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Liquid chromatographic assay for simultaneous determination of abacavir and mycophenolic acid in human plasma using dual spectrophotometric detection

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Abstract

A combined bio-analytical assay for abacavir, a reversed transcriptase inhibitor, and mycophenolic acid (MPA), based on reversed-phase liquid chromatography and both ultraviolet (UV) absorption and fluorescence detection, is reported. Both analytes are extracted from plasma with acetonitrile. After centrifugation, evaporation of the supernatant and reconstitution in water, the sample is injected into the chromatograph. Abacavir is detected using UV detection at 285 nm and MPA spectrofluorometrically at 345 and 430 nm for excitation and emission, respectively. The method has been validated in the 80–2000 ng/ml range for abacavir and in the 10–10 000 ng/ml range for MPA for 200-μl plasma samples. The lower limits of quantification are 80 and 10 ng/ml for abacavir and MPA, respectively. Precisions and accuracies are ≤8% in the valid concentration ranges of both analytes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Abacavir; Mycophenolic acid

1. Introduction

Abacavir (1592U89, Fig. 1A) is a novel nucleoside reverse transcriptase inhibitor for the treatment of human immunodeficiency virus (HIV-1) infection. The drug is safe and well tolerated in both adults (single oral dose of 100–1200 mg) [1] and children (4 or 8 mg/kg) [2]. The activity of the drug is caused by the intracellularly formed carbovir triphosphate [3]. Abacavir is metabolized into the pharmacological inactive 5′-glucuronide and the 5′-carboxylate and shows significant penetration into cerebrospinal fluid [4].

Mycophenolate mofetil (MPM) is a morpholinoethyl ester pro-drug of mycophenolic acid (MPA, Fig. 1B). Presently, the ester is an approved drug for immunosuppressive therapy after solid organ transplantation. In vivo, the ester is rapidly hydrolysed into the active acid; the acid is mainly deactivated by glucuronidation into the 7-*O*-glucuro-

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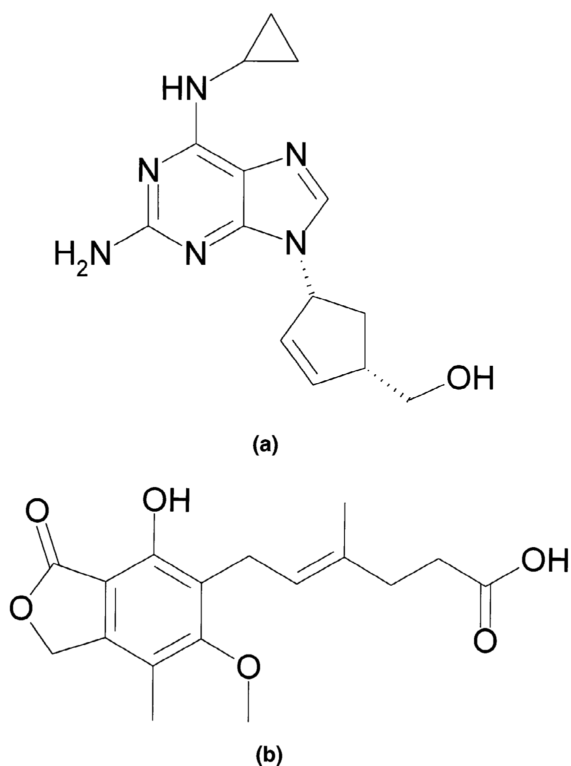

Fig. 1. Chemical structures of abacavir (A) and mycophenolic acid (B).

nide. In addition, the 7-*O*-glucoside, the acyl glucuronide and an unidentified metabolite of MPA have been observed [5,6].

Recently, abacavir and MPA showed a potent and synergistic effect on the inhibition of the replication of HIV-1 in stimulated peripheral blood mononuclear cells and in monocyte-derived macrophages at low MPA concentrations [7]. Therefore, a clinical study of the combined therapy of MPM and abacavir in both adults and children has been started for the exploration of this new therapeutical strategy in the treatment of HIV infection. For the pharmacokinetic evaluation of this study and further investigations into the combination of these drugs, the use of a combined assay for both, abacavir and MPA, is preferred.

