Journal of Chromatography, 491 (1989) 215–220
Biomedical Applications
Elsevier Science Publishers B.V , Amsterdam — Printed in The Netherlands

CHROMBIO. 4721

Note

Reversed-phase liquid chromatographic determination of 2′,3′-dideoxycytidine in human blood samples

MAURO MAGNANI*, LUIGIA ROSSI, MARZIA BIANCHI, LUIGI CUCCHIARINI and VILBERTO STOCCHI

Istituto di Chimica Biologica, Università degli Studi di Urbino, Via Saffi 2, 61029 Urbino (Italy)

(First received October 21st, 1988; revised manuscript received February 6th, 1989)

2′,3′-Dideoxycytidine (ddCyd) is the most active of a series of 2′,3′-dideoxynucleosides against the infectivity and cytopathic effect of the etiologic agent of acquired immune deficiency syndrome (AIDS), the HIV retrovirus [1–3]. Very recently, in the first phase 1 study, ddCyd has confirmed in vivo its potential applicability in HIV infections [4], but its half-life ($t_{1/2}$) was calculated to be 1.2 h with most of the drug eliminated by renal clearance. Similar data were also found in mice and monkeys [5] where a more accurate pharmacokinetic study has provided evidence for a biphasic elimination curve with the α-phase $t_{1/2}$ of only 11 min. This rapid elimination and the fact that at high concentrations ddCyd has toxic effects [4] make the monitoring of this drug a necessity when clinical trials are performed. In this paper we present a reversed-phase high-performance liquid chromatographic (HPLC) method that permits the determination of ddCyd and its possible deamination product in only 20 min. Human plasma or whole blood samples can be used indifferently since the method also permits the simultaneous separation of the nucleotides most commonly present in blood samples.

0378-4347/89/$03 50

EXPERIMENTAL

Materials

Nucleotide standards of the highest grade available were purchased from Sigma (St. Louis, MO, U.S.A.). 2′,3′-Dideoxycytidine was obtained from Fluka (Buchs, Switzerland) and 2′,3′-dideoxyuridine was from Aldrich (Milan, Italy).

Analytical-grade KH_2PO_4 was obtained from Merck (Darmstadt, F.R.G.), HPLC-grade methanol was from Fluka, and double-distilled water was prepared in the laboratory. Centricon™ 30 and CF 50A Amicon membranes were purchased from Amicon (Danvers, MA, U.S.A.) and 0.22 μm-filters were from Millipore (Bedford, MA, U.S.A.).

Extractions

Perchloric acid (PCA) extracts and alkaline extracts were obtained following the methods previously described [6]. Samples of human plasma were also ultrafiltered by using the Amicon Centricon 30 microconcentrators by centrifugation at 2000 *g* in a refrigerated Beckman GPR centrifuge for 30 min. In this case a 50-μl aliquot of the ultrafiltrate was used for HPLC analysis.

Chromatographic apparatus and conditions

The HPLC system used was from Varian (Palo Alto, CA, U.S.A.) and consisted of two Model 2010 pumps, a Model 2020 solvent programmer and a Model 2050 variable-wavelength detector. Integration of peak areas was obtained by means of an HP 3390A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.). A 5-μm Supelcosil LC-18 column (250 mm$\times$4.6 mm I.D., Supelco, Bellefonte, PA, U.S.A.) protected by a guard column (Pelliguard LC-18, 20 mm$\times$4.6 mm I.D., pellicular packing material, 40-μm particles) was used throughout these studies. The mobile phase used for the separation of ddCyd and the other nucleotides commonly present in blood samples consisted of two eluents: a 0.1 *M* KH_2PO_4 solution (pH 6.0) (buffer A) and a 0.1 *M* KH_2PO_4 solution (pH 6.0) containing 10% (v/v) of methanol (buffer B). All buffer solutions, as well as standards and sample solutions, were filtered through a 0.22-μm membrane filter (Millipore).

The elution conditions used to obtain the chromatograms were: 1 min 100% buffer A, 4 min up to 10% buffer B, 4 min up to 40% buffer B, 3 min up to 100% buffer B and hold for 6 min. The gradient was then returned to 100% buffer A in 1 min and the initial conditions restored in 5 min. The flow-rate was 1.3 ml/min and the detection wavelength was 272 nm. The analyses were performed at room temperature.

Quantitative measurements were carried out by injection of standard solutions of known concentration. The molar absorption value used for standard calibration of ddCyd at 272 nm was 9.1.

