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Citation for final published version:

Nannetti, Giulio, Pagni, Silvana, Parisi, Saverio G., Alberti, Alfredo, Loregian, Arianna and Palù, Giorgio 2016. Development of a simple HPLC-UV method for the determination of the hepatitis C virus inhibitor simeprevir in human plasma. Journal of Pharmaceutical and Biomedical Analysis 121 , pp. 197-203. 10.1016/j.jpba.2016.01.019 file

Publishers page: http://dx.doi.org/10.1016/j.jpba.2016.01.019
<http://dx.doi.org/10.1016/j.jpba.2016.01.019>

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Short communication

Development of a simple HPLC-UV method for the determination of the hepatitis C virus inhibitor simeprevir in human plasma

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Abstract

A simple high-performance liquid chromatography method for the determination of the hepatitis C virus protease inhibitor simeprevir in human plasma was developed and validated. The method involved a rapid and simple solid-phase extraction of simeprevir using Oasis HLB 1cc cartridges, an isocratic reversed-phase liquid chromatography on an XTerra RP18 (150 mm × 4.6 mm, 3.5

μm) column, and ultraviolet detection at 225 nm. The mobile phase consisted of phosphate buffer (pH 6, 52.5 mM) and acetonitrile (30:70, v/v). This assay proved to be sensitive (lower limit of quantification of 0.05 μg/ml), linear (correlation coefficients ≥ 0.99), specific (no interference with various potentially co-administrated drugs), reproducible (both intra-day and inter-day coefficients of variation ≤ 8.3%), and accurate (deviations ranged from – 8.0 to 1.2% and from –3.3 to 6.0% for intra-day and inter-day analysis, respectively). The method was applied to therapeutic monitoring of patients undergoing simeprevir treatment for hepatitis C and proved to be robust and reliable. Thus, this method provides a simple, sensitive, precise and reproducible assay for dosing simeprevir that can be readily adaptable to routine use by clinical laboratories with standard equipment.

Keywords: Simeprevir; TMC435; HCV protease inhibitor; Solid-phase extraction; HPLC-UV; Therapeutic drug monitoring.

1. Introduction

Simeprevir (SMV, also known as TMC435) is a novel inhibitor approved for the treatment of chronic hepatitis C virus (HCV) infection [1]. HCV infection affects more than 150 million people worldwide and represents the leading cause of liver failure and hepatocellular carcinoma [2].

For many years, the combination therapy with ribavirin and pegylated interferon α has been the standard of care, achieving a sustained virological response (SVR) in ≥80 % of patients with HCV genotypes 2 and 3 but in only ~50 % of subjects with HCV genotype 1, and often causing significant side effects [3, 4].

Major advances in the treatment of HCV infection have recently been obtained with the development of direct-acting antivirals (DAAs) that target viral nonstructural proteins. SMV is a once-daily oral HCV N3S/4A protease inhibitor for the treatment of chronic HCV genotype 1 infection as a component of combination antiviral therapy [5]. SMV has demonstrated high SVR rates in patients with HCV genotype 1 infection during phase II and III trials [5].

SMV is metabolized by the cytochrome P450 (CYP) system, primarily CYP3A, and is a substrate and/or an inhibitor of several drug transporters. Thus, it is prone to metabolic drug–drug interactions with drugs that are CYP3A inhibitors or inducers; in fact, coadministration of these drugs may increase or decrease plasma concentrations of SMV, respectively [6]. In addition, SMV is a mild inhibitor of CYP1A2, thus potentially affecting drugs that are mainly metabolized by this enzyme. Two populations at particularly high risk of drug-drug interactions due to complex, long-term therapeutic regimens are transplant recipients receiving immunosuppressants and HCV-HIV coinfected patients receiving antiretroviral therapy [7]. Because coadministration of SMV with these medications has the potential to significantly alter the drug exposure, a user-friendly method sufficiently sensitive and robust to measure plasma levels of SMV in different therapeutic regimens is needed, in order to maximize the efficacy of treatment and avoid drug-related toxicity.

To the best of our knowledge, only one method for quantifying SMV in plasma, which employs liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), has been published so far [8]. However, since MS/MS facilities are not always available in standard hospital laboratories, analytical methods employing UV detection need to be developed.

