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DETERMINATION OF CYTARABINE AND URACIL ARABINOSIDE IN HUMAN PLASMA AND CEREBROSPINAL FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of the antineoplastic agent cytarabine and its main metabolite uracil arabinoside in human plasma and cerebrospinal fluid is described. Complete separation from endogenous constituents was achieved by isocratic reversed-phase chromatography using phosphate buffer (0.05 *M*, pH 7.0) as the eluent. The limit of detection was 50 ng/ml. Day-to-day coefficients of variation were below 10%. The applicability of this rapid, simple and specific method for pharmacokinetic studies and monitoring of therapy was demonstrated.

INTRODUCTION

The pyrimidine analogue cytarabine (cytosine arabinoside; 1-β-D-arabinofuranosylcytosine; ara-C) is an antimetabolite that selectively inhibits DNA synthesis and is therapeutically effective in the treatment of acute myeloblastic leukaemia and other haematological malignancies [1—5].

Ara-C is rapidly deaminated in humans to uracil arabinoside (ara-U), a non-toxic compound [6, 7]. Owing to considerable individual variations in the pharmacokinetics of ara-C [8—11], the measurement of its concentration in plasma and cerebrospinal fluid is important for optimal dose scheduling [12]. Recently, extremely high doses of ara-C were successfully used for the treat-

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ment of acute leukaemia, refractory to conventional chemotherapy [13—15]. However, high doses of ara-C produce the risk of dangerously acute toxicity. Therefore, we monitored plasma levels of ara-C and its metabolite ara-U during and after infusions of conventional and high doses of ara-C using a rapid and sensitive high-performance liquid chromatographic (HPLC) method developed in our laboratory.

Several methods involving microbiological [16—21], radioenzymatic [22], radioimmunological assays [23—25] and some bioassay techniques [26, 27] have been employed for the analysis of the pharmacokinetics of ara-C. Some of these techniques are very sensitive (20 ng/ml). However, the analysis times are too long for clinical routine and the assays are subject to interferences from endogenous substances. An ultraviolet spectroscopic method for the determination of ara-C and ara-U in plasma lacks the sensitivity necessary for monitoring therapeutic concentrations in man [28]. The application of gas chromatography coupled with chemical ionization mass spectrometry [29] or the use of gas—liquid chromatography with a nitrogen-sensitive flame ionization detector and a coupled mass spectrometer [30] requires time-consuming steps of extraction and derivatization. Determination by paper chromatography [8, 31—33] or thin-layer chromatography [34] involves radioactive tracer technology (tritiated ara-C), which requires specialized handling and special permission for use in human studies.

HPLC has particular advantages for the analysis of ara-C and ara-U in that compounds may be analysed without initial derivatization. Wan et al. [8] emphasized the usefulness of HPLC but provided few details of the methodology of their assay. Kreis et al. [35] gave insufficient data about the scope and sensitivity of their HPLC assay. Bury and Keary [36] used cation-exchange chromatography for the determination of ara-C in human plasma, with a limit of sensitivity of 20 ng/ml. However, despite deproteinization of plasma samples with trichloroacetic acid the peak from endogenous plasma constituents was broad, allowing the measurement of ara-C as the tailing peak only. Ara-U could not be determined by this method.

This paper describes a rapid and simple reversed-phase HPLC assay for ara-C and ara-U suitable for drug monitoring and pharmacokinetic studies in human plasma and cerebrospinal fluid.

EXPERIMENTAL

Materials

Ara-C, ara-U, ara-C-5′-monophosphate, ara-C-5′-triphosphate and tetrahydrouridine were kindly supplied by Mack (Illertissen, G.F.R.). Tests for interfering peaks were performed with allopurinol, oxipurinol, uric acid (Henning, Berlin, G.F.R.), xanthine, hypoxanthine (Merck, Darmstadt, G.F.R.), cytidine (Sigma, St. Louis, MO, U.S.A.), methotrexate, leucovorin (Cyanamid, Munich, G.F.R.), 6-mercaptopurine, 6-thioguanine, 6-thiouric acid (Burroughs Wellcome, London, Great Britain) and doxorubicin (Farmitalia, Freiburg, G.F.R.). All other substances and solvents were of analytical-reagent grade and were used without further purification.

