ELSEVIER

Journal of Chromatography B, 742 (2000) 185–192

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Sensitive liquid chromatographic assay for amprenavir, a human immunodeficiency virus protease inhibitor, in human plasma, cerebrospinal fluid and semen

Rolf W. Sparidans[a,b,*], Richard M.W. Hoetelmans[b], Jos H. Beijnen[a,b]

[a]*Utrecht University, Faculty of Pharmacy, Division of Drug Toxicology, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[b]*Slotervaart Hospital/The Netherlands Cancer Institute, Department of Pharmacy and Pharmacology, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

Received 28 December 1999; received in revised form 28 February 2000; accepted 28 February 2000

Abstract

A sensitive bio-analytical assay for amprenavir, a human immunodeficiency virus protease inhibitor, based on reversed-phase liquid chromatography and fluorescence detection, is reported. The analyte is extracted from the matrix, plasma, cerebrospinal fluid (CSF) or semen, with chloroform using propyl-*p*-hydroxybenzoate as an internal standard. After centrifugation, evaporation of the organic phase and reconstitution in the eluent, the sample is injected into the chromatograph. The analyte is detected spectrofluorometrically at 270 and 340 nm for excitation and emission, respectively. The method has been validated in the 1–1000 ng/ml range for a 50-μl volume of plasma and in the 0.5–50 ng/ml range for a 100-μl volume of CSF and semen. The lower limit of quantification was 0.5 ng/ml in CSF and 1 ng/ml in both plasma and semen. Precision and accuracy both meet the current requirements for a bio-analytical assay and are <15% in the validated ranges. The assay was successfully used to obtain a concentration–time curve of amprenavir in plasma.

Keywords: Amprenavir; Protease inhibitor

1. Introduction

Amprenavir (141W94, VX-478, Agenerase, Fig. 1) is the fifth approved protease inhibitor, after indinavir, nelfinavir, ritonavir and saquinavir, for the treatment of human immunodeficiency virus (HIV) infected patients [1]. The pharmacokinetics and safety of this drug in HIV infected adults have recently been reported [2]. The drug was well tolerated up to 1200-mg oral doses, showed good absorption, a maximum concentration in plasma 1–2

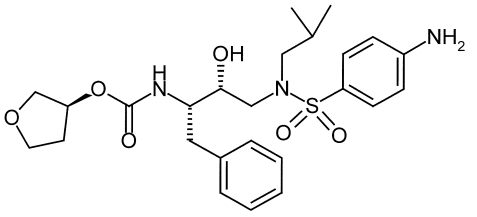

Fig. 1. Chemical structure of amprenavir.

*Corresponding author. Utrecht University, Faculty of Pharmacy, Division of Drug Toxicology, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands. Tel.: +31-30-2536-919; fax: +31-30-2535-180.

E-mail address: r.w.sparidans@pharm.uu.nl (R.W. Sparidans)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00163-8

h after administration and a long terminal half-life (8 h).

For an efficient long-term suppression of replication of the virus a sufficient amount of the anti-HIV agent has to penetrate into sanctuary sites of the human body [3]. The drug levels behind the classical sanctuary barriers like the blood–brain and blood–testes barriers can be significantly lower compared to the concentrations in plasma [4]. For example, for indinavir 210 n*M* was reported as the median concentration in cerebrospinal fluid (CSF) [5] while nelfinavir could not be detected in that matrix [lower limit of quantification (LLQ)=25 ng/ml] [6]. Because amprenavir and other HIV protease inhibitors are probably P-glycoprotein substrates, co-administration of a P-glycoprotein inhibitor may enhance the penetration of these drugs into sanctuary sites [7].

Bio-analytical assays for the five anti-HIV protease inhibitors, reported the past 3 years, are almost exclusively developed and validated for serum or plasma samples using reversed-phase liquid chromatography (RPLC) with ultraviolet detection after a pre-treatment comprising protein precipitation [8,9], liquid–liquid extraction [10–15] or solid-phase extraction [16–21]. Further, the LLQs of these assays are often in the 5–50 ng/ml range and are therefore possibly not always sensitive enough for an assay to be used for CSF or semen samples.