We already reported a bio-analytical assay for abacavir previously [8]. The chromatographic method was simple and was valid in the 20–2000 ng/ml range. The method was based on protein precipitation as a sample pre-treatment, followed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection which resulted in a lower limit of quantification (LLQ) as recently also reported by others [1–3,9].

A large number of bio-analytical assays for mycophenolic acid have already been reported, mostly based on reversed-phase liquid chromatography with UV absorbance detection, further, a homogeneous enzyme-immunoassay is commercially available [10–13]. If the active acid has to be determined chromatographically but not the glucuronide, a simple sample pre-treatment can be used, for example protein precipitation [14–17], liquid–liquid extraction [18] or a combination of both [19].

The liquid chromatographic methods for mycophenolic acid reported differ mainly from the existing abacavir assays with respect to the extraction procedure, the eluent used for reversed-phase liquid chromatography and the UV detection wavelength. The development and validation of a combined assay for abacavir and MPA is now reported in this paper using liquid chromatography with dual spectrophotometric detection.

2. Experimental

2.1. Chemicals

Abacavir was kindly provided by Glaxo Wellcome Research and Development (Stevenage, UK). MPA and naproxen were obtained from Sigma (St. Louis, MO, USA). The pharmaceutical agents used for testing analytical interference were obtained as pharmaceutical-grade reference material or as a solution for injection. Acetonitrile (gradient grade) was provided by Biosolve (Valkenswaard, The Netherlands) and phosphoric acid (85%, w/w, in water) by Baker (Deventer, The Netherlands). Sodium hydroxide (analytical grade) was supplied by Merck (Darmstadt, Germany) and water was laboratory-purified by reversed osmosis on a multi-laboratory scale. Blank, drug-free human plasma was obtained from the Bloedbank Midden Nederland (Utrecht, The Netherlands).

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 240-μl sample loop, a Spectroflow 757 absorbance detector (Kratos Analytical, Westwood, NJ, USA), and an FP-920 fluorescence detector (Jasco, Hachioji, Japan). 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 (100 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 40±2°C. The eluent comprised a mixture of 15% (v/v) acetonitrile and 85% (v/v) of a 25 m*M* sodium phosphate buffer (pH 7.8); the eluent flow-rate was 1.5 ml/min. The UV detection wavelength was 285 nm and the fluorescence detection wavelengths were 345 nm for excitation and 430 nm for emission, using a 40-nm slit width for emission.

2.4. Analytical procedure

A 200-μl plasma sample was transferred to a polypropylene reaction tube and 20 μl of 40 μg/ml naproxen, the internal standard tested, in water was added. After gently vortex-mixing, 450 μl acetonitrile was added and then the closed tube was thoroughly mixed by vortex-mixing. After centrifugation at $1.3\cdot10^3$ *g* for 10 min the supernatant was transferred to a conical glass tube and than evaporated to dryness by a nitrogen flow at 40°C. Next, the residue was reconstituted in 200 μl water by vortex-mixing and after centrifugation at $4.3\cdot10^3$ *g* for 5 min the sample was transferred to an injection vial with a 250-μl glass insert.

