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NEW ASSAY OF 5-FLUOROURACIL IN SERUM BY ISOTACHOPHORESIS

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SUMMARY

Blood levels of 5-fluorouracil are quantitatively determined by isotachophoresis. Serum is deproteinized, purified on an ion-exchange column and concentrated to 20 μl, and the drug is measured isotachophoretically. Down to 50 pmol (6.5 ng) of the drug can be determined in serum with a methodological error of $\pm$ 6%. The method can be used for routine control of patients undergoing therapy with the drug.

INTRODUCTION

5-Fluorouracil (5-FU) is one of the drugs most commonly used for palliative treatment of solid tumours of the breast and of the gastrointestinal tract[1,2]; it is used either alone or in combination with other chemotherapeutic agents[3–5]. Since its introduction some 20 years ago, various dose regimens and routes of administration have been used in an attempt to reduce side-effects and enhance the therapeutic response. Individual therapy is an important concept in clinical pharmacology, and several analytical methods for determining the serum concentration of 5-FU have been developed in efforts to improve and control the administration. The spectrophotometric method of Clarkson *et al.*[6] lacks both sensitivity and specificity, and the selective microbiological assay developed by us[7,8] (which is sensitive to 10 ng/ml in plasma) can be unsuitable when the patient is also receiving antibiotics. The gas chromatographic (GC) assay of Windheuser *et al.*[9], involves extended (22 h) dialysis to remove the drug from blood samples and is too complex for routine use. Other workers have described GC–mass spectrometry (GC–MS) methods, which, after extensive extraction procedures, are sensitive to 1–25 ng/ml of plasma[10,11]. Recently,

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a high-performance liquid chromatographic analysis for 5-FU in plasma has been reported; it is sensitive to 100 ng/ml of plasma[12].

Isotachophoresis is an electrophoretic technique by which charged molecules are separated quantitatively as discrete zones in a discontinuous electrolyte system[13–15], and it should be well suited to the quantitative determination of 5-FU.

EXPERIMENTAL

The isotachophoretic separations and determinations were made in an LKB 2127 Tachophor; the instrument and technique have been described by Arlinger[16]. Normal serum was obtained from 10 healthy blood donors and kept at −20° until used for multiple analysis. Serum from four patients receiving 250 mg of 5-FU orally were analysed by both isotachophoresis and the microbiological method[8].

Chemicals

5-FU was supplied by Roche, (Stockholm, Sweden). Tritiated 5-FU (1mCi/mmol) was purchased from the Radiochemical Centre (Amersham, Great Britain). HPMC (hydroxypropylmethylcellulose; Methocel 90 HG 15000 CPS) was obtained from the Dow Chemical Company (Midland, Mich., U.S.A.). All chemicals used were of analytical grade and commercially available, unless otherwise stated.

Leading electrolyte

Hydrochloric acid (5 m*M*) was titrated with 0.1 *M* Tris to the desired pH and was adjusted to contain 0.4% of HPMC.

Terminating electrolyte

Glycine (0.04 *M*) was titrated with saturated barium hydroxide solution to the desired pH.

Deproteinization of serum

Serum (1 ml) was diluted to 5 ml with 1% picric acid[17]. The precipitate formed was removed by centrifugation at 3000 *g* for 5 min. The supernatant liquid was brought to pH 11 (tested with paper) with 0.1 *M* potassium hydroxide and loaded on to an anion-exchange column.

Resin preparation and sample treatment

The resin (AG-1-X8; 200–400 mesh; $HCOO^-$) was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.); the column dimensions are given in Fig. 1. The resin was equilibrated with 5 volumes of 6 *M* formic acid for 1 h, then washed with 30 column volumes (CV) of glass-distilled water. The pH of the effluent was checked to be about 7 with test paper. The deproteinized serum sample was loaded on to the column, and 20 CV of glass-distilled water were passed through the column and discarded. 5-FU was then eluted with 5 CV (3 ml) of 0.05 *M* formic acid. The eluate was evaporated to dryness at 60° under a gentle stream of nitrogen (*ca.* 60 min), and the dry residue was dissolved in 20 μl of water; 1–10 μl of this solution were injected into the Tachophor.