Chromatographic methods with ultraviolet detection achieving a LLQ in the low ng/ml range for CSF samples have been reported for indinavir (2 ng/ml) [16] using a column switching technique and solid-phase extraction on a strong cation-exchange phase and for saquinavir (2.5 ng/ml) [21] using solid-phase extraction on C_2 columns. In addition, using high-performance liquid chromatography (HPLC) with tandem mass spectrometric detection, an LLQ of 1 ng/ml could be achieved for indinavir in human plasma [22].

The first bio-analytical assay applicable for the quantification of all five approved protease inhibitors was recently developed at one of our research facilities [19] and was also the first assay reported for amprenavir. This assay, based on solid-phase extraction as a sample pre-treatment and RPLC with ultraviolet detection facilitated routine monitoring of the concentrations of these protease inhibitors in plasma in their therapeutic ranges (0.1–10 μg/ml).

Because an assay for amprenavir in CSF and semen was not yet available we wanted to develop a simple bio-analytical assay for this drug in these matrices with a LLQ in the low ng/ml range. The fluorescent properties of this aromatic sulfonamide and an efficient sample pre-treatment procedure may facilitate this goal using RPLC with fluorescence detection.

2. Experimental

2.1. Chemicals

Amprenavir was kindly provided by Vertex Pharmaceuticals (Cambridge, MA, USA). Propyl-*p*-hydroxybenzoate (POB) and the pharmaceutical agents, used for testing analytical interference, were obtained as pharmaceutical grade reference material or as a solution for injection. Acetonitrile (gradient grade) and methanol (absolute for HPLC) were provided by Biosolve (Valkenswaard, The Netherlands) and phosphoric acid (85%, w/w, in water) by Baker (Deventer, The Netherlands). Sodium hydroxide (analytical grade) and chloroform (analytical-reagent grade) were supplied by Merck (Darmstadt, Germany). Water was purified in the laboratory by reversed osmosis on a multi-laboratory scale. Blank, drug-free human plasma was obtained from the Bloedbank Midden Nederland (Utrecht, The Netherlands); blank CSF and semen were collected and originated from patients at the Slotervaart Hospital (Amsterdam, The Netherlands). Semen was always centrifuged at $1.3 \cdot 10^3$ *g* for 5 min before use.

2.2. Equipment

Chromatographic analyses were performed using the following equipment: a P580 isocratic pump (Gynkotek HPLC, Germering, Germany), a Basic+ Marathon autosampler (Spark Holland, Emmen, The Netherlands), equipped with a 7739-005 injection valve (Rheodyne, Cotati, CA, USA) with a 100-μl sample loop, and an FP-920 fluorescence detector (Jasco, Hachioji, Japan). A Must multiport streamswitch (Spark Holland) was used as a programmable solvent selection valve and the column was thermostated in a water bath with the temperature being

controlled by a Thermomix 1420 heating device (B. Braun, Melsungen, Germany). Data were recorded on a Jotronics Pentium 166-32 Mb personal computer (Delfgauw, The Netherlands), equipped with a Chromelion chromatographic data system (Gynkotek HPLC).

2.3. Chromatographic conditions

Partial-loop injections (50 μl) were made on a Symmetry C_{18} column (100×4.6 mm, d_p=3.5 μm, average pore diameter=10 nm, Waters Chromatography, Milford, MA, USA) with a Symmetry C_{18} pre-column (20×3.8 mm, d_p=5 μm, Waters). The column temperature was maintained at 50±2°C. The eluent comprised a mixture of acetonitrile–25 m*M* sodium phosphate buffer (pH 6.8) (40:60, v/v); the eluent flow-rate was 1.5 ml/min. The fluorescence detection wavelengths were 270 nm for excitation and 340 nm for emission, using a 40-nm slit width for emission. Five minutes after each injection the column was flushed for 5 min at 1.5 ml/min with an acetonitrile–water (30:70, v/v) mixture; after equilibrating for ca. 8.5 min with the original eluent and flow, the next sample was injected.

2.4. Analytical procedure

A 50-μl plasma sample was transferred to a conical (non-silylated) glass tube and 100 μl water was added; alternatively, a 100-μl CSF or semen sample was used and supplemented with 50 μl of pooled drug-free plasma. Next, 50 μl of 1 μg/ml POB in water was added and the sample was mixed briefly using vortex-mixing. After adding 1 ml of chloroform, the tube was closed and shaken vigorously by vortex-mixing for ca. 20 s, followed by centrifugation at $4.3 \cdot 10^3$ *g* for 5 min. Next, the organic phase was transferred to a clean tube that was than evaporated to dryness by a nitrogen flow at 40°C. The residue was reconstituted in 150 μl of the eluent by vortex-mixing for ca. 20 s and transferred to an injection vial with a 250-μl glass insert.

