfinavir, ritonavir and saquinavir in human plasma by ion-pair high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. B* **719(1-2)**: 159–68 (1998).
 18. P. Villani, M. Pregnotato, S. Banfo, M. Rettani, D. Burrioni, E. Seminari, R. Maserati, and M.B. Regazzi. High-performance liquid chromatography method for analyzing the antiretroviral agent efavirenz in human plasma. *Ther. Drug Monit.* **21**: 346–50 (1999).
 19. C. Marzolini, A. Telenti, T. Buclin, J. Biollaz, and L.A. Decosterd. Simultaneous determination of the HIV protease inhibitors indinavir, amprenavir, saquinavir, ritonavir, nelfinavir and the non-nucleoside reverse transcriptase inhibitor efavirenz by high-performance liquid chromatography after solid-phase extraction. *J. Chromatogr. B* **740**: 43–58 (2000).
 20. S.P. Aries, B.M. Schaaf, U. Wilmsen, M. Schuett, M. Kurowski. Routine therapeutic drug monitoring in patients on protease inhibitors: impact on therapeutic decisions? Abstracts of the Fifth International Congress on Drug Therapy in HIV Infection. Glasgow, U.K., October 22–26, 2000, p. 264.
 21. R. Alric, M. Cociglio, J.P. Blayac, and R. Puech. Performance evaluation of a reversed-phase high-performance liquid chromatographic assay of valproic acid involving a “solvent-demixing” extraction procedure and precolumn derivatisation. *J. Chromatogr.* **224**: 289–69 (1981).
 22. M. Cociglio, D. Hillaire-Buys, and R. Alric. Prevalidation statistical design to assess analytical methods. Example of a quick liquid chromatographic assay of itraconazole in serum. *J. Chromatogr. B* **698**: 225–33 (1997).
 23. M. Legrand. Abstract 8.3. Presented at the 2nd International Workshop on Clinical Pharmacology of HIV Therapy, Noordwijk, The Netherlands April 2–4, 2001. <http://www.hivpharmacology.com>.
 24. M. Cociglio, R. Alric, and O. Bouvier. Performance analysis of a reversed-phase liquid chromatographic assay of lamotrigine in plasma. *J. Chromatogr.* **572**: 269–76 (1991).
 25. M. Cociglio, J. Bres, D. Sauvaire, R. Alric, and M. Richard. Pharmacokinetics of an indomethacin pro-drug: apyramide after intravenous administration in dog. *Euro. J. Drug Metab. Pharmacokinet.* **16(4)**: 275–80 (1991).
 26. R. Alric, L. Arce-Corales, J.P. Blayac, and R. Puech. Statistical analysis of performance of a liquid chromatographic assay of anticonvulsants. *Meth. Find Exp. Clin. Pharmacol.* **6**: 353–61 (1984).
 27. M. Cociglio, D. Sauvaire, and R. Alric. Liquid chromatographic assay of heptaminol in serum and its oral pharmacokinetics in the dog. *J. Chromatogr.* **307**: 351–59 (1984).
 28. V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, and A. Yacobi. Bioanalytical method validation—a revisit with a decade of progress. *Pharm. Res.* **17**: 1551–57 (2000).

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