2.5. Validation

Four stock solutions of 104 and 161 μg/ml abacavir and 1.21 and 1.43 mg/ml MPA in methanol were prepared (both with separate weighing) and were stored at −20°C. For calibration a dilution of 2.00 μg/ml abacavir and 9.98 μg/ml MPA in plasma, pooled from four individuals, was made from the 104 μg/ml abacavir and 1.21 mg/ml MPA stock solutions and stored at −20°C. Dilutions of this standard in plasma yielding 2, 10, 20, 100, 200 and 1000 ng/ml abacavir and 10, 50, 100, 499, 998 and 4988 ng/ml MPA calibration samples in plasma, respectively, were made daily and analyzed in duplicate for each analytical run. Least-squares linear regression, weighted by x^{-2} (reciprocal of the squared concentration) was employed for the calibration of both, abacavir, using the UV peak, and MPA, using the fluorescence peak signals. The powers of the weighting factors were determined by maximizing the log-likelihood functions and the linearity of the calibrations were tested using lack-of-fit tests; these statistical procedures were performed using the SPSS 7.5 software (SPSS, Chicago, IL, USA).

The 161 μg/ml abacavir and 1.43 mg/ml MPA stock solutions were used to obtain validation (quality control) samples in human plasma at 20, 82, 201 and 2010 ng/ml abacavir and 10, 97, 968 and 9680 ng/ml MPA, respectively. Plasma of four different individual donors was used. Precisions and accuracies were determined by six-fold analysis of each validation sample in three different analytical runs for all plasma concentrations. The assay precisions were obtained by one-way analysis of variance (ANOVA) [20]. The repeatability (intra-day precision) was 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. MS = Sum of squares/degrees of freedom; square = (observed concentration − GM)^2.

Six individual blank plasma samples from different individuals were processed to test the selectivity of the assay. The selectivity of the assay was also tested by investigating the chromatographic retention of several pharmaceutical compounds, including a few of their metabolites, potentially used by patients treated with abacavir and MPA. 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 water and injected into the chromatograph.

Validation samples containing 300 and 2000 ng/ml abacavir in plasma, originating from the validation of our previous reported assay for abacavir [8] were used for stability studies; these samples had been stored at −20 and −80°C, respectively, for 11 months.

For the determination of the extraction yield, calibration samples of abacavir and MPA in acetonitrile were made from the same stock as the calibration samples in plasma in the same concentration ranges and added to blank plasma 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 abacavir 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.

3. Results and discussion

For both, abacavir and MPA, a simple protein precipitation combined with reversed-phase liquid chromatography and UV detection has been used for bio-analytical assays previously. Only acetonitrile has been used as a precipitation agent for both analytes [8,14,16,17]. Thus far, MPA has always been eluted under acidic conditions (pH ≤ 4), using 20–45% of an organic modifier (methanol or acetonitrile) and a reversed-phase column. By using the existing reversed-phase liquid chromatographic system for abacavir (pH 7.0 and 15% acetonitrile) [8] the retention time for MPA was 24 min at this low modifier content thanks to the deprotonation of the carboxylic acid group at this pH. By further increasing the pH also the phenolic function may become (partially) deprotonated. As a result, the retention of MPA decreases further and the molecule becomes highly fluorescent [21], the ratio of the fluorescence yield at pH 7 and 8 is approximately 1:5, facilitating a more sensitive detection compared to UV absorbance. Finally, the eluent flow was increased from 1 to 1.5 ml/min to get an acceptable run time.

Examples of chromatograms at different concentrations are shown in Fig. 2. In each analytical run, calibration samples in the 20–2000 ng/ml range were used for the quantification of abacavir in the validation samples and in the 10–10 000 ng/ml range for MPA. A significant lack-of-fit ($P<0.05$) was observed for one of totally five calibrations for abacavir without I.S., for MPA without I.S. and for MPA with I.S., using peak areas of the UV response for abacavir and naproxen and the fluorescence response of MPA. This number of observations (one out of five) is not sufficient to consider the use of non-linear regression.