Kinetics of ddCyd influx into erythrocytes

Assays of ddCyd influx into erythrocytes was performed at 20°C in 1.5-ml polypropylene microcentrifuge tubes, using 'oil-stop' methodology essentially as described by Zimmerman et al. [7], except that unlabelled ddCyd was used and determined by HPLC after PCA extraction of the red blood cell pellet.

RESULTS

HPLC analysis

Fig. 1A shows the separation of the most important compounds present in a PCA extract of human blood (ATP, ADP, AMP, $NADP^+$ and NAD^+) together with ddCyd. ddCyd is completely separated from interfering compounds, and the gradient used does not cause a significant baseline drift. It should be pointed out that no ddCyd deamination occurred in plasma, even after incubation for 30 min at 37°C (not shown), in agreement with other results [5].

Extraction

Different extraction procedures were tried to find a method that is simple and rapid and provides quantitative recovery of ddCyd. Both the PCA extraction procedure and the alkaline extraction provide recoveries ranging from 85 to 88% for human blood samples. Significantly lower recovery was observed if the blood samples were not diluted as we have previously suggested, because of non-specific entrapment of the drug and other blood nucleotides in the protein precipitate and in the precipitate after neutralization.

We have found plasma ultrafiltration to be a suitable alternative procedure to the PCA and alkaline extractions: 0.5 ml of plasma can be used and recoveries are usually complete (Fig. 1B and C). The only serious drawback of this method is that it measures only the plasma ddCyd concentration and not the blood ddCyd concentration. In fact, as shown below, equilibrium is reached between the intra- and extracellular ddCyd in vitro after ca. 30 min, but in vivo this time seems to be too long to be useful because as stated above, the α-$t_{1/2}$ of ddCyd elimination is in the order of 11 min.

Kinetics of ddCyd influx into erythrocytes

Preliminary experiments on the determination of ddCyd in plasma obtained from blood samples to which the drug was added showed that the concentrations found were always higher than expected on the basis of a rapid equilibrium between plasma and erythrocytes (not shown). To understand better this unusual property of a nucleotide, we performed kinetic studies of ddCyd influx into erythrocytes (not shown). Equilibrium is reached after 30 min at room temperature.

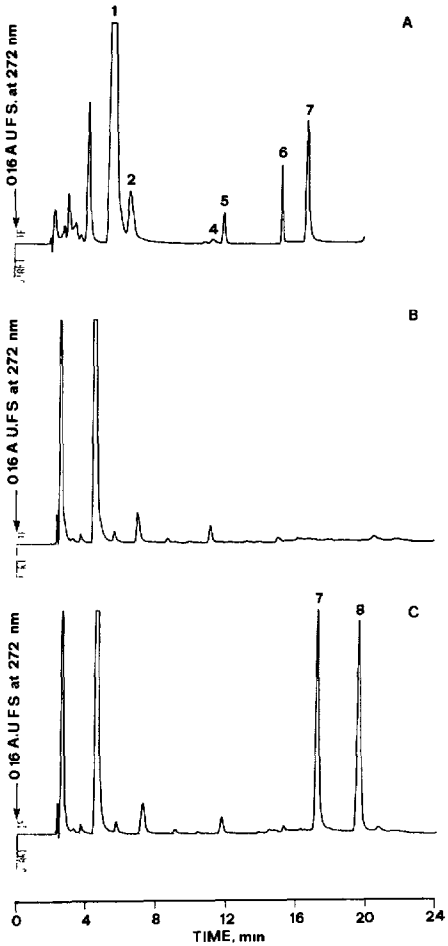

Fig. 1. Reversed-phase HPLC determination on a 5-μm Supelcosil LC-18 column (250 mm×4.6 mm I.D) of ddCyd added to a normal human blood sample and to human plasma. (A) ddCyd (120 nmol) was added to 1 ml of human blood, which was then extracted by the PCA procedure as described in Experimental. (B) Ultrafiltrate of human plasma. (C) Ultrafiltrate of human plasma to which 50 nmol/ml ddCyd and 2′,3′-dideoxyuridine were added. The chromatographic conditions were as described in Experimental; the detection wavelength was 272 nm. In each case 50 μl of extract or ultrafiltrate were used for the HPLC analysis. Peaks: 1=ATP; 2=ADP; 3=hypoxanthine (when present, the retention time is 8min); 4=AMP; 5=$NADP^+$; 6=NAD^+ 7=ddCyd; 8=2′,3′-dideoxyuridine.