Chromatography

The chromatographic studies were performed with a high-pressure liquid chromatograph equipped with a Gynkotek Model 600/200 HPLC pump (Gynkotek, Munich, G.F.R.), a modified ASI 45 automatic sample injector (Kontron, Eching, G.F.R.), an Uvikon 720 LC variable-wavelength ultraviolet detector (Kontron), an SP 4100 computing integrator (Spectra-Physics, Darmstadt, G.F.R.) and a BD9 two-channel electronic recorder (Kipp and Zonen, Kronberg/Ts., G.F.R.).

Chromatography was performed in the reversed-phase mode using as eluent a phosphate buffer (0.05 *M*, pH 7.0), which was prepared by mixing 1 l of 0.05 *M* disodium phosphate (8.99 g of $Na_2HPO_4 \cdot 2H_2O$ per litre of distilled water) with 704 ml of 0.05 *M* potassium phosphate (6.87 g of anhydrous KH_2PO_4 per litre of distilled water).

The chromatographic column was a stainless-steel tube (30 cm × 4 mm I.D.) filled with Spherisorb ODS, 5 μm (Latek, Heidelberg, G.F.R.). The column was packed by forcing a slurry of 4.2 g of the stationary phase material suspended in 25 ml of carbon tetrachloride into the tube under a pressure of 400 bar by means of a Type MS 80/8 high-pressure membrane pump (Orlita, Giessen, G.F.R.). In general, the efficiency of the column was about 10,000 theoretical plates for the ara-C peak. The column was discarded when the number of theoretical plates for ara-C fell to less than 1000.

The chromatograph was operated at a flow-rate of 1.6 ml/min and a pressure of 210 bar at room temperature. The eluent was monitored at 270 nm, the absorption maximum of ara-C in 0.05 *M* phosphate buffer at pH 7.0. The detector was set at 0.04 a.u.f.s. The areas under the peaks of interest were computed by the integrator.

Assay procedure

Samples of 1 ml of blood were collected in tubes containing 75 units of heparin (15 μl of Thrombophob; Nordmark, Hamburg, G.F.R.) and tetrahydrouridine (final concentration 10^{-3} *M*), a blood deaminase inhibitor. After centrifugation at 8000 *g* for 5 min (Microfuge 5412; Eppendorf, Hamburg, G.F.R.), plasma samples of 10—50 μl were immediately chromatographed or stored frozen at −16°C.

Samples of 1 ml of cerebrospinal fluid were collected in tubes containing tetrahydrouridine (final concentration 10^{-3} *M*).

As aliquots of plasma or cerebrospinal fluid were injected into the chromatographic system without further clean-up, the method of external standardization was used for quantitation. Every sample was analysed in duplicate, and a third analysis was performed if the peak areas of the compounds did not agree to within ± 5%. The calibration graphs (peak area against concentration) were straight lines for each compound up to 120 μg/ml. Therefore, calibration samples containing 1 μg of each substance per millilitre of plasma were analysed every ten chromatographic runs for external standardization.

Protein binding

For the determination of protein binding, ara-C and ara-U were measured in plasma and in the plasma ultrafiltrate of spiked samples of plasma. Ultrafiltration was performed using a Model MM 302 ultrafiltration system (Amicon, Düren, G.F.R.) and Type PM 10 Diaflo membranes. In the controls there was no adsorption of either substance to this membrane.