In this report, we describe the development and validation of the first HPLC-UV method for the determination of SMV in plasma of HCV-infected patients. The proposed technique uses a simple solid-phase extraction (SPE) procedure, an isocratic elution on a reversed-phase column, and UV detection. The method is sufficiently robust and sensitive to measure therapeutically relevant levels of SMV, employs standard equipment and is easy to set up, and thus its use is feasible in most hospital laboratories. In addition, we evaluated the stability of SMV to heat treatment that is recommended to inactivate HCV and/or HIV particles in biological samples and also to cover several common conditions to which samples can be subjected in the clinical setting.

2. Material and methods

2.1 Chemicals and reagents

SMV was obtained from Alsachim (Illkirch, France). Acetonitrile for HPLC (Gradient Grade, LiChrosolv) was from Merck (Darmstadt, Germany), methanol for chromatography was from Carlo Erba (Milan, Italy). All other chemicals were of analytical grade and purchased from J. T. Baker (Deventer, The Netherlands). Ultrapure water was purified by a Milli-Q apparatus (Millipore, Bedford, MA, USA). Control human plasma (with K_3EDTA as anticoagulant) was purchased from Roche (Milan, Italy; TaqScreen West Nile Virus COBAS kit).

2.2 Equipment and chromatographic conditions

The chromatographic system consisted of an Alliance 2695 Separation Module equipped with an online degasser and an automatic injector thermostated at 20°C, and a 2996 Photodiode Array Detector coupled with the Empower data acquisition software (version 2.0) (Waters, Milford, MA,

USA). Separations were performed on an XTerra RP18 (150 mm × 4.6 mm, particle size 3.5 μm; Waters) analytical column equipped with an XTerra RP18 (20 mm × 3.9 mm, particle size 5 μm; Waters) guard column. Both columns were maintained at 40°C. The isocratic mobile phase consisted of a mixture of acetonitrile-sodium phosphate buffer (pH 6.0, 52.5 mM) (70:30, v/v) and was filtered through a 0.2 μm nylon filter membrane (Millipore, Bedford, MA, USA) before use. The flow rate was 1.0 ml/min, and the assay runtime was 30 min. Absorbance was measured at 225 nm. An Extraction Manifold (Waters) liquid handling system was used to perform the sample preparation. A DRI-BLOCK DB-3 evaporator (Techne, Stone, United Kingdom) was used for drying the solid phase extraction (SPE) eluates.

2.3 Preparation of stock solutions, working solutions, calibration standards and quality control (QC) samples

A stock solution of 1 mg/ml SMV was prepared in 100% dimethyl sulfoxide (DMSO). The stock solution was diluted further with DMSO to obtain working solutions with concentrations of 500, 250, 100, 50, 25, 12.5, 5 and 2.5 μg/ml. The stock and working solutions were stored at –20°C. Plasma calibration standards at 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 μg/ml were prepared by 1:50 dilution of the respective working solution in control human plasma. Of note, the total added volume of organic solvent in all samples corresponded to 2% of biological sample in accordance to FDA guidelines [9] recommending that non-biological matrix (i.e., organic solvent) should correspond to only ≤ 2% of the volume of final biological samples. QC samples at the lower limit of quantification (LLOQ = 0.05 μg/ml), low (0.2 μg/ml), medium (1 μg/ml), high (5 μg/ml) and the upper limit of quantification (ULOQ = 20 μg/ml) concentration levels were prepared by diluting the working solutions in plasma. The QC samples were prepared in batches at the same occasion, stored at –20°C, and then thawed and thermized at 60°C for 60 min on the day of analysis. Because of potential degradation of SMV when exposed to daylight [8], all solutions and samples containing SMV were protected from daylight during preparation, storage, and analysis.

2.4 Sample pretreatment and preparation

Blood samples of patients (5 ml) were collected in tubes with K_3EDTA as anticoagulant and transported to the laboratory on ice. Plasma was immediately isolated by centrifugation at 3,000 rpm for 10 min at 4°C (Sigma Centrifuge, Model 2K15) and stored at –80°C until analysis. On the day of analysis, plasma samples were thawed and heated at 60°C for 60 min. The analysis of stability of SMV under these conditions is reported in the Section 3.3. Sample clean-up was achieved by a solid-phase extraction (SPE) procedure as follows. Aliquotes (0.5 ml) of heat-inactivated plasma samples were mixed with an equal volume of acetonitrile, vortexed for 1 min, and then centrifuged at 13,000 rpm for 5 min. Supernatants were transferred in clean tubes and diluted 1:1 with 5% NH_4OH. The samples were then loaded onto Oasis HLB 1cc cartridges (Waters, Milford, MA, USA). The cartridges were successively washed with 1 ml of 70% methanol (v/v). The analytes were eluted with 1 ml of methanol. The eluates were evaporated to dryness under a stream of nitrogen at 40°C. The dried extracts were reconstituted with 125 µl of mobile phase. The reconstituted samples were centrifuged at 13,000 rpm for 5 min at room temperature and 20 µl of the supernatant were injected onto the HPLC system. Blood samples were protected from daylight during sampling, storage and shipment, and sample preparation was performed under yellow light conditions.