Reproducibility, stability and sensitivity

Reproducibility was tested by preparing four separate extracts from the same blood sample. A coefficient of variation of 1% was found for ddCyd when the plasma ultrafiltration procedure was used and 2% for the PCA and alkaline extracts. ddCyd in blood is stable for several hours, both at room temperature

and in ice. No significant variations were observed over a period of 12 h. Stability was also checked by analysing the same PCA or alkaline extracts maintained at −20°C for two weeks without significant differences. The lowest ddCyd concentration detectable by this method is 0.01 m*M*.

DISCUSSION

ddCyd is in vitro the most active of a series of 2′,3′-dideoxynucleosides against the infectivity and cytophatic effect of HIV retrovirus, and it recently began a phase 1 clinical trial. However, pharmacokinetic studies [4,5] have shown that most of the drug is eliminated by renal clearance and that frequent repeated administrations are needed in order to maintain effective blood levels of this nucleoside. For these and other reasons a rapid and simple method for the detection of ddCyd in blood seems to be essential when patients are to be monitored or pharmacokinetic studies are done. Kelley et al. [5] have recently utilized plasma ultrafiltration and HPLC to quantify ddCyd and 2′,3′-dideoxyuridine. However, as we have shown in this paper, the plasma concentration probably does not represent the true blood concentration of the drug, since at the same time the drug is removed by renal clearance and an equilibrium is attained between plasma and red blood cells. As an alternative to this method, when the blood concentration of ddCyd is needed, we suggest the use of the PCA or alkaline extraction. These extraction procedures also permit the detection of all other nucleotides commonly present in human red blood cells and make the HPLC method proposed by Kelley et al. [5] unusable because of coelution of ddCyd with other UV-absorbing compounds. The HPLC method we propose overcomes all these problems because ddCyd and its deamination product, if present, are eluted completely separated from the other red blood cell nucleotides. The same HPLC procedure can also be used with plasma ultrafiltrates. As a routine procedure we prefer the PCA extraction method to the alkaline extraction, because although it requires the same length of time, it is considerably less expensive (the alkaline extraction involves the use of CF 50A Amicon ultrafiltration membranes). Particular attention should be given to the preparation of the PCA extract. We found that the ratio between blood and PCA is an important determinant of the drug recovery. If only one volume of blood is mixed with one volume of PCA the recovery should be of the order of 60%. If the concentration of the drug is high enough to be easily detected, a further dilution of blood, with respect to that proposed here, increases the recovery up to 95–98%. As an alternative, complete recovery of ddCyd can also be obtained using a 1:1 ratio of blood and PCA, but the protein precipitate and the perchloric precipitate after neutralization should be washed and combined with the sample.

CONCLUSION

The method described in this paper, based on PCA or alkaline extractions and reversed-phase HPLC, allows the simultaneous and accurate determination of ddCyd and 2′,3′-dideoxyuridine in human blood samples. The short analysis time and the absence of interferences make it useful for the study of the pharmacokinetics of this virustatic drug and for the monitoring of patients under treatment. Finally, the method can be used to determine ddCyd in plasma ultrafiltrates and also in other biological fluids.

ACKNOWLEDGEMENT

This work was supported by C.N.R. (Italy) P.F. "Biotecnologie e Biostrumentazione".

REFERENCES

1 H. Mitsuya and S. Broder, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 1911.
2 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.
3 H. Mitsuya and S. Broder, Nature, 325 (1987) 773.
4 R. Yarchoan, R.V. Thomas, J.P. Allain, N. McAtee, R. Dubinsky, H. Mitsuya, T.J. Lawley, B. Safai, C.E. Perno, R.W. Klecker, R.J. Wills, M.A. Fischl, M.C. McNeely, J.M. Pluda, M. Leuther, J.M. Collins and S. Broder, Lancet, i (1988) 76.
5 J.A. Kelley, C.L. Litterst, J.S. Roth, D.T. Vistica, D.G. Poplack, D.A. Cooney, M. Nadkarni, F.M. Balis, S. Broder and D.G. Johns, Drug Metab. Dispos., 15 (1987) 595.
6 V. Stocchi, L. Cucchiarini, M. Magnani, L. Chiarantini, P. Palma and G. Crescentini, Anal. Biochem., 146 (1985) 118.
7 T.P. Zimmerman, W.B. Mahony and K.L. Prus, J. Biol. Chem., 262 (1987) 5748.