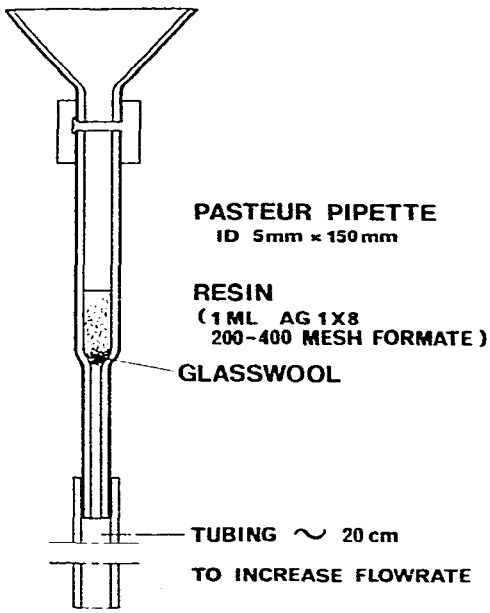

Fig. 1. Column used to extract 5-FU from serum. The funnel is attached to the top of the column with a piece of tubing.

Isotachophoretic conditions

During the investigation, capillaries of different lengths were used. For routine purposes, a 23-cm capillary (I.D. 0.5 mm) was satisfactory. The analysis time was less than 20 min at 18°. To reduce the running time, experiments were started at an elevated current (90 μA), which, before detection, was reduced to 40 μA. The light transmission at 254 or 278 nm was registered on a recorder (chart speed 5 cm/min).

Pre-requisites for analysis

5-FU cannot be quantitated by direct injection of serum into the Tachophor, but, after deproteinization with picric acid and concentration on the AG-1-X8 column, a distinct single zone with the same mobility as 5-FU in aqueous solution was obtained in the Tachophoretic runs. When 5-FU was added to such samples, this single zone was broadened. It was necessary to wash the ion-exchange column with 30 CV of water immediately before use in order to remove highly charged impurities from the resin. Such impurities interfere with the analysis and cause unduly long analysis times.

Optimal pH of the electrolytes

When 10 nmol of 5-FU were added to 1 ml of normal serum, a leading electrolyte at a pH 7.4–7.6 and 5 m*M* in chloride gave the widest zones. However, the best resolution was obtained with a pH of 7.45 in the leading electrolyte. By varying the pH of the terminating electrolyte between 9.0 and 10.0 it was found that, at the lower pH, the analysis time was increased without improving the separation.

Identification of the zone representing 5-FU

After deproteinization and ion-exchange chromatography, there are still several zones in the electropherogram of the 0.05 *M* formic acid fraction. In order to identify 5-FU in such a separation, it is advisable to inject the sample alone and then together with the pure drug, as shown in Fig. 2. Another technique is to analyse a blood sample from the patient before 5-FU has been administered and compare the electropherogram with those of samples containing the drug (Fig. 3).

