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Determination of stavudine, a new antiretroviral agent, in human plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A sensitive high-performance liquid chromatographic assay has been developed to determine the levels of a new antiretroviral agent, stavudine (2′,3′-didehydro-3′-deoxythymidine, d4T), in human plasma. Didanosine (2′,3′-dideoxyinosine, ddI) was used as the internal standard. The very selective sample pretreatment involved solid-phase extraction using silica gel columns. Chromatography was carried out on a μBondapak phenyl column, using a mobile phase of 0.005 *M* phosphate buffer (pH 6.8)–methanol (90:10, v/v) and ultraviolet detection at 265 nm. The method has been validated, and stability tests under various conditions have been performed. The detection limit is 10 ng/ml (using 500-μl human plasma samples). The bioanalytical assay has been used in a single pharmacokinetic experiment in a rat to investigate the applicability of the method *in vivo*.

INTRODUCTION

Stavudine (2′,3′-didehydro-3′-deoxythymidine, d4T) (Fig. 1) has been shown to be a potent inhibitor of the human immunodeficiency virus (HIV) [1–5], the causative agent of the acquired immune deficiency syndrome (AIDS). Zidovudine, the first dideoxynucleoside analogue, was introduced in 1987 [6], followed by didanosine (ddI) and dideoxycytidine (ddC) (Fig. 1), which have been extensively studied [7]. Stavudine is, therefore, the fourth in a series of dideoxynucleosides to be clinically studied. *In vitro* studies have demonstrated that the drug is less toxic than zi-

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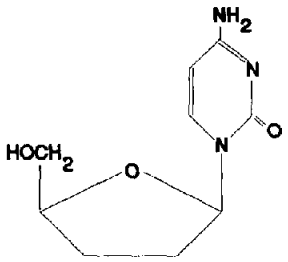

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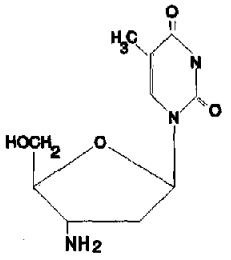

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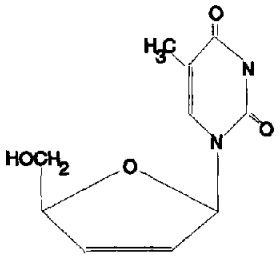

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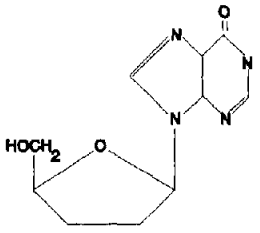

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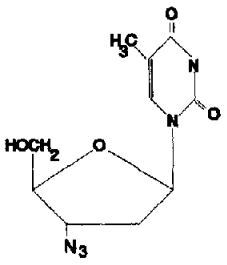

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Fig. 1. Structures of dideoxycytidine (1), 3′-amino-3′-deoxythymidine (2, a metabolite of zidovudine), stavudine (3), didanosine (4) and zidovudine (5).

dovudine to bone marrow stem cells, and that it does not accumulate as the (unactive) monophosphate form, like zidovudine does [8]. Phase I/II trials have just ended [8,9] and phase II/III trials are planned [9].

The determination of dideoxynucleosides in biological matrices has been reviewed by Riley *et al.* [10]. In general, the majority of the reports described liquid chromatographic assays, using liquid–liquid or liquid–solid extraction as sample pretreatment, followed by determination on reversed-phase C_{18} columns and UV detection [10].

The determination of stavudine in biological media has been described in a number of publications [11–14]. Boudinot *et al.* [11] described a procedure based on a protein precipitation method and direct injection of the supernatant into a high-performance liquid chromatographic (HPLC) column. This procedure is simple, but lacks a concentration step and has, therefore, a relatively high detection limit (50 ng/ml). Wong and Sawchuk [12] used liquid–liquid extraction, but achieved a recovery of only 46%. The HPLC method of Kaul *et al.* [13] was validated only for concentrations in the range 0.1–100 μg/ml in rat and monkey plasma. Furthermore, their internal standard (thymidine oxetane) is not commercially available, and the detection wavelength was not optimal for the determination of stavudine. Finally, Russell *et al.* [14] used an HPLC assay for a pharmacokinetic study with stavudine in animals. Like Kaul *et al.* [13], they used solid-phase extraction with C_{18} columns. However, no data on the validation of this method in human plasma have been reported.