2.5. Validation

Stock solutions of 457 and 280 μg/ml amprenavir in methanol were prepared (with separate weighing) and were stored at −20°C. For calibration a 4.57 μg/ml dilution in plasma, pooled from four individuals, was made from the first stock solution and stored at −20°C. Dilutions of this standard of amprenavir in plasma yielding 1, 4, 10, 41, 102, 406 and 1016 ng/ml amprenavir calibration samples in plasma, were made daily and analysed in duplicate for each analytical run. Least-squares linear regression, weighted by $x^{-1.5}$ (reciprocal of the concentration×1.5) was employed for the calibration, using the peak height ratio of amprenavir and the internal standard (I.S.) POB. The power of the weighing factor was determined by maximising the log-likelihood function and the linearity of the calibration was tested using a lack-of-fit test; both statistical procedures were performed using the SPSS 7.5 software (SPSS, Chicago, IL, USA).

From a 5.40 μg/ml dilution in plasma, originating from the second stock solution, validation (quality control) samples were prepared at 1, 10, 103, 1026 ng/ml, respectively in plasma and in the 0.5–51 ng/ml range for both CSF and semen; all samples were stored at −20°C. Plasma of four different individual donors and pooled CSF and semen were used. Precisions and accuracies were determined by six-fold analysis of each validation sample in three different analytical runs for all plasma concentrations, in one or three runs for CSF and in one run for semen samples. The repeatability (intra-day precision) is calculated according to

$$\text{Repeatability} = \frac{\sqrt{\text{ErrMS}}}{\text{GM}} \times 100\%$$

(ErrMS=error mean square, GM=grand mean) and the reproducibility (inter-day precision) according to

$$\text{Reproducibility} = \frac{\sqrt{(\text{DayMS} - \text{ErrMS})/n}}{\text{GM}} \times 100\%$$

(DayMS=day mean square, n=number of replicates in each run) for each individual concentration.

Six individual blank plasma samples from different individuals and blank CSF and semen samples were processed to test the selectivity of the assay. The selectivity of the assay was also tested by investigating the influence of several pharmaceutical compounds, including a few of their metabolites, potentially used by patients treated with amprenavir.

Reference solutions of 0.5 or 1 mg/ml of 3′-amino-3′-deoxythymidine, delavirdine, didanosine, efavirenz, fluconazole, folic acid, ganciclovir, indinavir, itraconazole, lamivudine, methadone, nelfinavir, nevirapine, oxazepam, pyrimethamine, pyrazinamide, ranitidine, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, trimethoprim, zalcitabine, zidovudine and zidovudine-glucuronide were diluted 1:50 (v/v) with eluent and injected into the chromatograph. Next, drugs interfering at the retention time of amprenavir were all diluted to 10 μg/ml in plasma and subjected, in duplicate, to the sample treatment procedure prior to injection.

The validation samples of 10 and 102 ng/ml amprenavir in plasma, 10 ng/ml in CSF and 10 and 51 ng/ml in semen were also used for stability studies; these samples were stored at 60°C for 1 h, at 25°C for 24 h, at 2°C for 7 days or at −20°C for 37 or 43 days. In addition, plasma samples were taken out the freezer to adapt at ambient temperature shortly to test the effects of extra freeze–thaw cycles ($n=3$).

For the determination of the extraction yield, calibration samples of amprenavir in methanol were made from the same stock as the calibration samples in plasma in the 1–1000 ng/ml range and added to blank chloroform extracts. The yield was calculated from this calibration and the original one in two separate analytical runs by dividing the slopes of the calibration lines.

The stability of amprenavir samples stored in the autosampler was tested by preparing a duplicate series of calibration samples as usual, however, the second series was injected during the next analytical run, approximately 24 h after the first series. The recovery was determined twice by dividing the slopes of the different calibration lines.

A 38-year-old HIV infected male was treated orally with a daily dose of 1200 mg amprenavir twice daily (bid) plus zidovudine (300 mg bid), lamivudine (150 mg bid), and delavirdine (600 mg bid). Blood samples were taken in heparinized tubes after ingestion of one dose and the plasma was separated by centrifugation prior to storage at −20°C. Prior to analysis, 100 μl of the sample was diluted to 1 ml with drug-free pooled plasma.