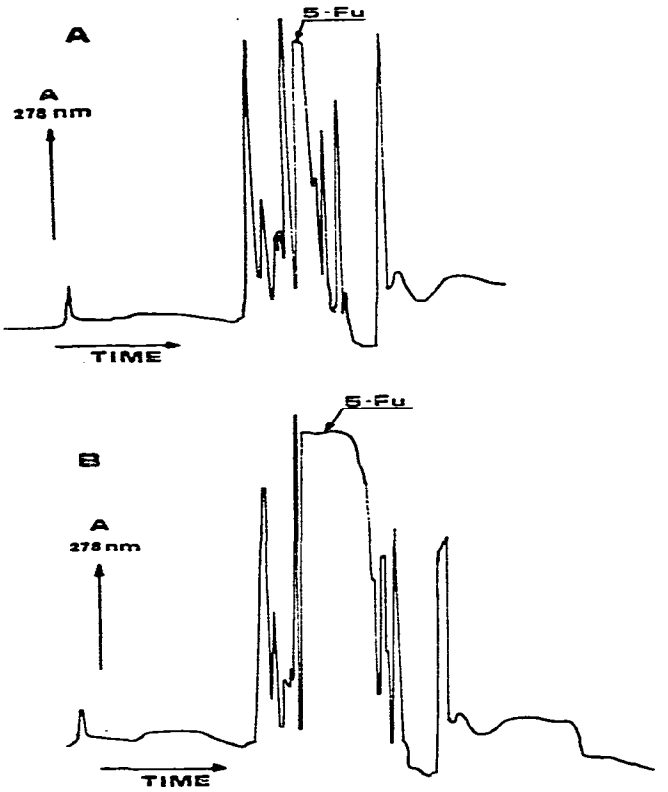

Fig. 2. A: Isotachopherogram of a sample corresponding to 1 ml of serum from a patient treated by continuous infusion of 5-FU (15 mg kg^{-1}) daily. B: Isotachopherogram from the same case after addition of 2 nmol of 5-FU to the original sample.

Quantification

The quantitative aspects of isotachophoresis have been discussed by Gower and Woledge[18]. The zone widths at the half peak-heights were determined to within 0.1 mm with a graticule after magnification (7 ×). The reproducibility of the isotachophoretic method was calculated from duplicate determinations on serum prepared as previously described, using the formula S.E. = $\sqrt{(di)^2/2n}$, where *di* is the difference between duplicate determinations.

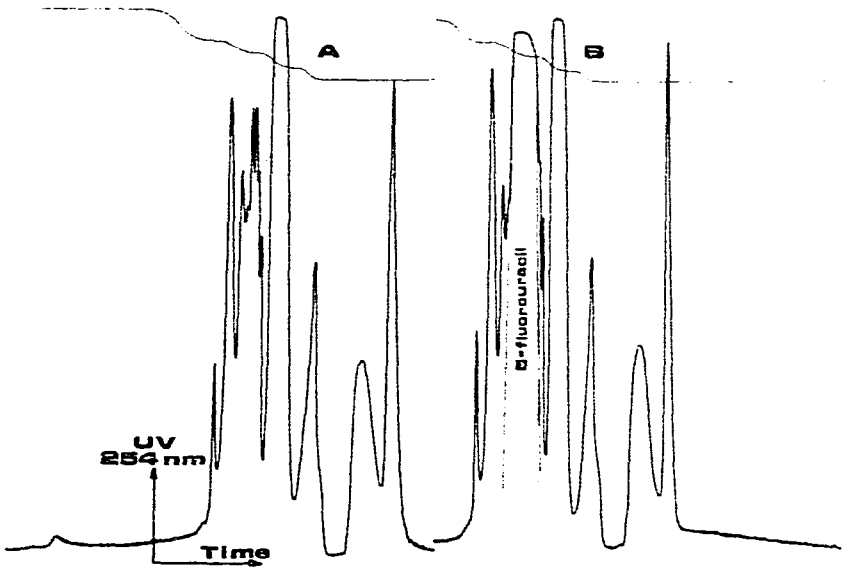

Fig. 3. A: Isotachopherogram of a serum sample from a patient before 5-FU was administered. The sample preparation was as described in the text. The leading electrolyte was 5 m*M* HCl (0.4% in HPMC) titrated with Tris to pH 7.45. The terminating electrolyte was 0.04 *M* glycine titrated with saturated $Ba(OH)_2$ to pH 9.4. Capillary 23 cm; current 45 μA; wavelength of recording 254 nm; temperature 18°. B: Isotachopherogram from the same patient 1 h after oral administration of 250 mg of 5-FU. The working conditions were as described above. The zone representing 5-FU is indicated.

