Journal of Chromatography, 534 (1990) 101–107
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5504

Determination of 3′-azido-3′-deoxythymidine, 2′,3′-dideoxycytidine, 3′-fluoro-3′-deoxythymidine and 2′,3′-dideoxyinosine in biological samples by high-performance liquid chromatography

NICOLE FRIJUS-PLESSEN, HANS CHRISTOPH MICHAELIS, HEIDI FOTH and GEORG FRIEDRICH KAHL*

Department of Pharmacology and Toxicology, University of Göttingen, Robert-Koch-Strasse 40, D-3400 Gottingen (F.R.G.)

(First received May 23rd, 1990, revised manuscript received July 26th, 1990)

ABSTRACT

A simple and fast high-performance liquid chromatographic assay for the determination of 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxycytidine (ddC), 3′-fluoro-3′-deoxythymidine (FT) and 2′,3′-dideoxyinosine (ddI) in complex biological matrices is described. The method allows rapid nucleoside determination using a phenyl column within extra- as well as intracellular media without further sample pretreatment and extraction procedures. The lower limit of detection is *ca* 0.05 μg/ml for each nucleoside, and the separation can easily be optimized for AZT, ddC, FT and ddI by variation of the methanolic part of the mobile phase and the detector wavelengths

INTRODUCTION

3′-Azido-3′-deoxythymidine (AZT), 2′,3′-dideoxycytidine (ddC), 3′-fluoro-3′-deoxythymidine (FT) and 2′,3′-dideoxyinosine (ddI) are synthetic nucleosides that exhibit antiviral activity against the human immunodeficiency virus (HIV) [1–3]. To study the elimination kinetics of these nucleoside derivatives in small laboratory animals as well as in cellular systems, sensitive and specific analytical methods for concentration determination are required.

For analysis of AZT in serum, an enzyme-linked immunosorbent assay as well as a time-resolved fluoroimmunoassay [4] has previously been described. Additionally, high-performance liquid chromatographic (HPLC) methods for analysis of AZT in plasma, serum, urine and cerebrospinal fluid have been reported [5–9]. For the determination of ddC concentrations in plasma and in urine, HPLC [10–13] or gas chromatographic–mass spectrometric (GC–MS) [14] methods have been established. To our knowledge, no assay for the determination of FT has yet been reported. The assay reported by Kalin and Hill [11] for analysis of ddC has also been applied for the measurement of ddI and 2′,3′-dideoxyadenosine (ddA)

0378-4347/90/$03.50

concentrations in plasma from mice and cats. Except for this assay, most of the HPLC methods reported require extensive sample preparation procedures, including liquid–liquid or C_{18} solid-phase extractions.

The aim of our study was to develop a simplified and fast HPLC assay for determination of AZT, ddC and FT concentrations in extra- and intracellular matrices, as well as in semisynthetic media used in rat liver perfusion systems. With this method, we obtained a lower limit of detection of *ca.* 0.05 μg/ml for AZT, ddC, FT and ddI in extracellular fluids. Except for ddI, all the nucleosides studied can also be quantified in intracellular media.

EXPERIMENTAL

Reagents and chemicals

AZT, ddC and ddI were obtained from Sigma (Deisenhofen, F.R.G.) and FT was a gift from Dr. Eckstein, Max-Planck-Institut für Experimentelle Medizin, (Göttingen, F.R.G.) Methanol (LiChrosolv grade) and all other chemicals were obtained from Merck (Darmstadt, F.R.G.) at the highest purity available.