RESULTS AND DISCUSSION

Plasma extraction

Attempts to extract ara-C and ara-U from aqueous solutions at different pHs with organic solvents (diethyl ether, dichloromethane, isooctane, *n*-butanol, isopropanol) gave poor recoveries even if the aqueous solutions were saturated with ammonium sulphate. The resolution of ara-C and ara-U from interfering plasma peaks was not improved by a preceding purification step, e.g., protein precipitation by trichloroacetic acid or ethanol. Protein removal by ultrafiltration using membranes which exclude substances with molecular weight >10,000 did not improve the separation. Therefore, plasma samples were used directly for HPLC without prior clean-up.

Chromatography

The effect of pH of 0.05 *M* phosphate buffer on the resolution of ara-C and are-U from plasma peaks was investigated. Optimal resolution was obtained at

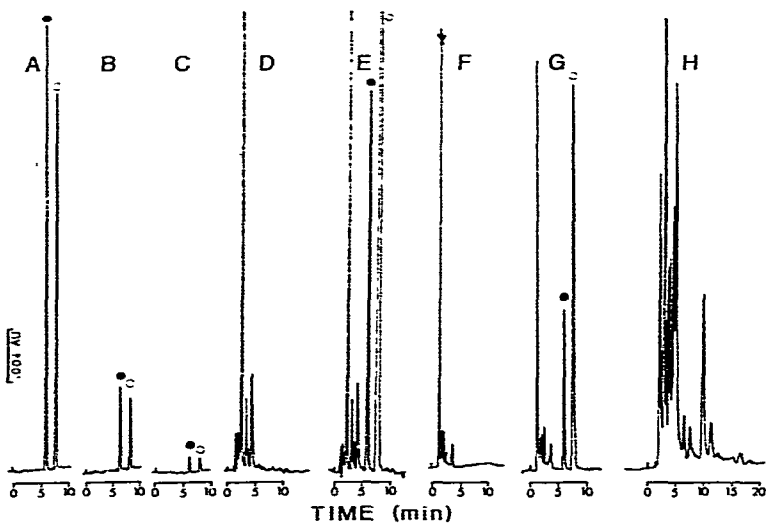

Fig. 1. High-performance liquid chromatograms of ara-C (●) and ara-U (○). Traces A—C; chromatograms of ara-C and ara-U dissolved in 0.05 *M* phosphate buffer (pH 7.0). Amounts chromatographed: (A) 5, (B) 1 and (C) 0.2 μg of each compound per millilitre. Traces D and E; chromatograms of plasma from a leukaemic patient, (D) before and (E) during infusion of ara-C. Traces F and G: chromatograms of cerebrospinal fluid from the same patient, (F) before and (G) at the end of intravenous infusion of ara-C. Trace H: chromatogram of drug-free urine, diluted 1:20 in distilled water. Stationary phase: Spherisorb ODS, 5 μm (30 cm × 0.4 cm I.D.). Mobile phase: 0.05 *M* phosphate buffer (pH 7.0). Sample volume: 20 μl. Flow-rate: 1.6 ml/min. Back-pressure: 210 bar. Room temperature. Detection at 270 nm.

pH 7.0. The use of pH 7 phosphate buffers at different molarities was investigated and it was found that the resolution was highest at values above 0.03 *M*. Of the several available reversed-phase materials, Spherisorb ODS, 5 μm, was the best stationary phase with respect to efficiency, permeability and stability. Therefore, chromatography was performed on a Spherisorb column (30 cm × 0.4 cm I.D.) with phosphate buffer (0.05 *M*, pH 7.0) as eluent. As shown in Fig. 1, the peaks of interest were sharp, symmetrical and well defined with respect to the baseline. Components of plasma (Fig. 1D and E) and cerebrospinal fluid (Fig. 1F and G) did not interfere in regions where ara-C and ara-U eluted. However, in urine samples the determination of both compounds was disturbed by endogenous components (Fig. 1H).