Accuracy of the method

The reproducibility of the technique was confirmed by running standard curves with increasing amounts of 5-FU in aqueous solution (Fig. 4a) and added to serum (Fig. 4b). The methodological error from duplicate determinations was $\pm$ 6.2% when 0.1–10 nmol of 5-FU were injected. The lower limit of quantification was 50 pmol.

Recovery

The over-all recovery of the method was determined by adding various amounts of 5-FU to 1-ml aliquots of normal serum. After deproteinization, ion-exchange chromatography and concentration, the yield in the first 3 ml was compared with a standard curve (as in Fig. 4a). The recovery was 84% (Fig. 4b).

The elution pattern of 5-FU from the AG-1-X8 resin was controlled by adding tritiated 5-FU ($1.8 \cdot 10^7$ cpm) to 1 ml of normal serum, which was then treated as described above. The tritiated 5-FU was eluted with 0.05 *M* formic acid, and the radioactivity of the fraction was counted by liquid scintillation (Fig. 5). The total recovery in the eluate was $1.7 \cdot 10^7$ cpm, or 94%. The first 3 ml of eluate contained 84% of the tritiated 5-FU applied to the column.

Blood samples from four patients with metastatic colorectal cancers receiving oral 5-FU therapy (250 mg) were analysed in duplicate by the proposed method and by microbiological assay. As shown in Fig. 6, there was excellent agreement between the two estimates (correlation coefficient = 0.99).

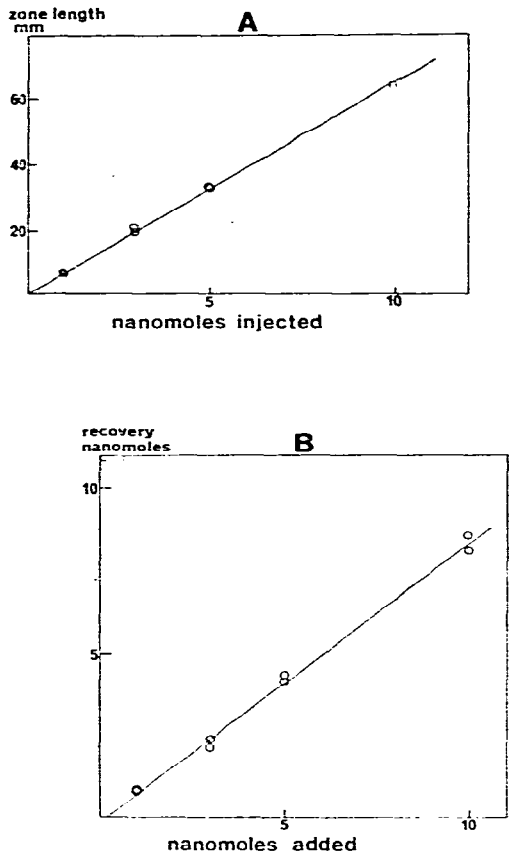

Fig. 4. A: Standard curve of 5-FU in aqueous solution. B: Standard curve of 5-FU added to 1 ml of serum. After deproteinization, extraction and concentration, the samples were dissolved in 20 μl of water, and 10 μl were injected into the Tachophor. Working conditions as described in the text.

RESULTS AND DISCUSSION

Hitherto, methods for determination of 5-FU have been time-consuming, expensive or too complex for routine hospital use. The results of the present study show that isotachophoresis offers a unique method for qualitative as well as quantitative determination of very low levels of charged molecules. In some instances, *e.g.*, the analysis of cerebrospinal fluid proteins, it is possible to inject the sample directly into the instrument without any pre-treatment[19]. However, because other compounds disturb the analysis and the concentration of 5-FU in serum from treated patients is low, it is necessary to concentrate and purify the sample before it is introduced into the Tachophor. Serum is therefore deproteinized, and the 5-FU is then extracted on an ion-exchange column. Highly charged impurities can, however, be eluted from the resin itself, and, if present in the concentrated sample, they cause an unduly protracted analysis. These compounds are probably poorly polymerized sulphonated styrenes, as discussed by Greenland *et al.*[20]. By extensive washing of the resin immediately before use, the influence of these impurities can be avoided. This phenomenon (not previously described) seems to be of general impor-