Apparatus and HPLC conditions

The HPLC system consisted of a Merck/Hitachi L-6200 intelligent pump (Merck, Darmstadt, F.R.G.) coupled to a six-port injector (Rheodyne, Cotati, CA, U.S.A.), a Phenyl Hypersil NC-04 column, particle size 5 μm, 250 mm × 4 mm I.D. (Bischoff, Leonberg, F.R.G.), and a Merck/Hitachi 655 A variable-wavelength UV monitor (Merck) equipped with a 2.2-μl flow-cell and operated at 271 nm (ddC), 250 nm (ddI) or 267 nm (AZT, FT) A Merck/Hitachi D-2000 chromato-integrator was used for printing the chromatograms and for data calculation. The mobile phase for separation of either extra- or intracellular media was sodium acetate solution (1 4 g/l) with 25% (AZT), 3% (ddC), 10% (FT) and 15% (ddI) methanol, and was adjusted to pH 6.55. For human plasma samples, best separation results could be obtained using a methanol concentration of 20% (AZT) and 3% (FT). The flow-rate was 1.0 ml/min and the column temperature was ambient

Sample treatment

Rat hepatocyte incubation samples. The incubation buffer was MEM Eagle's Medium pH 7.6 (Sigma, Deisenhofen, F.R.G.). At the end of incubation, the cells were immediately separated from the medium by centrifugation (15 s; 17 000 *g*) through an oil layer (silicone oil AR 200, Wacker Chemie, Munich, F R.G.). A 50-μl aliquot of the cell-free supernatant (extracellular sample) was directly injected onto the HPLC column.

The cellular pellet (9 μl) was suspended in 100 μl of 0.1 *M* phosphate buffer (pH 7.4) in methanol (1:1, v/v). Cell lysis was performed by freezing and thawing three times, using liquid nitrogen. The lysate was centrifuged for 5 min at 17 000 *g*

and a 25-μl aliquot of the supernatant (intracellular sample) was injected onto the HPLC column.

Rat liver perfusion samples. The semisynthetic perfusion medium (pH 7.4) consisted of bovine erythrocytes (hematocrit 0.33), 15 mg/ml bovine serum albumin and 1 mg/ml glucose in Krebs–Ringer bicarbonate buffer. Samples were prepared by centrifugation at 17 000 *g* for 5 min, and 50-μl aliquots of the supernatant were injected onto the HPLC column.

Human plasma samples. A 1-ml volume of plasma was diluted with 1 ml of saturated ammonium sulphate solution for protein precipitation. After vortex-mixing for 30 s, the samples were centrifuged (2 min; 3000 *g*) and 50-μl aliquots of the supernatant were injected onto the HPLC column.

Calibration curves, precision and recovery studies

For calibration, standard solutions spiked with concentrations ranging between 0.267 and 2.67 μg/ml (AZT), 0.211 and 1 69 μg/ml (ddC), and 0.49 and 1 96 μg/ml (FT) were prepared from blank perfusion medium. The coefficient of correlation and the regression equations were calculated using least-squares linear regression analysis by correlating the peak height (μV) against the corresponding spiked concentrations (μg/ml). The intra- and inter-day precisions of the assay were estimated by measuring spiked standards with 1.068 μg/ml (AZT), 0.844 μg/ml (ddC) and 0.98 μg/ml (FT) of each nucleoside at day one ($n = 10$) and on the six (AZT), five (ddC) and ten (FT) following days. The recovery of drug after plasma protein precipitation was determined by comparing aqueous standard solutions (AZT, 5.34 μg/ml; ddC, 4.22 μg/ml; FT, 4.90 μg/ml; ddI, 4.72 μg/ml) with human plasma samples spiked with the same concentrations. A 1-ml volume of each standard was diluted with 1 ml of water, and aliquots of 50 μl were injected. The plasma samples were assayed as described above, and the recovery was calculated from the peak-height ratios (plasma sample to standard solution). Arithmetic means and standard deviations were calculated from ten samples for each drug.

Application of the method

The assay was used to determine concentrations of AZT, ddC and FT in intra- and extracellular medium of isolated rat hepatocytes after incubation and during isolated rat liver perfusions, an experimental model suitable for the evaluation of pharmacokinetic parameters.