An alternative, highly sensitive HPLC method for the quantification of stavudine in human plasma has been developed. A highly sensitive assay is needed, because stavudine is used at relatively low doses (40 mg twice daily) [8,9]. As with didanosine, there is no evidence for the presence of metabolites [8,9], so we concentrated on the parent compound.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Model 510 pump (Waters Assoc., Milford, MA, USA), a Spectra 200 programmable wavelength detector (Spectra Physics, Santa Clara, CA, USA), a Model 8880 automatic sample injection device (Spectra Physics) and a Model 4270 integrator (Spectra Physics). The analytical column was a μBondapak Phenyl (300 mm × 3.9 mm I.D.; particle size 10 μm) (Waters Assoc.) protected by a LiChroCART 4-4 RP-8 guard column (4 mm × 4 mm I.D.; particle size 5 μm) (Merck, Darmstadt, Germany). Analytical runs were processed by the Autolab Software Winner 386 system (Spectra Physics). UV spectra of stavudine solutions in the eluent were recorded with an SP8-400 UV–VIS spectrophotometer (Pye Unicam, Cambridge, UK).

Chemicals

Stavudine and didanosine (2′,3′-dideoxyinosine, ddI) were generously provided by Bristol-Myers Squibb (Wallingford, CT, USA). 3′-Amino-3′-deoxythymidine (metabolite of zidovudine) was purchased from Sigma (St. Louis, MO, USA). Zidovudine was extracted from Retrovir capsules (Wellcome Pharmaceuticals, Utrecht, Netherlands) as described before [15], and dideoxycytidine (ddC) was obtained through Hoffmann-La Roche (Mijdrecht, Netherlands). Methanol was purchased from Chrompack (Middelburg, Netherlands), and potassium dihydrogenphosphate p.a. and disodium hydrogenphosphate dihydrate p.a. from Merck. Deionized water was used throughout. Blank plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, Netherlands).

Drug solutions

Stock solutions of stavudine (1 μg/μl), 3′-amino-3′-deoxythymidine (1 μg/ml), didanosine (1 μg/μl), zidovudine (1 μg/ml) and dideoxycytidine (1 μg/ml) were prepared by dissolving the appropriate amount of the drug, accurately weighed, in methanol. For the construction of calibration curves fresh solutions were used.

Sample preparation

For the preparation of the standard samples, stock solutions of stavudine and didanosine were diluted with methanol. To achieve stavudine calibration concentrations of 10–10 000 ng/ml, appropiate amounts of the various diluted stock solutions were added to 10-ml polypropylene tubes (International Medical Products, Zutphen, Netherlands). To each tube, 50 μl of a diluted stock solution of didanosine (5 ng/μl) were added as internal standard. The solutions were evaporated to dryness under a nitrogen stream at 60°C. Next, 500 μl of plasma were added to each tube and mixed on a vortex mixer for 10 s and subjected to solid-phase extraction. Prior to this, C_{18} extraction columns (3 ml capacity; Bakerbond SPE, J. T. Baker, Phillipsburg, NJ, USA) or silica gel extraction columns (3 ml capacity; Bondelut, Analytichem International, Rotterdam, Netherlands) were placed on a vacuum elution manifold (Baker 10-SPE system) and were conditioned with 2 ml of methanol and rinsed twice with 2 ml of water. Care was taken that the columns did not run dry. Next, 500 μl of the plasma samples were brought onto the columns and drawn into them by applying reduced pressure. The columns were washed with 1 ml of distilled water and allowed to dry for 10 min under continuous vacuum suction. Elution of the absorbed analytes was performed with two 0.5-ml volumes of methanol into Eppendorf tubes and evaporated to dryness under a gentle stream of nitrogen at 60°C. The residues were redissolved in 200 μl of mobile phase, mixed on a vortex mixer for 30 s and centrifuged for 5 min at 800 *g*. The supernatants were brought into autosampler vials and injected into the HPLC system.

