Journal of Chromatography, 276 (1983) 395—401
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1758

SIMULTANEOUS ANALYSIS OF 5′-DEOXY-5-FLUOROURIDINE AND 5-FLUOROURACIL IN PLASMA BY ANALYTICAL ISOTACHOPHORESIS

BENGT GUSTAVSSON*, OLLE ALMERSJÖ, MAGNUS BERNE and
JOHAN WALDENSTRÖM

Department of Surgery, Östra sjukhuset, and Department of Clinical Chemistry, University of Gothenburg, S-416 85 Gothenburg (Sweden)*

(First received December 16th, 1982; revised manuscript received April 1st, 1983)

SUMMARY

Analytical isotachophoresis was used for the determination of 5-fluorouracil and 5′-deoxy-5-fluorouridine in plasma. The inclusion of spacers in the system greatly improved the separation and quantitation. The method can be employed for simultaneous measurements of different fluorinated pyrimidines used in clinical practice.

INTRODUCTION

5′-Deoxy-5-fluorouridine (5′-dFUR) is a recently synthesized fluoropyrimidine nucleoside (Roche 21-9738) reported in preliminary investigations to have an antineoplastic activity in vivo against several rat and murine tumor lines superior to that of 5-fluorouracil (5-FU), 2′-deoxy-5-fluorouridine and ftorafur [N_1-(2′-furanidyl-5-fluorouracil] [1, 2]. The results observed with 5′-dFUR show a dramatic reduction in host toxicity. Further, an excellent activity has been observed after per oral administration [3].

The mechanism through which 5′-dFUR induces its antineoplastic activity is not known. In vitro studies have not revealed evidence for the formation of an active novel metabolite in sensitive Ehrlich ascites tumor cells [4]. In investigations of its cellular metabolism it has been found that 5′-dFUR is converted to 5-FU by the enzyme uridine phosphorylase. There is a greater activity of this enzyme in tumor tissues than in normal tissues. No doubt 5′-dFUR is an interesting new fluoropyrimidine. The present method was worked out as a tool for pharmacokinetic studies of this new 5-FU derivative.

We have recently reported that 5-FU can be analysed in body fluids by isotachophoresis [5, 6]. The principle of isotachophoresis has been known for

0378-4347/83/$03.00 © 1983 Elsevier Science Publishers B.V.

many years, but the technique has not been used much until recently [7].

The tachophor used was equipped with an UV absorbance detector which gives an immediate response to an absorbing zone, and because of the high resolution of the detector the quantitative evaluation of the UV signal is easy. By using non-UV-absorbing compounds as discrete spacers, the identification and quantitation of fluorinated pyrimidines in biological samples can be improved.

In this paper we describe a specific and sensitive assay for 5-FU and 5′-dFUR in plasma by analytical isotachophoresis.

MATERIAL AND METHODS

Chemicals

5-FU and 5′-dFUR were supplied by Roche AB, Stockholm, Sweden. HPMC (hydroxypropylmethylcellulose), Methocel 90HG, 15,000 CPS, was obtained from Dow Chemical Company, Midland, MI, U.S.A. The resin Ag 1-X8 (formate), 200–400 mesh, was purchased from Bio-Rad Labs., Richmond, CA, U.S.A. All other chemicals used were of analytical grade and commercially available.

Instrumentation

The isotachophoretic separations were performed in an LKB 2127 Tachophor (LKB Instruments, Stockholm, Sweden). The UV signal (254 nm) was recorded at a chart speed of 6–10 cm min^{-1}. The separations were carried out in a 23-cm standard capillary.

Pretreatment of plasma samples

A 1-ml volume of plasma was diluted to 5 ml with picric acid [5]. The precipitate formed was removed by centrifugation at 3000 *g* for 5 min. The supernatant was brought to pH 11 by the addition of 0.1 mol/l potassium hydroxide solution. The Ag 1-X8 resin was equilibrated with 5 volumes of 6 mol/l formic acid for 1 h. Pasteur pipettes (150 × 5 mm) were used as columns. The resin was washed with 30 column volumes of glass-distilled water. The flow-rate was approximately 1 ml/min. The deproteinized plasma sample was loaded onto the column, then 20 column volumes of glass distilled water were passed through the column and discarded. 5-FU and 5′-dFUR were eluted with 5 column volumes (3.5 ml) of 0.1 mol/l formic acid and evaporated to dryness at 60°C under a gentle stream of nitrogen (approx. 60 min). Ten columns can be handled simultaneously. The evaporated sample was dissolved in 20 µl of water and 1–4 µl were then injected into the Tachophor. A second injection with spacer solution, 1–3 µl, was then performed.

Quantitation

In isotachophoresis the zone length is directly proportional to the amount of a compound in equilibrium with the leading electrolyte [8].