3. Results and discussion

In order to obtain a more sensitive bio-analytical assay for amprenavir than the existing one (LLQ= 25 ng/ml) in plasma [19] with a LLQ of ca. 1 ng/ml and applicable to other matrices like CSF and semen several adaptations were required. (1) Another detection method was required to improve the sensitivity; (2) a sample pre-treatment procedure applicable to all matrices had to be used; (3) a calibration valid for all different matrices was preferred; (4) for CSF and semen only a small sample volume could be used.

Different additional compounds in acetonitrile–water mixtures as eluents, for example an ion-pairing agent (heptanesulfonic acid) or a change of the pH (2.2–6.8), showed no influence on the retention of both, amprenavir and POB. When the fluorescent properties of amprenavir were fully exploited, 0.1 ng/ml was achieved as the limit of detection in an aqueous solution ($S/N=3$); which should be sufficient for a LLQ in the low ng/ml range.

The development of the sample pre-treatment procedure was started by investigating protein precipitation of plasma and centrifugation of semen prior to injection and the direct injection of CSF. Unfortunately, the endogenous interferences urged further sample pre-treatment, and therefore a liquid–liquid extraction procedure was tested. Chloroform and ethyl acetate were investigated and compared as extraction solvents, both solvents extracted amprenavir completely (>95%), however, chloroform was preferred for further use because less endogenous peaks were observed in the chromatograms after extractions with this solvent. In order to facilitate the use of calibration samples in plasma for the measurement of CSF and semen samples, plasma was added to these matrices prior to further treatment. Initially, the low sample volumes led us to use polypropylene micro tubes during the sample treatment, however, a significant loss of the analyte (not the I.S.) and poor precision was observed compared to the use of glass tubes.

Examples of chromatograms at different concentrations are shown in Fig. 2. The column was automatically flushed with a strong eluent after each analysis in order to remove late eluting endogenous