Chromatography

Chromatographic analysis was performed at ambient temperature with a mobile phase of 900 ml of 0.005 *M* phosphate buffer (pH 6.8) and 100 ml of methanol. Prior to use, the mobile phase was filtered under reduced pressure through a 0.2-μm cellulose acetate filter (Sartorius, Spruyt-Hillen, Utrecht, Netherlands). The absorbance was monitored at 265 nm. The flow-rate was maintained at 1.0 ml/min. Aliquots of 100 μl were injected into the chromatograph.

Validation

The accuracy and precision of the method were determined by assaying replicates of plasma samples containing known concentrations of stavudine. The recovery of stavudine from the solid-phase extraction was determined and calculated by comparing observed concentrations in extracted plasma samples with those in unextracted aqueous standard solutions.

Stability studies

Drug-free plasma samples (2.5 ml) were spiked with an aliquot of 50 μl of a stock solution of stavudine in methanol (50 ng/μl) to give an initial concentration of 1 μg/ml. These samples were stored for 30 min at 60°C, for 24 h at 25°C, for 7 days at 8°C, or for 21 days at −30°C. After the storage period the samples were analysed immediately.

Pharmacokinetic experiment in a rat

A dose of 0.5 mg of stavudine, dissolved in 2.0 ml of 0.9% saline solution, was given as a rapid intravenous bolus injection in the tail in 30 s into a male rat (WAG/Rij; weight 372 g; food *ad libitum*). At t = 0, 0.5, 3, 7, 15, 30, 60, 90, 120 and 180 min, 300-μl blood samples were withdrawn from the carotid artery and collected in an Eppendorf tube, containing 10 μl of heparin (equivalent to 50 I.U.), and immediately centrifuged for 5 min to obtain the plasma fraction. Plasma samples were frozen at −30°C. Analyses were carried out with 100 μl of plasma.

RESULTS AND DISCUSSION

Chromatography and detection

In a review of reported methods for the biodetermination of dideoxynucleoside antiretroviral agents, Riley *et al.* [10] stated that the majority of investigators use liquid chromatographic assays with reversed-phase C_{18} columns and UV detection. An analytical phenyl column was used only once [16] for the determination of 2′,3′-dideoxy-

adenosine (ddA) and didanosine. In our laboratory chromatography on a phenyl column is preferred for the determination of antiretroviral drugs as it allows the separation of dideoxynucleosides in one system (Fig. 2). Furthermore, in combination with pretreatment by solid-phase extraction on silica gel columns, it led to less interference from endogenous compounds (see below).

For the determination of stavudine and didanosine (internal standard), the UV detection was 265 nm, because this is the wavelength of maximum absorption of stavudine (molar absorptivity 5555) in the eluent.

Sample pretreatment

Four reports on the biodetermination of stavudine have been published [11–14]. The most acceptable HPLC method was reported by Kaul *et al.* [13] and Russell *et al.* [14], who used solid-phase extraction of rat, monkey and mouse plasma with a C_{18} extraction column. Human plasma samples, pretreated and analysed according to this method [13,14], gave many interferences. These were diminished by the use of a phenyl column as the analytical column, but a co-eluting compound at 10 min interfered (Fig. 3A). Further improvement was accomplished with solid-phase extraction on silica gel columns preceding the analysis on a phenyl column (Fig. 3B). This combination is attractive as it is based on sample pretreatment and analysis defined by different chromatographic interactions. A typical elution profile of a spiked plasma sample of 100 ng/ml stavudine, after solid-phase extraction on a silica gel extraction column, is shown in Fig. 4A and B.