In this paper the zone widths at half the peak height was determined when more than 100 pmol were injected. In the range 10–100 pmol of a compound

the peak heights of the ions were measured. Zones representing 5-FU and 5′-dFUR were measured and quantitated by comparison with standard curves [5].

Reproducibility and linearity

Calibration curves were constructed by adding increasing amounts of 5-FU and 5′-dFUR to pooled plasma from healthy blood donors. The addition of 5-FU and 5′-dFUR was followed by immediate deproteinization. Day-to-day variations were tested by repeat runs of pooled plasma samples. The within-day variation was determined in the same way. Further, plasma from cancer patients treated by intravenous bolus injections or slow intravenous infusions was used for analysis. For these samples 5′-dFUR 0.1—20 nmol were added to 1 ml of plasma.

RESULTS

Extraction procedure

5-FU and 5′-dFUR cannot be quantitatively separated by isotachophoresis when whole plasma is injected into the Tachophor. Deproteinization with picric acid and ion-exchange chromatography were therefore used as purification steps. The recovery of known amounts of 5-[^{3}H]FU and 5′-dFUR subjected to the extraction procedure was 88 ± 0.8% and 78 ± 1.2%, respectively, as calculated from ten separate experiments.

Determination of optimal isotachophoretic conditions

In aqueous solution 5-FU and 5′-dFUR is well separated with 5 mmol/l leading electrolyte at a pH interval of 7.0—8.6. However, in order to obtain a perfect separation of 5-FU and 5′-dFUR in plasma the pH of the leading electrolyte is critical. Two different leading electrolyte systems were finally selected. In samples where other compounds such as allopurinol, hypoxanthine and oxypurinol are to be quantitated together with fluorinated pyrimidines a pH of 8.4 was selected (Table I). In samples where only 5-FU and 5′-dFUR are to be quantitated a pH of 7.6 is convenient. The terminator finally selected was 40 mmol/l of recrystallized glycine with barium hydroxide added to pH 9.4.

TABLE I

ELECTROLYTE SYSTEM FOR THE SEPARATION OF 5-FLUOROURACIL AND 5′-DEOXY-5-FLUOROURIDINE

	Leading electrolyte	Terminating electrolyte
Anion	Cl^-	Glycine*
Concentration	0.005 *M*	0.04 *M*
Counterion	Tris	Ba^{2+}
pH	8.4	9.4
Additive	0.25% HPMC	None

*Glycine recrystallized.

During the investigation different lengths of capillary were used. For routine purposes a capillary 23 cm long (I.D. 0.5 mm) was satisfactory. The analysis time was 20—23 min at 18°C. To reduce the separation time, the experiments were started at an elevated current (95 μA) which was reduced to 45 μA prior to detection. As spacers, 4-morpholineethanesulfonic acid (MES), 4-morpholinepropanesulfonic acid (HEPES), and tris(hydroxymethyl)methylaminopropanesulfonic acid (TAPS) were used (Table II).

TABLE II

SPACER COMPOUNDS FOR SEPARATION OF 5-FLUOROURACIL AND 5'-DEOXY-5-FLUOROURIDINE

The leading electrolyte, 0.005 *M* HCl in 0.25% HPMC, was titrated with Tris to pH 8.4. The terminating electrolyte, 0.04 *M* glycine, was titrated with saturated $Ba(OH)_2$ to pH 9.4.

	pK_a
4-Morpholineethanesulfonic acid (MES)	6.5
4-Morpholinepropanesulfonic acid (MOPS)	7.20
N-2-(Hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)	7.5
4,2-(Hydroxyethyl)1-piperazineethanesulfonic acid (HEPES)	7.55
Tris(Hydroxyethyl)methylaminopropanesulfonic acid (TAPS)	8.40

Calibration and quantitation

Fig. 1 shows an isotachopherogram of a normal plasma sample where known amounts of 5-FU and 5'-dFUR have been added to the plasma pretreated as described above. To identify 5'-dFUR in this experiment the sample was rerun together with pure 5'-dFUR (Fig. 2) using the non-UV-absorbing spacers. The resolution of the separation was greatly improved. In Fig. 3 a plasma sample from a patient receiving 5-FU combined with allopurinol is demonstrated. It was empirically found that the peak height was proportional to the 5-FU and 5'-dFUR concentration in the range 10—80 pmol. This had earlier been demonstrated for hypoxanthine [9].

Reproducibility

The within-day coefficient of variation was calculated from the results of ten separate runs at two different concentrations of 5-FU and 5'-dFUR, 1.0 and 20.0 μmol/l. The coefficients of variation for 5-FU and 5'-dFUR were 7% and 8%, respectively, for the low concentration and 4% and 5%, respectively, for the high concentration.