2.5 Recovery

The overall recovery of SMV from human plasma was determined at the LLOQ, low-, medium-, high-QC, and ULOQ level by comparing the peak area response of extracted plasma samples (four samples for each concentration level) with that obtained by direct injection of the same amount of drug diluted in mobile phase (four samples for each concentration level). The extraction recovery was calculated using the ratio of the response and the concentration factor of the assay (500:125)

and was expressed as a percentage of the response of the calculated amount of SMV diluted in mobile phase and directly injected onto the HPLC, which corresponds to 100% recovery.

2.6 Analytical method validation

The validation of the assay was based on the FDA guidelines for Bioanalytical Method Validation [9]. Assay validation involved linearity, specificity, accuracy, precision, limit of detection (LOD), and lower limit of quantification (LLOQ) determination. Intra-day and inter-day precision values were estimated by assaying plasma samples spiked with five different concentrations of SMV five times on the same day and on four separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of deviation between nominal and measured concentration (% bias). Analytic interferences from endogenous substances were investigated by testing ten different lots of blank human plasma. The method specificity was also investigated by analyzing both plasma spiked with potentially co-administered drugs and patient samples.

2.7. Stability studies

Since photochemical degradation of SMV in plasma under daylight conditions was previously reported [8], all stability experiments were performed under yellow light conditions. The stability of SMV in plasma under the thermization process (60°C for 60 min) was assessed as follows: four series of calibration samples at the nine concentrations reported above (0.05–20.0 μg/ml) and of samples at the LLOQ, low-, medium-, high-QC, and ULOQ level were prepared. Two series were heated at 60°C for 60 min, while the thermization procedure was omitted in the other two. The four series were then subjected simultaneously to SPE and analyzed. The slope of the calibrations curves was compared as well as the SMV levels in QC samples. Further studies of the stability of SMV in plasma included: (a) after three freeze-thaw cycles; (b) storage at room temperature (RT) for 96 h; and (c) storage at –80°C for 3 months. For each tested condition, six series of LLOQ, low-, medium-, high-QC, and ULOQ plasma samples were prepared. Three series were

immediately analyzed, while the three remaining series were subjected to the storage or treatment conditions under examination. The SMV levels in samples were compared. Additionally, the stabilities of dried extracts and of extracts reconstituted in mobile phase were analyzed: dried extracts (i.e., after SPE) containing SMV at LLOQ, low-, medium-, high-QC, and ULOQ concentration were analyzed in triplicate either immediately after preparation, or after being stored at –20°C for 120 h. Processed samples (i.e., reconstituted in mobile phase) were analyzed in triplicate immediately after preparation and after being left for 96 h in the autosampler thermostated at 20°C. The results were compared.

3. Results and discussion

3.1 Method development

To establish an efficient method for SMV extraction from plasma, several purification methods were compared. Initially, a liquid–liquid extraction method employing acetonitrile was attempted for sample preparation, which was based on a previously published study [8]. However, the chromatography resulting from this method showed significant interference between background plasma peaks and the SMV peak (data not shown). Liquid-liquid extraction methods with different solvents (i.e., methanol, ethylacetate, ether, chloroform, dichloroethane, and dichloromethane) were then tested, but they gave in general low recovery and/or low clean-up efficiency (data not shown). Therefore, solid phase extraction (SPE) was attempted. Among the SPE cartridges that we tested (Oasis HLB, Oasis MAX, Oasis WAX, and Oasis MCX cartridges from Waters; Bond-Elut phenyl boronic acid, Bond-Elut C18 cartridges from Varian; Si-Sax cartridges from Agilent), the Waters Oasis HLB cartridges proved to be the most adequate in terms of extraction efficiency and reliability. However, when SPE was performed with the standard procedure recommended by the producer, a very low recovery (around 20-30%) was observed. No significant increase in recovery was obtained by using the 3cc or 6 cc cartridges instead of the 1cc cartridges. Thus, we worked to improve the SPE procedure by introducing several modifications.