Recoveries from extraction columns

In order to determine the recoveries of stavudine from the solid-phase extraction columns,

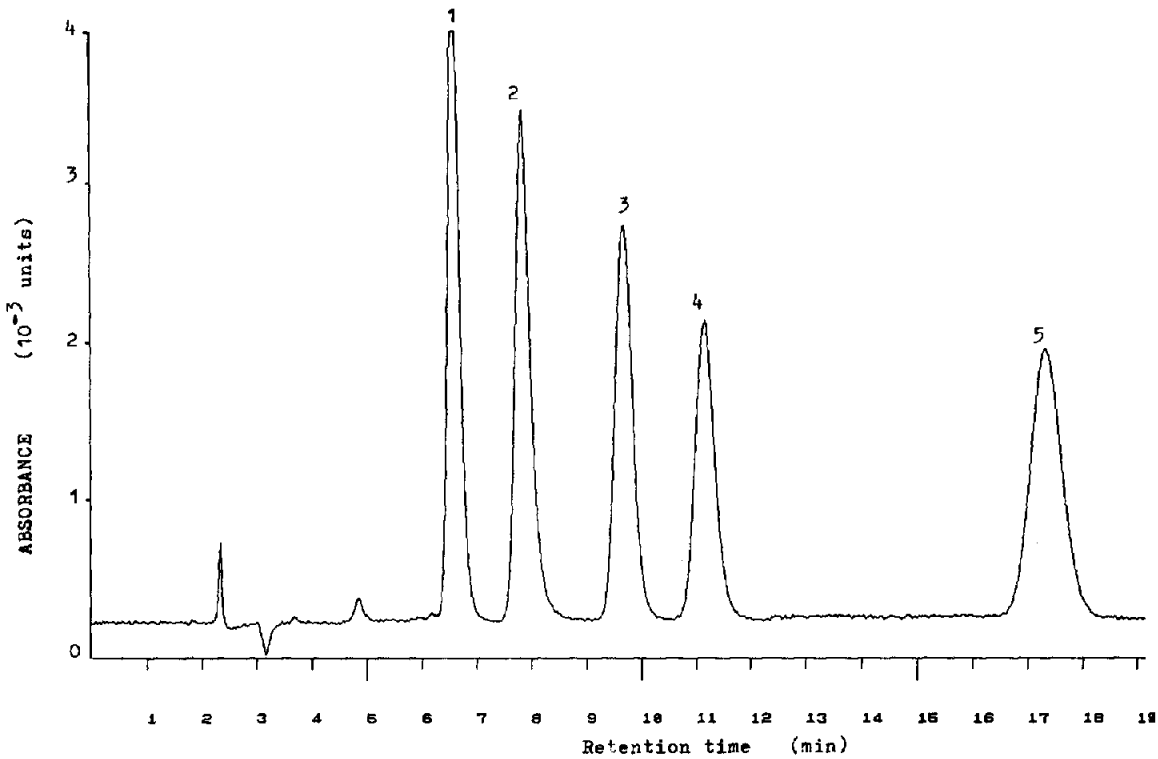

Fig. 2. Chromatogram of a mixture of dideoxycytidine (1), 3'-amino-3'-deoxythymidine (2), stavudine (3), didanosine (4) and zidovudine (5) (all concentrations 1 μg/ml, 20 ng absolute amount). Chromatographic conditions: see text.