Application to biological samples

Blood samples were taken from patients with gastrointestinal cancer during 5-FU therapy. The concentration of 5-FU after intravenous bolus injection of 15 mg kg^{-1} body weight reached its highest level 5 min after injection and varied between 120 and 260 μmol/l (four patients), which is in good agreement with the concentrations reported after the same dose schedule analysed by gas chromatography—mass spectrometry [10].

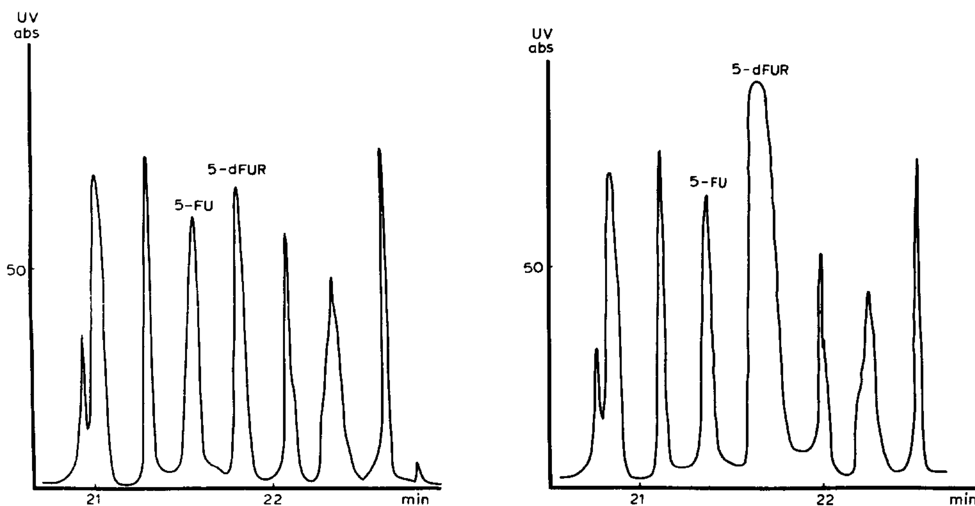

Fig. 1. Isotachopherogram of plasma sample; 2 μl were injected into the Tachophor (corresponding to 0.1 ml of plasma). The leading electrolyte was 0.005 *M* HCl in 0.25% HPMC titrated with Tris to pH 8.4. The terminating electrolyte, 0.04 *M* glycine, was titrated with saturated $Ba(OH)_2$ to pH 9.4. Capillary 43 cm long, current 45 μA. The wavelength of 254 nm was recorded and the temperature was 18°C. A 2-μl volume of spacer solution (see Table II) was also injected.

Fig. 2. Isotachopherogram of the same plasma sample as in Fig. 1 together with injection of 1 nmol of pure 5′-dFUR. The working conditions were the same as in Fig. 1.

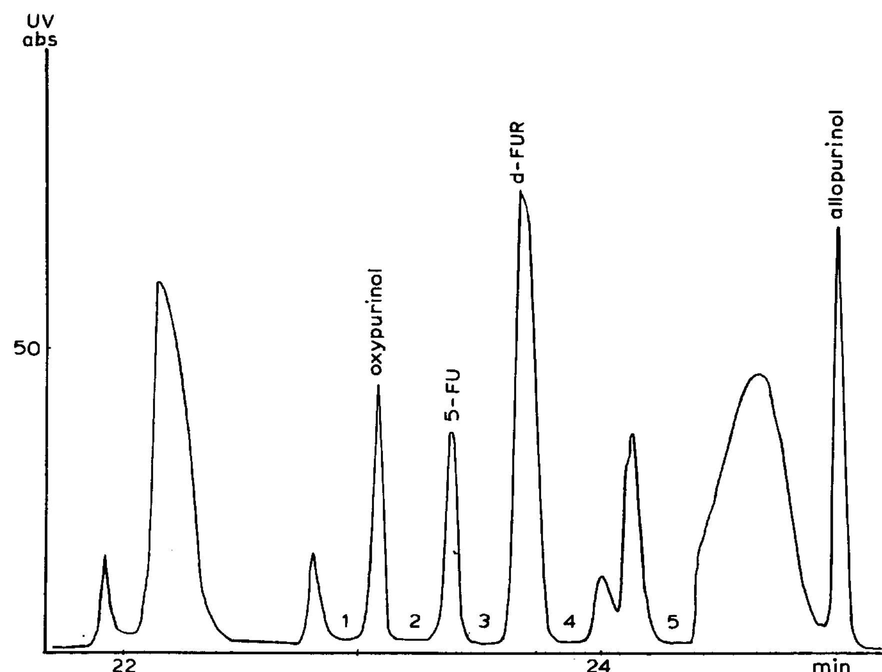

Fig. 3. Isotachopherogram of plasma sample from a patient treated with a continuous infusion of 5-FU, 15 mg kg^{-1} 24 h^{-1} and allopurinol 100 mg × 3 per os. A 2-μl volume was injected (corresponding to 0.1 ml of plasma) together with 3 μl of spacer solution (see Table II).