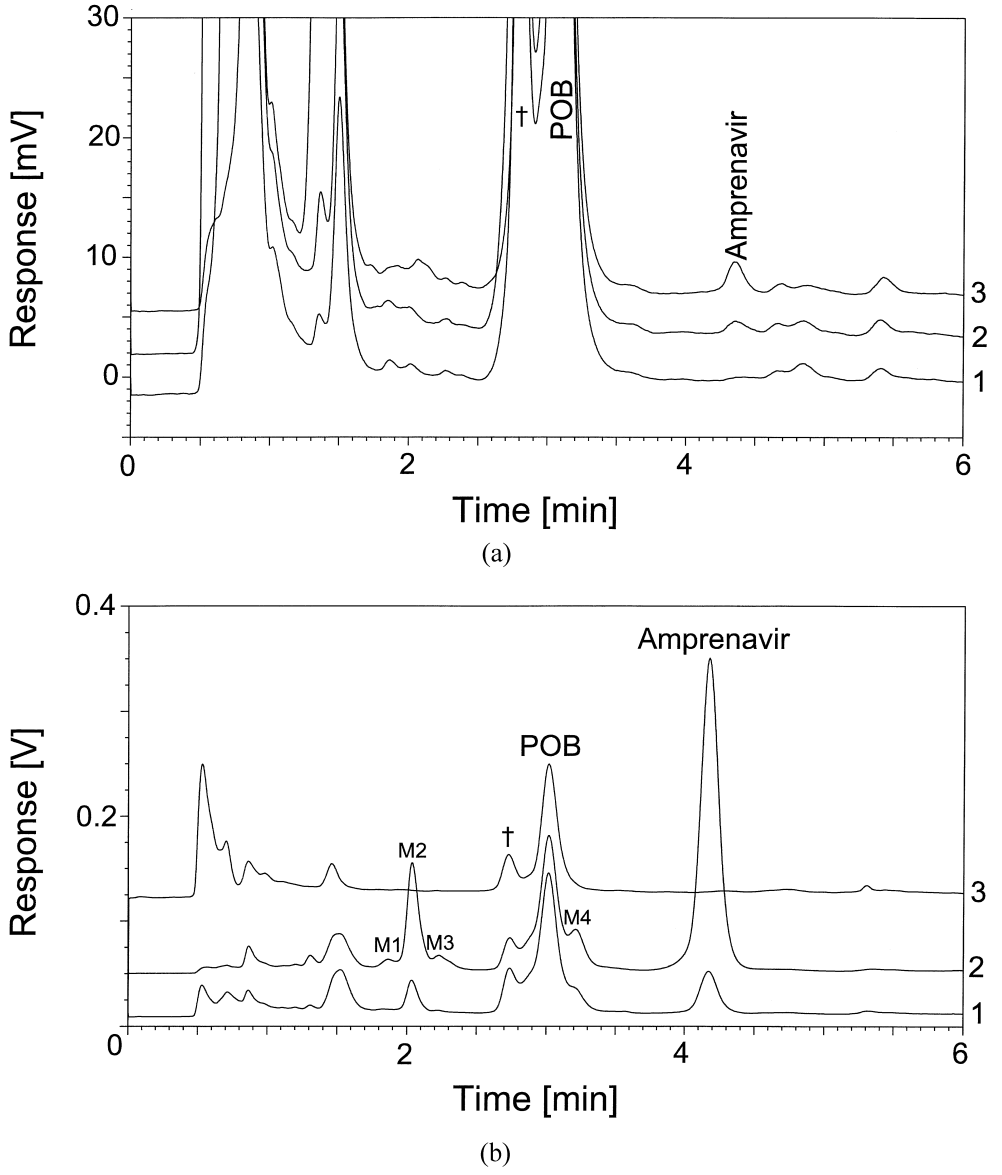

Fig. 2. Chromatograms of amprenavir. (a) Blank drug-free plasma (1); 0.5 ng/ml amprenavir spiked to CSF (2); 2 ng/ml amprenavir spiked to semen (3). (b) 0.52 μg/ml amprenavir in a patient sample after 10-fold dilution (1); 4.1 μg/ml amprenavir in a patient sample after 10-fold dilution (2); blank drug-free plasma (3). (M1–M4) Possible metabolites; (†) contaminant.

compounds from the column. This washing step showed to be efficient in removal of these compounds and the extra time-consumption was no problem during analysis overnight. In an analytical run, calibration samples in the 1–1000 ng/ml range were used for quantification of the validation samples. No significant lack-of-fit ($P>0.95$) was observed in six of the first seven calibration lines using peak heights of analyte and I.S. Peak heights were preferred for quantification because this resulted in less variation of the response at the level of the LLQ than when peak areas were used.

Table 1
Overall results of amprenavir analysis of the validation samples in different matrices

c (ng/ml)	Repeatability (%)	Reproducibility (%)	Accuracy (%)	*n*
Plasma				
1	11	6	91	18
10	3	2	114	18
103	4	1	92	18
1026	4	1	106	18
CSF				
0.5	6	3	92	16
2	5	–[a]	96	6
10	9	3	95	18
51	4	–[a]	90	6
Semen				
0.5	12	–[a]	129	6
1	3	–[a]	105	6
2	8	–[a]	93	5
10	3	–[a]	101	6
51	2	–[a]	97	6

[a] Not measured inter-daily due to lack of sample matrix.

The results obtained with the validation samples (precision and accuracy at each level in the different analytical runs) are listed in Table 1. The lowest level, 1 ng/ml for plasma and 0.5 ng/ml for CSF samples, proved to be the LLQ. Only in semen at the lowest concentration of amprenavir (0.5 ng/ml) the demands for a bioanalytical assay (precision and the accuracy $\leq$20% for the LLQ and $\leq$15% at higher concentrations [23]) were not met. The LLQ of amprenavir in semen was therefore 1 ng/ml.

In six individual blank plasma samples, no interferences in the chromatograms were observed which could influence the quantification of amprenavir in the validated concentration range. Furthermore, three individual blank CSF samples, an individual semen sample, a pooled CSF sample and a pooled semen sample showed no contribution to the amprenavir response of the assay. From the potentially co-administered drugs tested six showed retention times in the 1–6 min range in the present analytical system: abacavir (1.0 min), delavirdine (4.1 min), efavirenz (5.4 min), indinavir (4.2 min), oxazepam (2.3 min) and pyrimethamine (2.4); the retention times of POB and amprenavir were 2.9 and 4.0 min, respectively. Thus, all tested drugs could be distinguished from amprenavir; however, two of them could not be totally separated from the analyte. Therefore, delavirdine (a non-nucleoside reverse transcriptase inhibitor) and indinavir (another protease inhibitor), the drugs potentially interfering with amprenavir in the present chromatographic system, were subjected to the whole assay including sample pretreatment at concentrations of 10 μg/ml samples in plasma. This resulted in a response of 22 and 11 ng/ml amprenavir, respectively, for both drugs at this concentration. Because the maximum concentrations to be expected for delavirdine and indinavir in plasma are 19 μg/ml delavirdine and 7 μg/ml indinavir [4], these drugs may interfere with amprenavir concentrations below 2 and 0.4 μg/ml, respectively, in this assay.