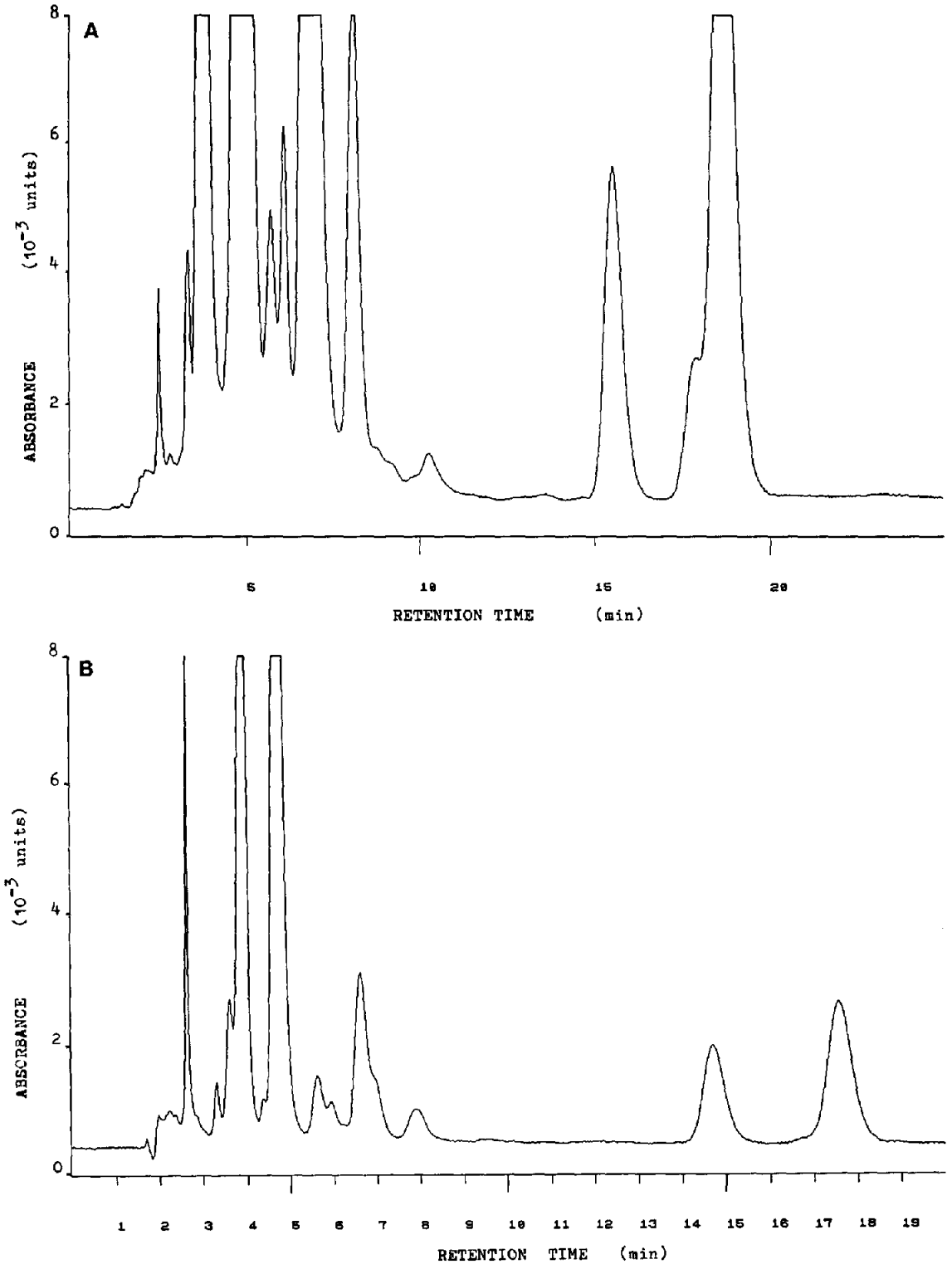

Fig. 3. Typical chromatograms obtained from the analysis of extracted human plasma. (A) Blank plasma sample after solid-phase extraction with a C_{18} extraction column; (B) blank plasma sample after solid-phase extraction with a silica gel extraction column. Chromatographic conditions: see text.

TABLE I

RECOVERIES OF STAVUDINE FROM SPIKED HUMAN PLASMA SAMPLES

Stavudine concentration (ng/ml)	Recovery[a] (%)	C.V.[b] (%)	n[c]
50.35	101.8	4.1	3
503.5	92.5	0.3	3
5035	93.7	0.9	3

[a] Recovery is expressed as the ratio (x 100%) of the observed analyte concentration and the corresponding concentration obtained from the HPLC analysis of the unextracted aqueous samples.

[b] C.V. = coefficient of variation.

[c] n = number of replicates.

aqueous and plasma samples of 50, 500 and 5000 ng/ml were prepared. Three replicates of each plasma standard concentration were extracted and analysed. The recovery was defined as the ratio of the observed analyte concentrations of the standard serum samples to those of the unextracted aqueous sample. Values ranged from 92.5 to 101.8% (Table I).