First, since SMV is known to be extensively bound to plasma proteins (> 99.9%) [5], that could explain the low recovery, the SPE was preceded by a protein precipitation step with acetonitrile and subsequent basification by diluting the sample 1:1 with 5% NH_4OH (while no improvement was observed by acidifying the sample with 4% H_3PO_4). Second, the condition and equilibration steps of the Oasis HLB cartridges were omitted, since they were found to affect the final recovery; in addition, the elimination of these steps allowed shortening and simplifying the overall extraction procedure. Third, a washing step with 70% methanol instead of the 5% concentration suggested by the producer, greatly improved the sample clean-up giving a cleaner baseline at the retention time of SMV without decreasing the drug recovery. Fourth, elution with 1 ml of methanol was found to be crucial since lower elution volumes significantly decreased SMV recovery; elution with acidified methanol did not further increase the final recovery. All these modifications, when combined, allowed to obtain a good sample clean-up along with a high and constant recovery of SMV (see Fig. 1 and Section 3.2).

Different HPLC columns were tested for their ability to separate SMV from background peaks. The columns that were tested included a Waters Atlantis dC18 (150 mm × 3.9 mm, 5 μm) column, a Simmetry C18 (75 mm × 4.6 mm, 3.5 μm), and finally an XTerra RP18 (150 mm × 4.6 mm, 3.5 μm) column, which provided the best separation efficiency. For the mobile phase, different acetonitrile/phosphate buffer ratios and different phosphate buffer concentrations were tested. The mobile phase that gave optimal separation from plasma endogenous peaks was a 70:30 mixture of acetonitrile and phosphate buffer (v/v). The optimum pH of the mobile phase buffer was determined by changing pH every 0.5 from pH 2 to pH 7. Finally, pH 6.0 phosphate buffer (52.5 mM) for the mobile phase appeared to be optimal to separate SMV. Isocratic elution was preferred, as gradient elution requires control by a gradient HPLC pump system, re-equilibration time, perfect solvent mixing, etc. Column temperature, flow rate, and detection wavelength (i.e., 225 nm) were all optimized to give the final instrumentation conditions described in Section 2.2. The autosampler was thermostated at 20°C, as sample precipitation was observed at lower

temperatures. The stability of SMV under these conditions was investigated (see Section 3.3). Representative chromatograms of a control human plasma sample and spiked samples at the LLOQ and ULOQ level are shown in Fig. 1.

3.2 Method validation: linearity, recovery, sensitivity, accuracy and precision, selectivity and specificity

A 9-point calibration standard curve of SMV in plasma, ranging from 0.05 to 20 μg/ml, was prepared in triplicate in four independent runs. The calibration curves were linear over the tested concentration range, with correlation coefficients of 0.997 - 0.999.

The mean recovery obtained for SMV during the extraction from plasma ranged from 85.9 to 90.3% in the tested concentration range (0.05-20 μg/ml), with the CV ranging from 1.3 to 5.7%. These results indicate that the extraction method developed here achieves a high degree of efficiency and reproducibility.

The LLOQ, defined as the lowest concentration in the standard curve that back-calculates with good accuracy and precision (bias from -8 to 6%, CV = ≤8.3%, n = 20; see Table 1), was 0.050 μg/ml. A typical chromatogram of an LLOQ sample is shown in Fig. 1B. This LLOQ is well below the C_{trough} values observed in patients (0.96 μg/ml) [10] and thus provides sufficient sensitivity for routine analysis of human plasma samples in the clinical setting. The LOD, defined as the concentration giving a signal-to-noise ratio of 3, was 0.02 μg/ml.

The analytical accuracy and precision were evaluated by assaying LLOQ, ULOQ and QC samples in five replicates on each of four different days. The accuracy (expressed as % bias) and precision (expressed as % CV) data are summarized in Table 1. The intra-day and inter-day deviations (% bias) from the nominal concentrations were always ≤8.0% and ≤6.0%, respectively. The CVs for intra-day and inter-day data ranged from 1.4 to 7.2% and from 1.0 to 8.3%, respectively. Thus, both precision and accuracy were <15%, according to guidelines [9].

Furthermore, these values are similar to the values previously reported for the LC-MS/MS method measuring SMV concentration in plasma [8]. These results indicate that the method we developed achieves a high degree of reproducibility and accuracy.