The stability of amprenavir in the biological samples was investigated in addition to the reported data at high concentrations in plasma samples by van Heeswijk et al. [19]. The results of storage of different concentrations of amprenavir at different temperatures in different matrices are given in Table 2. No significant loss of the analyte was observed for amprenavir in all three matrices under all conditions tested; three extra freeze–thaw cycles, however, seemed to slightly decrease the concentration of amprenavir in plasma.

The recovery of amprenavir after overnight storage in the autosampler at ambient temperature is

Table 2
Recovery of amprenavir ($n=4$) in biological samples after storage under different conditions

Temperature (°C)	Storage time	Recovery (%)				
		103 ng/ml Plasma	10 ng/ml Plasma	10 ng/ml CSF	10 ng/ml Semen	51 ng/ml Semen
60	1 h	103±6	108±6	93±11	–[b]	–[b]
25	24 h	97±5	109±7	93±6	102±1	–[b]
−2	7 days	95±8	105±2	95±4	–[b]	–[b]
−20[a]	7–14 days	84±6	102±3	89±8	–[b]	96±5
−20	37 days			105±5	99±6	–[b]
−20	43 days	97±3	105±9			

[a] The samples were also subjected to three additional freeze–thaw cycles.
[b] Not measured due to lack of sample matrix.

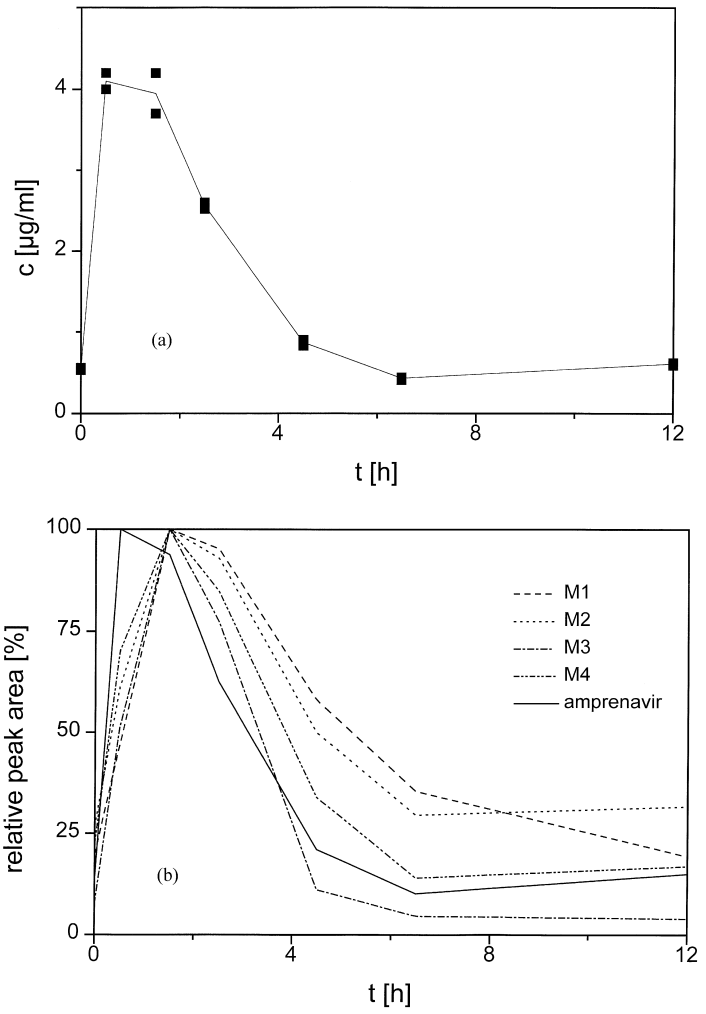

Fig. 3. (a) Concentration–time curve of amprenavir in plasma in a 38-year-old male; dose: 1200 mg bid. (b) Response of potential metabolites (Fig. 2B), relative to their maximum, in this patient compared to amprenavir.