Specificity

No interfering peaks were found in plasma samples to which were added drugs that are usually coadministered for the treatment of acute leukaemia, e.g., methotrexate, 6-mercaptopurine, 6-thioguanine (6-thiouric acid), doxorubicin and allopurinol (oxipurinol, xanthine, hypoxanthine). Cytidine, tetrahydrouridine and the intracellularly occurring ara-C metabolites ara-C-5′-monophosphate and ara-C-5′-triphosphate were eluted together with components of plasma. Twenty-five drug-free blood samples obtained from healthy volunteers were processed and there was no evidence that normal components of plasma interfered with the determination of ara-C and ara-U.

Identification

Retention times were used for the identification of ara-C and ara-U. The variation of retention time was less than 5%, as demonstrated with spiked samples of plasma.

Quantitation

Unknown substances were evaluated by measuring their peak areas relative to that of the external standard. For ara-C and ara-U a linear relationship between peak area and concentration was obtained for the range 0.05—120 μg/ml. The correlation coefficient of each regression was better than 0.99.

Precision

Within-run precision was established in a drug-free plasma pool. Ara-C and ara-U were added at seventeen different concentrations within the range 0.05—120 μg/ml. For each concentration the within-run precision of ten consecutive runs was determined with a coefficient of variation of less than 5%. The day-to-day precisions as determined on ten consecutive days for frozen samples of plasma at levels of 0.2 and 1.0 μg/ml were found to be 9.3 and 7.0%, respectively.

Accuracy

The recoveries from blood and plasma were 93—104% by comparison with the peak areas obtained by direct injection of the pure compounds.

Sensitivity

The sensitivity of the assay allowed the quantitation of 50 ng/ml of ara-C and ara-U in a 1-ml blood sample with a precision of better than 5%.

Speed of analysis

The analysis is performed within 15 min: 5 min for centrifugation of blood and 10 min for chromatography.

Stability of standards

Ara-C and ara-U were found to be stable in human blood and plasma in the presence of tetrahydrouridine (10^{-3} *M*) and heparin during a 4-h incubation at 37°C. The same was true on storage for 8 h in 0.05 *M* phosphate buffer (pH 7.0) at room temperature. Frozen samples of plasma (0.2 and 1 μg/ml) were stable for at least 10 months.

Stability of the column

Baseline separations of ara-C and ara-U from plasma were obtained for up to 200 injections of 20-μl samples of plasma.

Protein binding

Plasma protein binding of ara-C was found to be 14.8 ± 4.7% (mean ± S.E.) for the concentration range 0.1—100 μg/ml. This value is close to that reported by Van Prooijen et al. [37], who obtained 13.3 ± 2.2% by ultrafiltration and ultracentrifugation. Protein binding of ara-U was 5.1 ± 4.9% (mean ± S.E.).

Applicability

The HPLC method described was used for the determination of ara-C and ara-U in plasma of leukaemic patients who were receiving conventional or high doses of ara-C. A representative plot of plasma concentration versus time for a patient following a 6-h infusion of 120 mg of ara-C (70 mg/m^2) is shown in Fig. 2A. Plasma pharmacokinetics of ara-C and ara-U for a patient who was treated with infusions of high doses of ara-C (2.6 g/m^2 · 2 h) are shown in Fig. 2B.

At the end of infusions the concentration of ara-C in cerebrospinal fluid was found to be 10—15% of simultaneous plasma concentrations.

The method described in this paper is rapid, sensitive and specific. In comparison with the method of Bury and Keary [36] our procedure requires no clean-up procedures before chromatography. By using reversed-phase chromatography instead of cation-exchange chromatography, baseline separations of ara-C and ara-U from plasma constituents are obtained. The analysis is very rapid and results are obtained within 15 min. The assay can be run using as little as 100 μl of plasma, making it appropriate for clinical use even with paediatric patients.