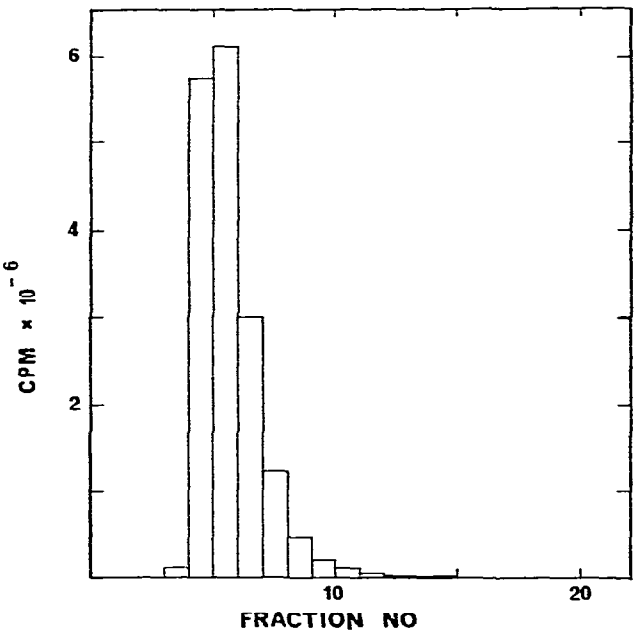

Fig. 5. Histogram showing the elution pattern for 5-FU in serum; column as in Fig. 1. Tritiated 5-FU ($1.8 \cdot 10^7$ cpm) was added to 1 ml of serum. After deproteinization with 4 ml of 1% picric acid and centrifugation, the supernatant solution at a pH of 11 was chromatographed. The total recovery was 94% as determined by liquid scintillation. One fraction volume represents 0.5 ml of eluate.

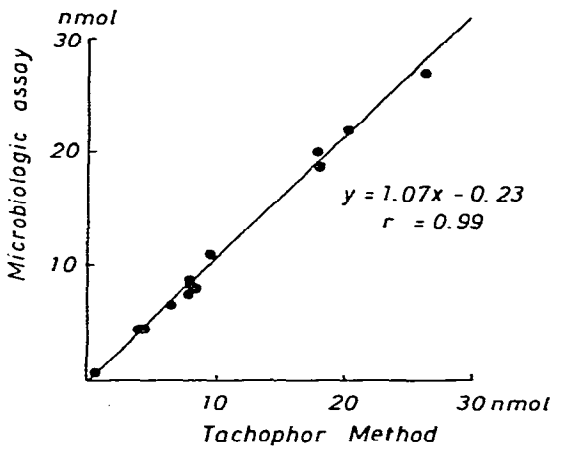

Fig. 6. Comparison of contents (determined by isotachophoresis and a microbiological method) of 5-FU in sera from four patients receiving the drug orally. For details see text.

tance in all situations when biological samples are extracted on ion-exchange columns before isotachophoretic separation.

The over-all recovery was 84% (range 72–86%) when only the first 3 ml of eluate with the 0.05 *M* formic acid were collected. The investigation with tritiated 5-FU (Fig. 2) shows that the recovery can be increased and the accuracy of the

method improved if a further 1–2 ml of eluate are collected. It is, however, convenient to evaporate only 3 ml of the 0.05 *M* formic acid, thus sacrificing some accuracy; otherwise, it is necessary to reduce the volume in two steps to make it possible to dissolve the residue in as little as 20 μl of water. The construction of standard curves by adding known amounts of 5-FU to serum before elution and analysis should minimize the analytical error (Fig. 4b).