109%. The calculated yield of the extraction of amprenavir is 92%.

The assay has been employed to obtain a concentration–time plot from a patient treated with amprenavir (Figs. 2B and 3a). Four additional peaks, showing a concentration–time profile analogous to amprenavir (Fig. 3b), were observed; these peaks may therefore be due to metabolites of amprenavir. Semen and CSF samples will be measured in clinical (pharmacokinetic) investigations in the near future.

4. Conclusions

A precise and sensitive assay for amprenavir in human plasma, CSF and semen has been developed and can be used in clinical pharmacokinetic investigations. The sensitivity of the assay is ca. 25-fold improved compared to the existing methodology. Only if delavirdine or indinavir is co-administered, the assay can not be used if their concentrations are at least 10–20-fold higher than those of amprenavir. Further, repetitive freeze–thaw cycles should be avoided for plasma samples.

References

[1] J.C. Adkins, D. Faulds, Drugs 55 (1998) 837.
[2] B.M. Sadler, C.D. Hanson, G.E. Chittick, W.T. Symonds, N.S. Roskell, Antimicrob. Agents Chemother. 43 (1999) 1686.
[3] R.M.W. Hoetelmans, Antiviral Ther. 3 (Suppl. 4) (1998) 13.
[4] R.M.W. Hoetelmans, AIDS Rev. 1 (1999) 167.
[5] C. Matin, A. Sonnerborg, J.O. Svensson, L. Stahle, AIDS 13 (1999) 1227.
[6] F. Aweeka, A. Jayewardene, S. Staprans, S.E. Bellibas, B. Kearney, P. Lizak, T. Novakovic-Agopian, R.W. Price, J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 20 (1999) 39.
[7] J.W. Polli, J.L. Jarrett, S.D. Studenberg, J.E. Humphreys, S.W. Dennis, K.R. Brouwer, J.L. Woolley, Pharm. Res. 16 (1999) 1206.
[8] R.M.W. Hoetelmans, M. van Essenberg, M. Profijt, P.L. Meenhorst, J.-W. Mulder, J.H. Beijnen, J. Chromatogr. B 23 (1998) 119.
[9] D.M. Burger, M. de Graaff, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 703 (1997) 235.
[10] P.W. Hugen, C.P. Verweij-van Wissen, D.M. Burger, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 727 (1999) 139.
[11] W. Li, R.W. Coombs, A.C. Collier, V.A. Raisys, Ther. Drug Monit. 21 (1999) 360.
[12] B.W. Granda, G.M. Giancarlo, L.L. von Moltke, D.J. Greenblatt, J. Pharmacol. Toxicol. Methods 40 (1998) 235.
[13] A.L. Jayewardene, F. Zhu, F.T. Aweeka, J.G. Gambertoglio, J. Chromatogr. B 707 (1998) 203.
[14] K.C. Marsh, E. Eiden, E. McDonald, J. Chromatogr. B 704 (1997) 307.
[15] H.R. Ha, F. Follath, Y. Bloemhard, S. Krahenbuhl, J. Chromatogr. B 694 (1997) 427.
[16] L. Zhong, K.C. Yeh, J. Chromatogr. B 734 (1999) 63.
[17] J.M. Poirier, P. Robidou, P. Jaillon, Ther. Drug Monit. 21 (1999) 404.
[18] M.L. Foisy, J.P. Sommadossi, J. Chromatogr. B 721 (1999) 237.
[19] R.P.G. van Heeswijk, R.M.W. Hoetelmans, R. Harms, P.L. Meenhorst, J.W. Mulder, J.M.A. Lange, J.H. Beijnen, J. Chromatogr. B 719 (1998) 159.
[20] S. Frappier, D. Breilh, E. Diarte, B. Ba, D. Ducint, J.L. Pellegrin, M.C. Saux, J. Chromatogr. B 714 (1998) 384.
[21] R.M.W. Hoetelmans, M. van Essenberg, P.L. Meenhorst, J.-W. Mulder, J.H. Beijnen, J. Chromatogr. B 698 (1997) 235.
[22] E.J. Woolf, B.K. Matuszewski, J. Pharm. Sci. 86 (1997) 193.
[23] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGliveray, J.P. Skelly, A. Jacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.