RESULTS AND DISCUSSION

The evaluation of the elimination kinetics and of the metabolic fate of AZT, ddC, FT and ddI requires a sensitive and specific assay that permits analysis within complex intracellular matrices. Additionally, in view of a large number of samples usually needed for kinetic studies it is desirable to have an assay without

time-consuming sample pretreatment. The reported HPLC methods are very sensitive and specific for determination of AZT or ddC concentrations in human samples, but most of them require liquid–liquid or solid-phase extraction pretreatment. In addition, in our experience, HPLC separation on commonly used C_{18} analytical columns was not successful for determination of nucleoside concentrations in intracellular media because of interferences. Therefore, we performed extra- as well as intracellular analysis by using a phenyl column. Under these conditions, the separation was free from interferences from sample components for all the investigated nucleosides except ddI, which could be analysed in extracellular medium and in human plasma samples. However, when intracellular medium was used an interfering peak was still present Although no purification step is performed prior to injection, we have observed no decrease in the column life in the course of up to several hundred injections.

Fig 1 shows chromatograms of perfusion medium (I) spiked with 0.54 μg/ml AZT (A), 0.42 μg/ml ddC (B) or 0.49 μg/ml FT (C), or without drug (II). No interferences with the nucleoside peaks occurred. The lower limit of detection at a signal-to-noise ratio of 3:1 was *ca.* 0.05 μg/ml of sample volume. The calibration curves were linear over the concentration ranges studied. The least-squares linear regression line has a slope of 6286.1 (AZT), 4445.2 (ddC) and 2509.1 (FT) and an *y*-intercept (were y = peak height, μV; x = concentration, μg/ml) of 197.5 (AZT), 111.6 (ddC) and 66.1 (FT). The coefficient of correlation was $r = 0.9996$ (AZT), $r = 0.9991$ (ddC) and $r = 0.9996$ (FT). The recovery (mean ± S.D.; n = 10) of the plasma protein precipitation procedure was 82.5 ± 0 7% (AZT), 102.2 ± 3.9% (ddC), 97.9 ± 0.9% (FT) and 98.4 ± 6% (ddI).

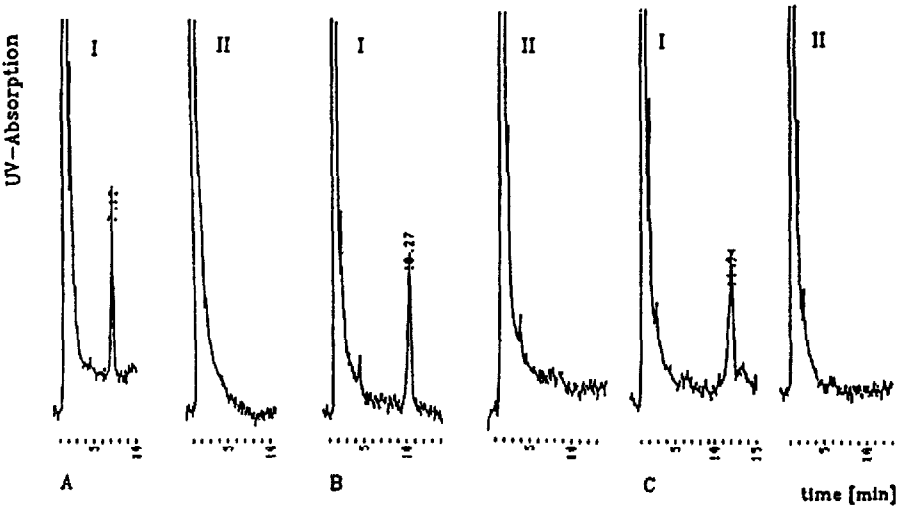

Fig 1 Chromatograms of perfusion medium (I) spiked with 0 54 μg/ml AZT (A), 0.42 μg/ml ddC (B) or 0 49 μg/ml FT (C); (II) the corresponding blank chromatograms