The results obtained with the validation samples (precisions and accuracies at each level in the different analytical runs) are listed in Table 1, for both with and without using the I.S.. The validation results without I.S. were better for the repeatability of MPA and the accuracy of measurements for both analytes. This differences are probably due to the higher variation of the recovery of naproxen and the increased peak area of this I.S. for the calibration samples at the highest concentrations, respectively. The lowest concentration of MPA in plasma, 10 ng/ml, proved to be the LLQ for this analyte. This sensitivity is equivalent to the LLQ of the LC method with mass spectrometric detection of Shaw et al. [16]; however, a lower LLQ of the present assay may be possible because the lower limit of detection

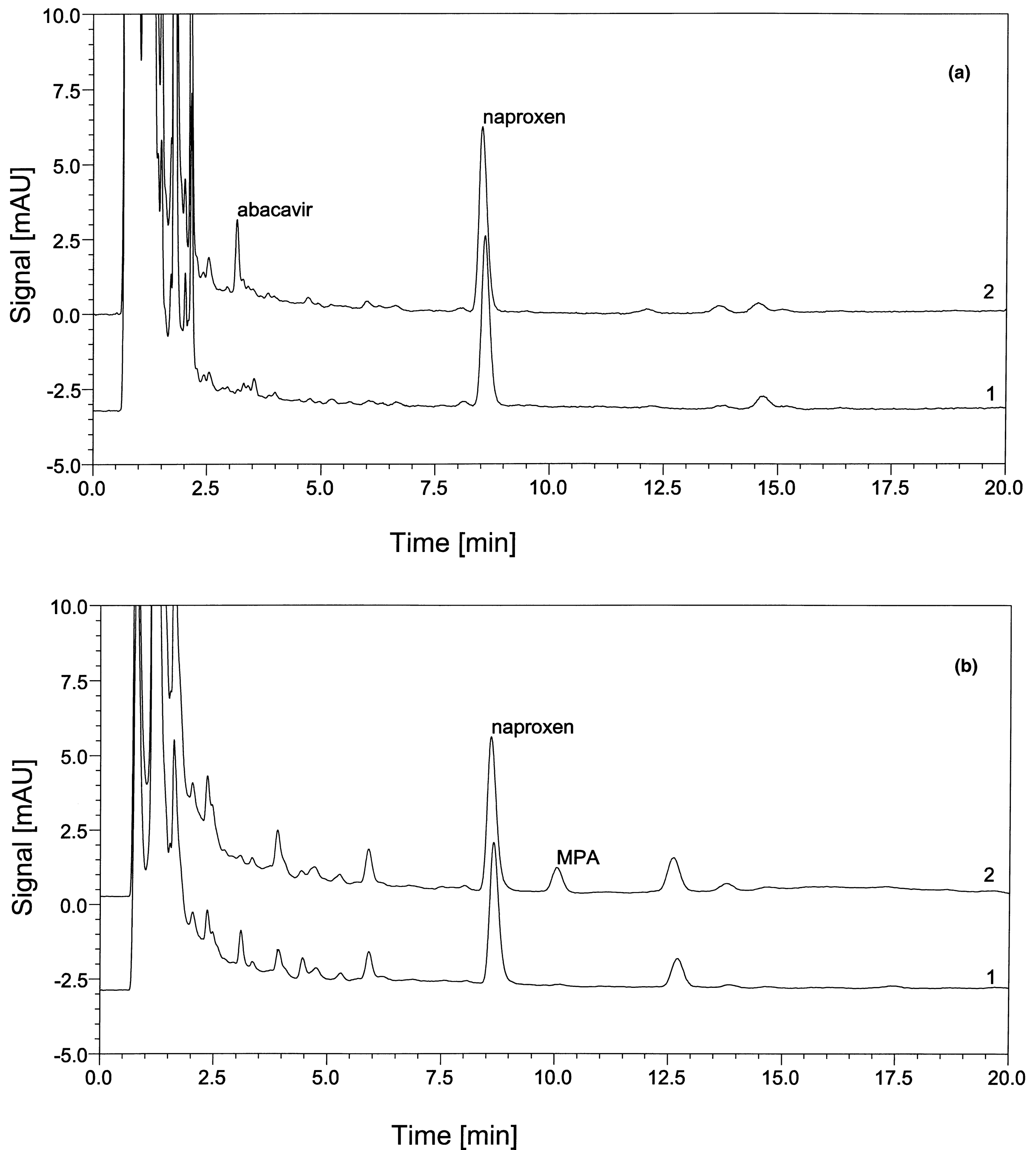

Fig. 2. Chromatograms of a blank plasma sample (1) and a validation sample spiked with 82 ng/ml abacavir and 10 ng/ml MPA (2). (A) UV signal; (B) fluorescence signal.