Although 5-FU has been quantitated by comparison of standard curves, the specificity was further evaluated by comparing the microbiological assay and the proposed isotachophoretic technique (Fig. 6). The sensitivity of the proposed method was found to be 50 pmol/ml of serum, which corresponds well with the lower sensitivity of available GC–MS methods. However, it seems possible to increase the sensitivity of the isotachophoretic method by a factor of at least 10 by improving the quantitation routine. This might be done by means of log–linear conversions and integration of the areas under the UV absorbance curves[18].

For best resolution, it has been found that the pH in the leading electrolyte is of fundamental importance. Thus, we have found that a small zone appears as a shoulder on the 5-FU zone; if the pH of the leading electrolyte exceeds 7.6, this zone cannot be separated from the 5-FU zone. The optimal separation was achieved with a leading electrolyte of pH 7.45.

During the work, it was noted that both peak height and zone width were optimum at pH about 7.45. Variations in pH in either direction resulted in decreased zone width an poorer reproducibility. These effects are not entirely explained by the increased degree of dissociation of 5-FU with increased pH. A possible reason could be that a steric change also takes place and affects the mobility.

The present work has shown that 5-FU in serum can be identified and quantitated by analytical isotachophoresis. The technique is simpler than available GC–MS methods, and the sensitivity is at least as good. The method can be used for the routine control of serum levels of 5-FU after the administration of this drug. The isotachophoretic technique opens new possibilities for determination of drugs and their metabolites in biological samples, especially in situations where many substances are to be measured on a few samples. The extraction technique and the isotachophoretic separation should be readily adaptable to the study of 5-FU and its metabolites in other biological materials. A method for determination of 5-FU and its metabolites in tumours and host tissues is being developed.

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REFERENCES

1 F. J. Ansfield, S. Backman and G. Ramirez, *Oncology*, 26 (1972) 114.
2 C. Heidelberger, *Cancer Res.*, 30 (1970) 1549.
3 E. Miller, *J. Surg. Oncol.*, 3 (1971) 309.
4 F. J. Ansfield, *J. Amer. Med. Ass.*, 190 (1964) 686.
5 G. Ramirez, B. C. Korbitz, H. L. Davis *et al.*, *Cancer Chemother. Rep.*, (1969) 243.
6 B. Clarkson, A. O'Connor, L. Winston and D. Hutchison, *Clin. Pharmacol. Ther.*, 5 (1964) 581.

7 O. Almersjö, Å. Brandberg and B. Gustavsson, *Cancer Lett.*, 1 (1975) 113.
8 Å. Brandberg, O. Almersjö, E. Falsen, B. Gustavsson, L. Hafström and G.-B. Lindblom, *Acta Pathol. Microbiol. Scand.*, 85 (1977) 227.
9 J. J. Windheuser, J. L. Sutter and E. Aven, *J. Pharm. Sci.*, 61 (1972) 572.
10 J. L. Cohen and P. B. Brennan, *J. Pharm. Sci.*, 62 (1973) 572.
11 B. L. Hillcoat, M. Kawai, P. B. McCullock, J. Rosenfeld and C. K. O. Williams, *Brit. J. Clin Pharmacol.*, 31 (1976) 135.
12 J. L. Cohen and R. E. Brown, *J. Chromatogr.*, 151 (1978) 237.
13 H. Haglund, *Sci. Tools*, 17 (1970) 2.
14 R. J. Routs, *Thesis*, Univ. Eindhoven, 1971.
15 A. J. P. Martin and F. M. Everaerts, *Proc. Roy. Soc. Ser. A*, 316 (1970) 493.
16 L. Arlinger, *Prot. Biol. Fluids, Proc. Colloq.*, 22 (1975) 661.
17 T. G. Rajugnelan, S. L. Moore and N. H. Stein, *J. Biol. Chem.*, 241 (1966) 4940.
18 D. C. Gower and R. C. Woledge, *Sci. Tools*, 24 (1977) 17.
19 K. G. Kjellin, L. Hallander, U. Moberg, *J. Neurol. Sci.*, 26 (1975) 617.
20 R. D. Greenland, W. Roth, T. Gieske and I. A. Michaelson, *Anal. Biochem.*, 62 (1974) 305.