Standard curves

The linearity, accuracy and precision at different concentrations of the analyte are listed in Tables II and III. The detection limit at a signal-to-noise ratio of 3 is 10 ng/ml, using 500-μl plasma samples. All standard curves had correlation coefficients greater than 0.994 and showed low variability in the calculated slopes. The fact that the y-intercepts were very close to zero reflects the observation that there was little interference from endogenous plasma constituents.

Stability data

The stability data of stavudine at various conditions are shown in Table IV. Under all conditions tested, stavudine is stable at concentrations of at least 91% of the initial concentration (1 μg/ml). These conditions are important in the hand-

TABLE II

EQUATIONS OF CALIBRATION LINES FOR THE DETERMINATION OF STAVUDINE IN HUMAN PLASMA

y = peak-area ratio between stavudine and the internal standard didanosine; x = stavudine concentration (ng/ml).

Concentration range (ng/ml)	Equation	r^2	n
10–100	$y = 0.00384(\pm 0.00016)x - 0.00329(\pm 0.01205)$	0.9946	5
100–1000	$y = 0.00341(\pm 0.00006)x + 0.06747(\pm 0.04274)$	0.9991	5
1000–10 000	$y = 0.00337(\pm 0.00006)x - 0.01340(\pm 0.45133)$	0.9990	5
Overall equation			
10–10 000	$y = 0.00336(\pm 0.00002)x + 0.04422(\pm 0.24073)$	0.9996	13

TABLE III

ACCURACY OF THE DETERMINATION OF STAVUDINE IN HUMAN PLASMA

Concentration added (ng/ml)	Concentration found (ng/ml)	n	C.V. (%)	Accuracy (%)
50.35	45.91	3	2.9	91.2
503.5	508.4	3	0.9	101.0
5035	4839	3	0.7	96.1