Specificity based on endogenous interfering peaks was evaluated in ten different lots of commercial control human plasma. No significant interfering peaks from human plasma were found at the retention time of SMV (see Fig. 1A). The anti-HCV drugs ribavirin, interferon, α, daclatasvir, sofosbuvir, and telaprevir did not interfere with the analytical method. Other drugs which were tested included: abacavir, acyclovir, ampicillin, amprenavir, atazanavir, carbenicillin, chloramphenicol, daptomycin, didanosine, efavirenz, erythromycin, fluconazole, foscarnet, ganciclovir, gentamicin, imipenem, indinavir, kanamycin, lamivudine, linezolid, lopinavir, nevirapine, posaconazole, rifampicin, ritonavir, saquinavir, stavudine, streptomycin, teicoplanin, telaprevir, tenofovir, tetracycline, vancomycin, voriconazole, zalcitabine, and zidovudine. None of these drugs was found to interfere with the assay. The absence of analytic interference in patient plasma samples was also confirmed by the use of the peak purity testing system and the library matching of the Empower software.

3.3 Stability data

Good stability of SMV in human plasma under some conditions (i.e., 72 h at room temperature, 1184 days at –20°C, 64 days at -70°C, and after 6 freeze-thaw cycles) has been previously shown [8]. However, to the best of our knowledge, no detailed investigation has been previously conducted on SMV stability during the thermization procedure (56-60°C for 30-60 min) that is recommended to heat-inactivate HCV and/or HIV particles in biological samples for safety issues [11, 12]. Our paper reports the first detailed study on SMV stability upon such a heat treatment. The slope of the calibration curves of SMV determined with samples subjected to the thermization procedure (60°C for 60 min) was similar (mean variation of -6.1 ± 0.3%) to that of the calibration curves obtained with non-heated samples. In addition, Table 2 shows the back-calculated values

of both thermized and non-thermized samples using calibration curves established with samples subjected or not to the same treatment. Considering the experimental variability (Table 1), these results indicated that such a procedure does not affect SMV concentrations within the considered concentration range.

We also investigated the SMV stability in human plasma under conditions that clinical samples commonly experience. Moreover, we tested the stability of SMV both in dried and in reconstituted extracts. Our results (Table 3) confirmed the good stability of SMV in plasma previously reported [11] after repeated freeze/thaw cycles and under short-term storage (3 days) at room temperature and long-term storage (3 months) at -80°C. In addition, SMV was stable both in dried extracts kept at -20°C for 5 days and in extracts reconstituted in mobile phase and kept at 20°C for 4 days (Table 3).

3.4 Analysis of patient samples

This method has been applied to measure SMV plasma concentrations in samples received by our diagnostic Unit from HCV-infected patients and has proved to be robust and sensitive enough for routine therapeutic drug monitoring of SMV plasmatic levels. Fig. 2 shows two representative chromatograms of plasma samples from HCV-positive patients receiving SMV. In these samples, observed SMV plasma concentrations were 3.56 µg/ml and 2.59 µg/ml, respectively, which are within the concentration range reported by others [10].

4. Conclusions

A simple HPLC assay with UV detection for the measurement of SMV concentrations in human plasma was established and validated according to the FDA recommendations [9], and was shown to be specific, sensitive, and accurate over a concentration range of 0.05-20 µg/ml. Analysis of the stability of SMV under various conditions showed that the drug concentration remains stable in plasma stored at room temperature for 3 days or at -80°C for up to 3 months as well as in samples

undergoing three freeze-thaw cycles and thermal virus inactivation for 60 min at 60°C. The drug is also stable in processed samples, both in dried extracts kept at –20°C for 5 days and in reconstituted samples for at least 4 days at 20°C. The applicability of the method and the appropriateness of the validated concentrations ranges have been demonstrated in the analysis of plasma samples of HCV-infected subjects. This simple HPLC method can be conveniently used for routine therapeutic drug monitoring of SMV in conventional hospital laboratories wherein LC–MS/MS is not available.

Conflict of interest

The authors disclose no conflicts.

Acknowledgements

This work was funded by Azienda Ospedaliera of Padua, by the University of Padua (Progetto di Ricerca di Ateneo 2014 grant n. CPDA141311 and MURST EX60%) to A.L., and by the Italian Ministry for Universities and Scientific Research (PRIN 2010-11, grant n. 2010Z9FLM8) to G.P.

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Figure Legends

Fig. 1. Representative chromatograms obtained after extraction of (A) spiked plasma sample containing SMV 20 μg/ml (ULOQ), (B) spiked plasma sample containing SMV 0.05 μg/ml (LLOQ), and (C) blank plasma sample.

Fig. 2. Chromatograms of plasma samples from HCV-infected patients treated with SMV in combination with (A) sofosbuvir and ribavirin; or (B) sofosbuvir. The measured concentration of SMV was 3.56 μg/ml in sample (A) and 2.59 μg/ml in sample (B).