The method is sufficiently sensitive to monitor ara-C and its metabolite at the low concentrations usually found in blood when conventional doses of ara-C are applied to patients. Plasma levels of ara-C can be monitored during intravenous infusion so that a proper infusion rate can be used for establishment of cytotoxic concentrations of ara-C for leukaemic cells. In addition,

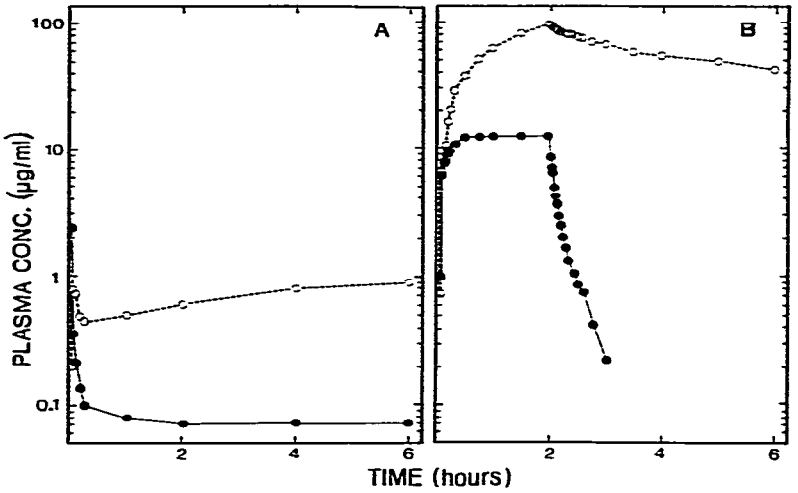

Fig. 2. Time course of plasma concentrations of ara-C (●) and ara-U (○) during and after infusions of ara-C. A, Continuous 6-h infusion of 120 mg of ara-C (70 mg/m²), which was preceded by an initial loading dose of 40 mg of ara-C (bolus injection). B, Continuous 2-h infusion of a high dose of ara-C (2.6 g/m²). Each point represents the mean value of three infusions performed at 12-h intervals.

rapid monitoring of ara-C in blood and cerebrospinal fluid is a valuable means for avoiding toxic concentrations during the infusion of high doses of ara-C.

Our assay has been in routine use for more than 1 year. It is simpler than the other methods available for the analysis of ara-C in clinical samples and can be applied to research, pharmacokinetic studies and monitoring of therapy.

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REFERENCES

1 M.Y. Chu and G.A. Fischer, Biochem. Pharmacol., 11 (1962) 423.
2 R.R. Ellison, J.F. Holland, M. Weil, C. Jacquillat, M. Boiron, J. Bernard, A. Sawitsky, F. Rosner, B. Gussof, R.T. Silver, A. Karanas, J. Cuttner, C.L. Spurr, D.M. Hayes, J. Blom, L.A. Leone, F. Haurani, R. Kyle, J.L. Hutchinson, R.J. Forcier and J.H. Moon, Blood, 32 (1968) 507.
3 T.S. Gee, K.-P. Yu and B.D. Clarkson, Cancer, 23 (1969) 1019.
4 I. Fleming, J. Simone, R. Jackson, W. Johnson, T. Walters and C. Mason, Cancer, 33 (1974) 427.
5 W.B. Kremer, Ann. Int. Med., 82 (1975) 684.
6 G.W. Camiener and C.G. Smith, Biochem. Pharmacol., 14 (1965) 1405.
7 W.A. Creasey, R.J. Papac, M.E. Markiw, P. Calabresi and A. Welch, Biochem. Pharmacol., 15 (1966) 1417.