Table 1
Overall results of the validation samples ($n=18$) if the assay is used with[a] and without I.S. for both analytes

Nominal concentration (ng/ml)	Repeatability (%)		Reproducibility (%)		Accuracy (%)	
Abacavir						
2010	2	*3*	1	*1*	94	*84*
201	3	*5*	2	*1*	92	*90*
82	6	*6*	1	*1*	101	*94*
20	24	*24*	23	*25*	64	*69*
MPA						
9680	2	*4*	1	*1*	101	*91*
968	2	*5*	1	*1*	103	*101*
97	3	*6*	2	*1*	103	*100*
10	4	*6*	3	*5*	97	*90*

[a] Italics indicate the validation parameters for calibration with using the I.S.

($S/N=3$) is ≤ 1 ng/ml MPA if the most sensitive settings of the fluorescence detector are utilized. The concentration range between 1 and 10 ng/ml MPA was not validated because this range was not expected to be clinical relevant. For abacavir the demands for a bioanalytical assay (precision and the accuracy $\leq 20\%$ for the LLQ and $\leq 15\%$ at higher concentrations [22]) were not met at the lowest validated concentration (20 ng/ml); the LLQ of abacavir in plasma was therefore set at 80 ng/ml. This sensitivity is sufficient for a large majority of clinical samples containing abacavir.

In six individual blank plasma samples, no interferences in the chromatograms were observed which could influence the quantification of abacavir or MPA in the validated concentration ranges. From the potentially co-administered drugs tested none showed retention times in the 2–20 min range in fluorescence chromatograms of the present analytical system and four in the UV chromatograms: trimethoprim (6.1 min), nevirapine (8.8 min), ranitidine (5.3 min) and zidovudine (2.4 min); the retention times of abacavir and MPA were 3.1 (UV) and 8.7 min (fluorescence), respectively. Thus, all tested drugs except nevirapine are separated from both, abacavir and MPA; nevirapine, however, showed no fluorescent response.

The recovery of abacavir after storage for 11 months at -20 and -80°C was 90 ± 2 and $85\pm2\%$ at 300 ng/ml and 86 ± 2 and $85\pm2\%$ at 2000 ng/ml, respectively ($n=4$). All other relevant stability data of abacavir in plasma have been published previously [8], abacavir was stable in plasma at -20°C for 51 days, at 25°C for 3 days, at 37°C for 6.5 h and at 60°C for 4.5 h. In the last 5 years also a lot relevant stability data of MPA in plasma have been published. MPA was shown to be stable in plasma at -20°C [23] for 11 months and at 25°C for 28 h [18]; however, the MPA concentration in clinical samples at ambient temperature can be influenced by the conversion of the MPA–glucuronide into MPA of 1.2–2‰ per week if the glucuronide–MPA ratio is very high [24]. Further, both analytes showed to be stable in plasma during four extra freeze–thaw cycles [8,15].

The recovery in extracted samples after overnight storage in the autosampler at ambient temperature is 100% for abacavir and 102% for MPA. The calculated yield of the extraction is 96% for abacavir and 98% for MPA.

4. Conclusions

A convenient simultaneous assay for abacavir and MPA has been developed using HPLC with dual spectrophotometric detection. Alternatively, a programmable UV-absorbance or a photodiode array detector can be used; however, interference of nevirapine should then be further eliminated if this drug is co-administered. Samples of abacavir in plasma should be stored less than 11 months.

Finally, the assay can be used in the near future

for clinical studies of the combination of both drugs in both adults and children.

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