Table 1

Intra-day and inter-day accuracy and precision results.

Nominal concentration (μg/ml)	Intra-day (*n* = 5)			Inter-day (*n* = 4)		
	Mean measured concentration (μg/ml)	Accuracy[a] (% bias)	Precision (% CV)	Mean measured concentration (μg/ml)	Accuracy (% bias)	Precision (% CV)
0.05 (LLOQ)	0.046	-8.0	7.2	0.053	6.0	8.3
0.2 (low QC)	0.191	-4.5	3.9	0.211	5.5	2.2
1.0 (medium QC)	1.012	1.2	2.1	0.967	-3.3	3.1
5.0 (high QC)	4.989	-0.2	6.1	5.202	4.0	1.0
20.0 (ULOQ)	19.397	-3.0	1.4	20.730	3.7	3.2

[a]Accuracy = [(measured concentration – nominal concentration)/nominal concentration] x 100

Table 2

Back-calculated values of both thermized (T) and non-thermized (NT) plasma samples spiked with SMV using calibration curves established with samples subjected or not to heat treatment (60°C for 60 min)

Nominal concentration (µg/ml)	T *vs* T (µg/ml)	Accuracy (%)	T *vs* NT (µg/ml)	Accuracy (%)	NT *vs* T (µg/ml)	Accuracy (%)
0.05 (LLOQ)	0.053	6.0	0.046	-8.0	0.047	-6.0
0.2 (low QC)	0.196	-2.0	0.213	6.5	0.193	-3.5
1.0 (medium QC)	1.039	3.9	0.976	-2.4	1.055	5.5
5.0 (high QC)	4.988	-0.2	4.877	-2.5	5.132	2.6
20.0 (ULOQ)	20.765	3.8	19.886	-0.6	20.893	4.5

T *vs*. T: mean concentration of thermized LLOQ, QC, and ULOQ samples back-calculated with the calibration curves established with thermized samples; T *vs*. NT: mean concentration of thermized LLOQ, QC, and ULOQ samples back-calculated with the calibration curves established with non-thermized samples; NT *vs.* T: mean concentration of non-thermized LLOQ, QC, and ULOQ samples back-calculated with the calibration curves established with thermized samples.

Table 3

Stability of SMV in plasma samples and in dried or reconstituted extracts under different treatment/storage conditions.

	Nominal concentration (μg/ml)				
	0.05	**0.2**	**1.0**	**5.0**	**20.0**
(A) In plasma subjected to three freeze/thaw cycles					
Mean measured conc. at *t=0* (μg/ml)	0.049	0.193	1.111	4.865	19.665
Mean recovered conc. (μg/ml)	0.053	0.214	1.053	4.999	20.002
Deviation (%)	8.2	10.9	-5.22	2.8	1.7
CV (%)	7.5	5.9	3.3	0.3	1.2
(B) In plasma stored at room temperature for 72 h					
Mean measured conc. at *t=0* (μg/ml)	0.051	0.202	0.976	5.133	20.743
Mean recovered conc. (μg/ml)	0.046	0.218	0.988	5.288	19.968
Deviation (%)	-9.8	7.9	1.2	3.0	-3.7
CV (%)	7.1	6.4	0.9	1.6	1.9
(**C) In plasma stored at – 80°C for three months**					
Mean measured conc. at *t=0* (μg/ml)	0.049	0.189	1.122	5.088	20.421
Mean recovered conc. (μg/ml)	0.045	0.198	1.133	4.966	20.988
Deviation (%)	-8.2	4.8	1.0	- 2.4	2.8
CV (%)	6.5	3.2	1.9	2.8	3.1
(D) In dried extracts stored at – 20°C for 120 h					
Mean measured conc. at *t=0* (μg/ml)	0.058	0.218	0.954	5.265	20.955
Mean recovered conc. (μg/ml)	0.054	0.205	1.003	5.304	19.742
Deviation (%)	-6.9	-6.0	5.1	0.7	-5.8
CV (%)	5.7	0.1	3.8	4.0	2.9
(E) In reconstituted extracts stored at 20°C for 96 h					

Mean measured conc. at *t=0* (μg/ml)	0.049	0.209	0.977	4.966	19.876
Mean recovered conc. (μg/ml)	0.053	0.198	1.044	5.087	20.566
Deviation (%)	8.2	-5.3	6.9	2.4	3.5
CV (%)	6.7	7.8	2.0	3.8	1.6