8 S.H. Wan, D.H. Huffman, D.L. Azarnoff, B. Hoogstraten and W.E. Larsen, Cancer Res., 34 (1974) 392.
9 D.H.W. Ho and E. Frei, Clin. Pharmacol. Ther., 12 (1971) 944.
10 R. van Prooijen, E. van der Klein and C. Haanen, Clin. Pharmacol. Ther., 21 (1977) 744.
11 A.L. Harris, C. Potter, C. Bunch, J. Boutagy, D.J. Harvey and D.G. Grahame-Smith, Brit. J. Clin. Pharmacol., 8 (1979) 219.
12 H.E. Skipper, F.M. Schnabel, L.B. Mellett, J.A. Montgomery, L.J. Wilkoff, H.H. Lloyd and W. Brockman, Cancer Chemother. Rep., 54 (1970) 431.
13 S.A. Rudnick, E.C. Cadman, R.L. Capizzi, R.T. Skeel, J.R. Bertino and S. McIntosh, Cancer, 44 (1979) 1189.
14 Ch. Karanes, S.N. Wolff, G.P. Herzig, G.L. Phillips, H.M. Lazarus and R.H. Herzig, Blood (Suppl.), 54 (1979) 191a.
15 H. Breithaupt, H. Pralle, Th. Eckhardt, M. von Hattingberg, J. Schick and H. Löffler, Cancer, submitted for publication.
16 R.F. Pittillo and D.E. Hunt, Proc. Soc. Exp. Biol. Med., 124 (1966) 636.
17 H.H. Buskirk, Proc. Tissue Culture Assoc., 20 (1969) 23.
18 J. Borsa, G.F. Whitmore, F.A. Valeriote, D. Collins and W.R. Bruce, J. Nat. Cancer Inst., 42 (1969) 235.
19 L.J. Hanka, S.L. Kuentzel and G.L. Neil, Cancer Chemother. Rep., 54 (1970) 393.
20 B.M. Mehta, M.B. Meyers and D.J. Hutchison, Cancer Chemother. Rep., 59 (1975) 515.
21 L.B. Mellett, P.J. Wyatt and C. Woolley, Res. Commun. Chem. Pathol. Pharmacol., 20 (1978) 379.
22 R.L. Momparler, A. Labitan and M. Rossi, Cancer Res., 32 (1972) 408.
23 T. Okabayashi, S. Mihara, D.B. Repke and J.G. Moffatt, Cancer Res., 37 (1977) 619.
24 T. Okabayashi, S. Mihara and J.G. Moffatt, Cancer Res., 37 (1977) 625.
25 T. Okabayashi, S. Mihara, D.B. Repke and J.G. Moffatt, Cancer Res., 37 (1977) 3132.
26 B.C. Baguley and E.-M. Falkenhaug, Cancer Chemother. Rep., 55 (1971) 291.
27 H.C. van Prooijen, G. Vierwinden, J. van Egmond, J.M.C. Wessels and C. Haanen, Eur. J. Cancer, 12 (1976) 899.
28 R.L. Furner, R.W. Gaston, J.D. Strobel, S. El Dareer and L.B. Mellett, J. Nat. Cancer Inst., 52 (1974) 1521.
29 C. Pantarotto, A. Martini, G. Belvedere, A. Bossi, M.G. Donelli and A. Frigerio, J. Chromatogr., 99 (1974) 519.
30 J. Boutagy and D.J. Harvey, J. Chromatogr., 146 (1978) 283.
31 K. Uchida and W. Kreis, Biochem. Pharmacol., 18 (1969) 1115.
32 M.R. Dollinger, J.H. Burchenal, W. Kreis and J.J. Fox, J. Biochem. Pharmacol., 16 (1969) 689.
33 D.H.W. Ho, N.S. Brown, J. Benvenuto, K.B. McCredie, D. Buckels and E.J. Freireich, Clin. Pharmacol. Exp. Ther., 22 (1977) 371.
34 W. Kreis, A. Greenspan, T. Woodcock and C. Gordon, J. Chromatogr. Sci. 14 (1976) 331.
35 W. Kreis, C. Gordon, C. Gizoni and T. Woodcock, Cancer Treat. Rep., 61 (1977) 643.
36 R.W. Bury and P.J. Keary, J. Chromatogr., 146 (1978) 350.
37 H.C. van Prooijen, G. Vierwinden, J. Wessels and C. Haanen, Arch. Int. Pharmacodyn. Ther., 229 (1977) 199.