TABLE I

INTRA- AND INTER-DAY PRECISIONS FOR AZT, ddC AND FT

Precision	*n*	Spiked concentration (μg/ml)	Found concentration (mean ± S.D.) (μg/ml)	C.V. (%)
AZT				
Intra-day	10	1.068	1.044 ± 0.053	5.1
Inter-day	6	1.068	1.020 ± 0.040	3.9
ddC				
Intra-day	10	0.844	0.740 ± 0.063	4.9
Inter-day	5	0.844	0.770 ± 0.047	6.1
FT				
Intra-day	10	0.980	0.989 ± 0.047	7.5
Inter-day	10	0.980	0.950 ± 0.027	2.8

The mean concentrations, standard deviations and coefficients of variation for intra- and inter-day precision are listed in Table I.

This method was used to determine AZT, ddC and FT concentrations in extra- and intracellular media during isolated hepatocyte incubation and isolated rat liver perfusion. As an example, Fig. 2 shows an intracellular concentration *versus* time curve obtained in incubations of isolated rat hepatocytes at 21.2 μg/ml ddC. Chromatograms (A) before and (B) after 15-min incubation at lower concentrations of ddC (6.12 μg/ml) are depicted in Fig. 3. The ddC concentration calculated from chromatogram B was 0.173 μg/ml. HPLC determination of intracellular AZT and FT (chromatograms not shown) yielded similar separation results.

The lower limit of detection of ddI in human plasma is *ca.* 0.025 μg/ml. In comparison, Kalin and Hill [11] observed a limit of detection of 0.2 μg/ml using an analytical C_{18} column.

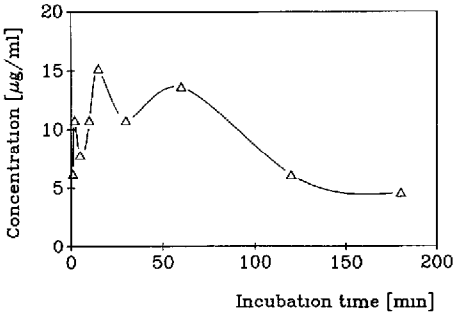

Fig. 2. Intracellular ddC concentration *versus* time curve obtained from isolated rat hepatocyte incubation with 21.2 μg/ml ddC

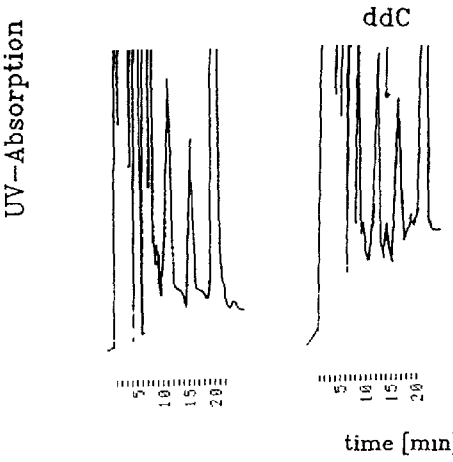

Fig 3. Chromatograms of intracellular medium samples withdrawn before (left) and 15 min after (right) starting an incubation with 6 12 μg/ml ddC The ddC concentration value, calculated from this chromatogram, was 0.173 μg/ml

In Fig. 4, chromatograms of human plasma (I) without and (II) spiked with 0.081 μg/ml AZT, 0.106 μg/ml ddC, 0.123 μg/ml FT and 0.078 μg/ml ddI are shown.