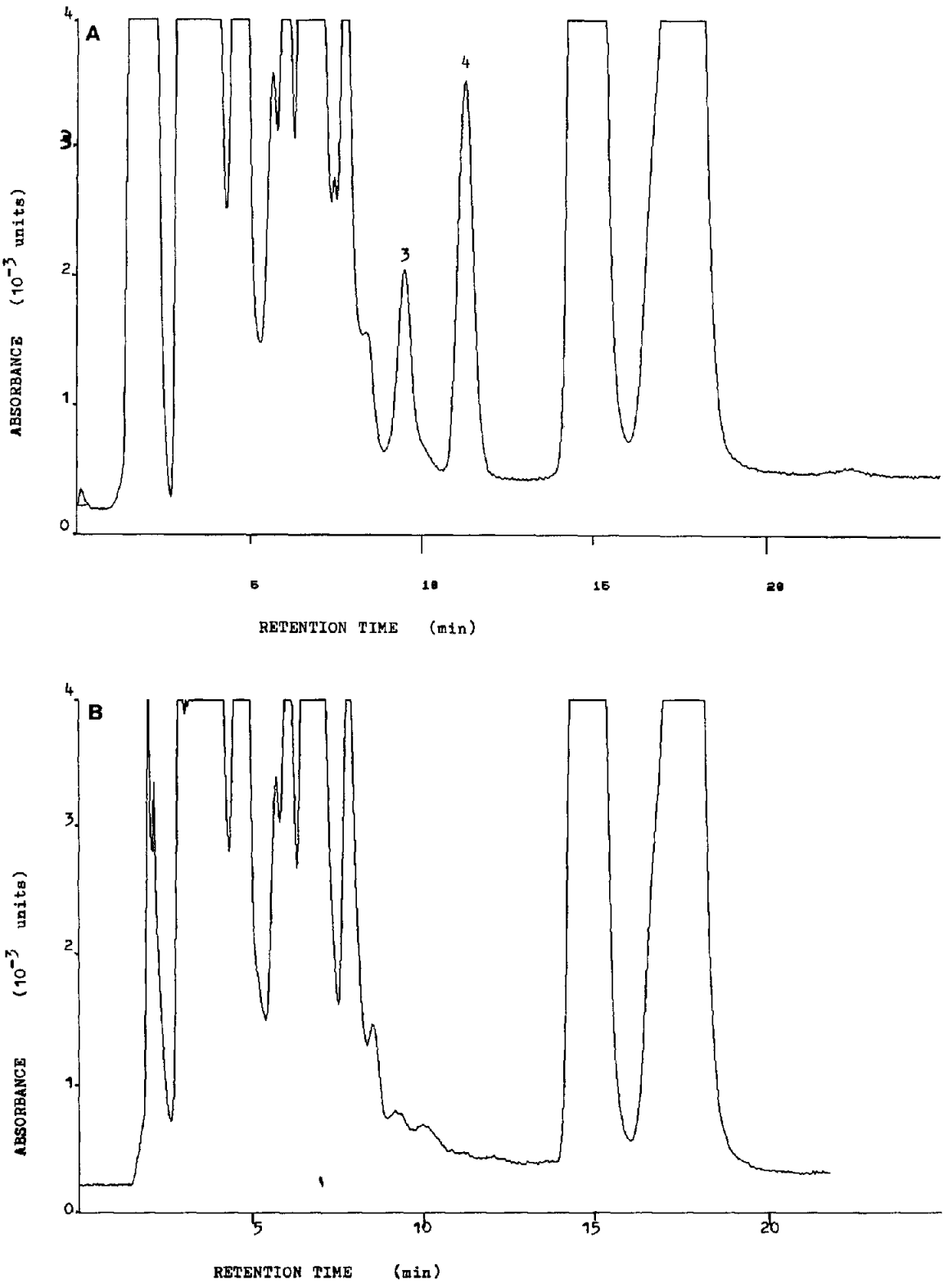

Fig. 4. Typical chromatograms obtained from the analysis of extracted human plasma. (A) Plasma sample spiked with stavudine (100 ng/ml) (3) and didanosine (500 ng/ml) (4) as internal standard: retention times are 9.0 and 10.5 min, respectively; (B) blank plasma sample. Chromatographic conditions: see text.

TABLE IV

STABILITY OF STAVUDINE IN SPIKED HUMAN PLASMA SAMPLES

Storage condition	Recovery[a] (%)	C.V. (%)	n
30 min at 60°C	95	0.6	4
24 h at 25°C	91	1.6	4
7 days at 4°C	97	1.3	4
21 days at −30°C	94	2.7	4

[a] Recovery is defined as the percentage of the initial stavudine concentration (1000 ng/ml).

ling of plasma samples in pharmacokinetic studies.

Pharmacokinetic experiment

The applicability of the assay to pharmacokinetic studies was demonstrated in an experiment with a rat. The plasma–concentration curve after an intravenous dose of 0.5 mg stavudine is presented in Fig. 5.

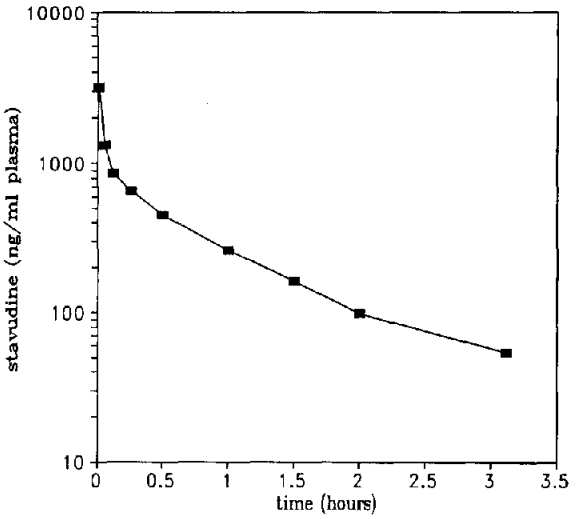

Fig. 5. Plasma concentration–time curve of stavudine (dose 0.5 mg intravenously) in one rat.

CONCLUSION

This HPLC assay for stavudine in human plasma samples, pretreated with a solid-phase extraction on silica gel columns, is a sensitive and validated method for the determination of this new antiretroviral drug. The assay will be used in our hospital for pharmacokinetic studies of stavudine in HIV-infected patients.

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