DISCUSSION

The results of the present study indicate that analytical isotachophoresis is a sensitive and specific method which can be employed for simultaneous measurements of different fluorinated pyrimidines and oxipurines. As previously reported, prepurification of biological samples is necessary to obtain an optimal separation. We have earlier reported that deproteinization with picric acid followed by ion-exchange chromatography is a simple and accurate procedure for this purpose [5].

In isotachophoresis the resolved zones are forced to run in immediate contact with each other. This can lead to practical difficulties as reported in the original method description [5]. As early as 1965 Vestermark [11] drew attention to this problem and suggested addition of intermediate-mobility compounds to the sample solution.

Briefly, spacing can be obtained by using a continuous gradient or by using discrete spacers [12]. In principle, a discrete spacer is a single compound chosen to have a mobility intermediate to those of two sample compounds of interest. In this method we have used non-UV-absorbing compounds and found that this technique is extremely useful in order to improve identification and quantitation of 5-FU and 5′-dFUR, especially in samples with low (< 100 pmol) concentrations, or in samples where other purines or pyrimidines are to be quantitated. By using spacers, the pH in the leading electrolyte will not be as critical as reported in our original method to obtain optimal separation. Thus, as demonstrated in Fig. 3, the spacer MOPS and TES "framed" the 5-FU zone while the spacer HEPES moved directly behind the 5′-dFUR zone. It was further found that in patients given allopurinol, the parent drug and its metabolite oxipurinol were eluted in the same fraction as the fluorinated pyrimidines. It thus seems likely that the isotachophoretic technique described should be readily adapted for simultaneous analysis of not only fluorinated pyrimidines. In fact, Oerlemans et al. [13] recently reported that isotachophoresis offers a simple and rapid way to determine urinary purines and pyrimidines. These authors further concluded that the high reproducibility (day-to-day) variation and repeatability), and short analysis time justify the conclusion that isotachophoresis offers interesting possibilities for the analysis of UV-absorbing compounds both for experimental and diagnostic purposes.

Until now, to our knowledge, only one method for the determination of 5′-dFUR has been reported. Using high-performance liquid chromatography Armstrong and Diasio [4] reported that using a 50-μm C_8 reversed-phase column with a 5-μm reversed-phase precolumn, 5-FU and 5′-dFUR could be separated in plasma. The retention time for 5′-dFUR was 46 min.

This present study demonstrates that isotachophoresis offers possibilities for the determination of 5′-dFUR and other fluorinated pyrimidines in plasma. Since 5′-dFUR represents an interesting new fluoropyrimidine with clinical potential the described method could be used for further studies of the physiological disposition of this drug.

ACKNOWLEDGEMENT

This study was supported by the Swedish Cancer Society.

REFERENCES

1 M.J. Kramer, P.W. Trown, R. Cleeland, A.F. Cook and E. Grunberg, Proc. Amer. Ass. Cancer Res., 20 (1979) 20.
2 R.D. Armstrong and R.B. Diasio, Proc. Amer. Ass. Cancer Res., 20 (1979) 260.
3 W. Bollag and H.R. Hartmann, Eur. J. Cancer, 16 (1980) 427.
4 R.D. Armstrong and R.B. Diasio, Cancer Res., 40 (1980) 3333.
5 B. Gustavsson, A. Baldesten, P.O. Hasselgren and O. Almersjö, J. Chromatogr., 179 (1979) 151.
6 B. Gustavsson and A. Baldesten, in A. Adam and C. Schots (Editors), Biochemical and Biological Applications of Isotachophoresis, Elsevier Amsterdam, 1980, pp. 89—94.
7 L. Arlinger, J. Chromatogr., 91 (1974) 785.
8 L. Arlinger, Protides Biol. Fluids, 22 (1975) 737.
9 B. Gustavsson, R.M. Olsson and J. Waldenström, Anal. Biochem., 122 (1982) 1.
10 C. Finn and W. Sadée, Cancer Chemother. Rep. Part I, 59 (1975) 279.
11 A. Vestermark, Report from the Department of Biochemistry, University of Stockholm, No. 5, 1966.
12 A. Kopwillem, W.G. Merriman, R.M. Cuddebach, A.J.K. Smolka and M. Bier, J. Chromatogr., 118 (1976) 35.
13 F. Oerlemans, C. De Bruyn, F. Mikkers Th. Verheggen and F. Everaerts, in F.M. Everaerts (Editor), Analytical Isotachophoresis, Elsevier, Amsterdam, 1981, pp. 189—194.