Compared with the HPLC assays reported, our method offers the advantage of rapid nucleoside determination in complex biological matrices, *e.g.* in intracel-

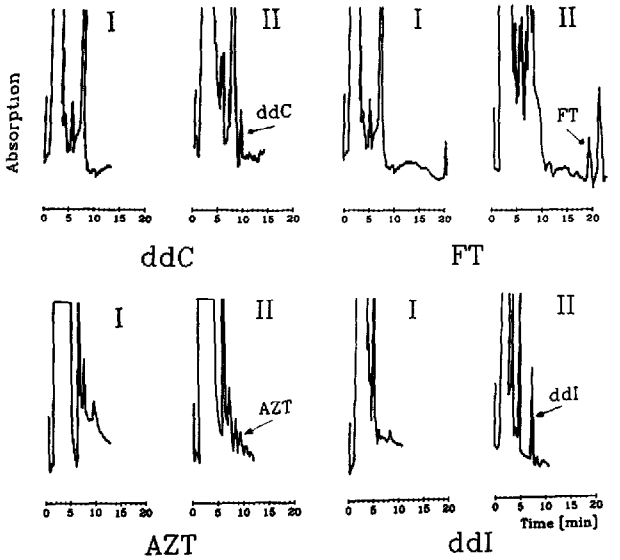

Fig. 4. Chromatograms of human plasma samples (I) without and (II) spiked with 0 106 μg/ml ddC 0 123 μg/ml FT, 0 081 μg/ml AZT and 0.078 μg/ml ddI

lular medium, without further sample pretreatment, and the limit of detection is *ca.* ten-fold lower than reported for ddC in a comparable assay without extraction procedures [11]. The assay is suitable for sensitive measurement of numerous nucleosides and can easily be optimized for the determination of AZT, ddC and FT by variation of the methanolic component of the mobile phase and the detector wavelengths. AZT, ddC, FT and ddI concentrations in spiked human plasma samples can be determined after precipitation of proteins with ammonium sulphate. This method could be applied for the analysis of four different nucleosides, and therefore might be suitable for rapid and specific determination of other nucleosides.

ACKNOWLEDGEMENT

This study was supported by the Bundesminister für Forschung und Technologie (BMFT) (Bonn, F.R.G.), FKZ III-003-89.

REFERENCES

1 E. Matthes, Ch Lehmann, D Scholz, M von Janta-Lipinski, K Gaertner, H. A Rosenthal and P. Langen, *Biochem Biophys Res Commun.*, 148 (1987) 78.
2 P. Herdewijn, J. Balzarini, E De Clercq, R Pauwels, M Baba, S Broder and H. Vanderhaeghe, *J Med. Chem.*, 30 (1987) 1270
3 H. Mitsuya and S Broder, *Proc. Natl Acad. Sci U.S A* , 83 (1986) 1911.
4 S M. Tadepalli and R. P Quinn, *J Acquired Immune Defic Syndr* , 3 (1990) 19
5 R. Kupferschmidt and R W. Schmid, *Clin Chem.*, 35 (1989) 1313
6 S. S Good, D. J. Reynolds and P de Miranda, *J. Chromatogr.*, 431 (1988) 123.
7 R W Klecker, Jr , J M Collins, R. Yarchoan, R. Thomas, J F Jenkins, S. Broder and C. E Myers, *Clin Pharmacol. Ther* , 41 (1987) 407
8 M. A Hedaya and R. J Sawchuk, *Clin. Chem.*, 34 (1988) 1565
9 H Irth, R Tocklu, K. Welten, G. J de Jong, U A. Brinkmann and R W Frei, *J. Chromatogr.*, 491 (1989) 321
10 R. W. Klecker, Jr., J M Collins, R C Yarchoan, R. Thomas, N McAtee, S Broder and C. E. Myers, *J. Clin. Pharmacol* , 28 (1988) 837.
11 J. R Kalin and D Hill, *J. Chromatogr* , 431 (1988) 184
12 M. C Starnes and Y.-C. Cheng, *J Biol Chem* , 262 (1987) 988
13 D. A. Cooney, M. Dalal, H Mitsuya, J B McMahon, M Nadkarni, J Balzarini, S Broder and D G Johns, *Biochem. Pharmacol.*, 35 (1986) 2065
14 L. E Gustavson, E. K Fukuda, F A Rubin and A. W Dunton, *J Acquired Immune Defic. Syndr.*, 3 (1990) 28