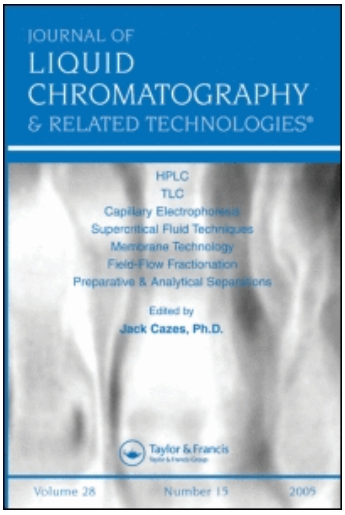

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Extraction Procedures for the HPLC Determination of 5-Fluorouracil in Biological Samples

M. J. del Nozal[a]; J. L. Bernal[a]; P. Marenero[a]; A. Pampliega[a]
[a] Department of Analytical Chemistry, Faculty of Sciences University of Valladolid, Valladolid, Spain

To cite this Article Nozal, M. J. del , Bernal, J. L. , Marenero, P. and Pampliega, A.(1994) 'Extraction Procedures for the HPLC Determination of 5-Fluorouracil in Biological Samples', Journal of Liquid Chromatography & Related Technologies, 17: 7, 1621 — 1636
To link to this Article: DOI: 10.1080/10826079408013184
URL: http://dx.doi.org/10.1080/10826079408013184

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(7), 1621-1636 (1994)

EXTRACTION PROCEDURES FOR THE HPLC DETERMINATION OF 5-FLUOROURACIL IN BIOLOGICAL SAMPLES

M. J. DEL NOZAL, J. L. BERNAL, P. MARINERO, AND A. PAMPLIEGA
Department of Analytical Chemistry
Faculty of Sciences
University of Valladolid
E-47005, Valladolid, Spain

ABSTRACT

An extraction procedure was optimized for the reversed-phase high performance liquid chromatographic determination of 5-Fluorouracil in rabbit plasma, liver, kidney, lung and heart samples. The extraction conditions are adapted to each type of matrix in order to achieve high extraction yields (*ca.* 90%) and interference-free chromatographic peaks.

INTRODUCTION

5-Fluorouracil (5-Fu) is widely used as an antitumour drug, so the determination of residual concentrations of this substance in various body parts following administration is of great interest in order to optimize therapeutic doses and identify potential metabolic pathways or side effects. Most of the revised literature on this substance is concerned with clinical, pharmaco-kinetic and therapeutic aspects rather than with its analytical determination. Lately, special attention has been paid to the extraction and determination of this drug in plasma samples in preference to tissue samples, available literature on which is rather scant.

Sample pretreatment procedures used in the determination of 5-Fu are quite varied in nature, but most involve a long string of steps, one of which is common to most: deproteination, which can be accomplished by using a precipitating agent [1–4], membrane ultrafiltration [5] and ion-exchange [6], chiefly. A second step involves extraction from the matrix with an appropriate mixed solvent (*e.g.* *n*-propanol/water [7], *n*-propanol/ether [8], chloroform/methanol [9] or chloroform/propanol [10]).

After the drug is isolated, it is normally quantified by high performance liquid chromatography (HPLC).

As a rule, the proponents of such procedures fail to mention recoveries or any differences arising from the type of matrix involved —many authors use a single, scarcely efficient procedure for every type of sample [11]. This prompted us to develop extraction/clean-up procedures providing high drug recoveries from such matrices as plasma, liver, kidney, lung and heart, using *n*-propanol/ether mixtures as extractants and optimizing the working conditions for each type of matrix —the goodness of each procedure was checked by using HPLC. All samples employed in this work were from experimental rabbits.

EXPERIMENTAL

Chemicals

5-Fluorouracil (5-Fu) was purchased from Sigma (St Louis, MO). Ammonium dihydrogen phosphate and all other chemicals used to prepare buffers were analytical-reagent grade and supplied by Merck (Darmstad, Germany). The water employed was purified by passage through a Nanopure II system from Barnstead (Newton, MA). All solvents used were HPLC-grade and obtained from SDS (Peypin, France).

Apparatus

The chromatographic set-up used consisted of a CD4000 multi-solvent partitioning pump and an SM4000 variable-wavelength UV-visible detector, both from LDC Analytical (Riviera Beach, FL), in addition to a JCL6000 Chromatography Data system from Jones Chromatography (Colorado).

An ultrasonic bath, a vibromatic stirrer, a centrifuge (all three from Selecta, Spain) and a rotavapor from Büchi (Italy) were also used.

Chromatographic conditions

The column used was 25 long × 0.46 cm ID and packed with Spherisorb 5 ODS resin of 5-μm particle size from Phenomenex (Torrance, CA). The mobile phase was 0.05 M phosphate buffer of pH 3.5 and was pumped at a flow-rate of 1 ml/min. Samples were injected by means of a Marathon autosampler from Spark Holland (Emmen, The Netherlands) furnished with a fixed-volume (20-μl) loop. Detection was performed at 254 nm.

Experimental animals

The animals used in the experiments were untreated rabbits from which plasma and tissue samples were withdrawn on sacrificing.

Plasma samples were collected in tubes containing sodium heparin that were centrifuged for 10 min and frozen until analysis. Tissue samples were ground, added physiological serum and frozen.

All experiments involving these animals were carried out in compliance with accepted norms.

Extraction/clean-up

Experiments were always performed in duplicate by using two groups of samples of the same weight for each type of matrix, to which known amounts of 5-Fu were added. One group was used to obtain the chromatogram background and the other to assess recovery.

Preliminary experiments involving ultrafiltration and solid–liquid extraction with cartridges containing various materials (C_{18}, C_8, NH_2, CN and PH functions) provided poor recoveries, so solvent extraction with various mixtures was assayed next. Thus, an amount of 0.5 g of tissue or 1 ml of plasma was added the extractant; the mixture was immersed in an ultrasonic bath for an appropriate time and then centrifuged in order

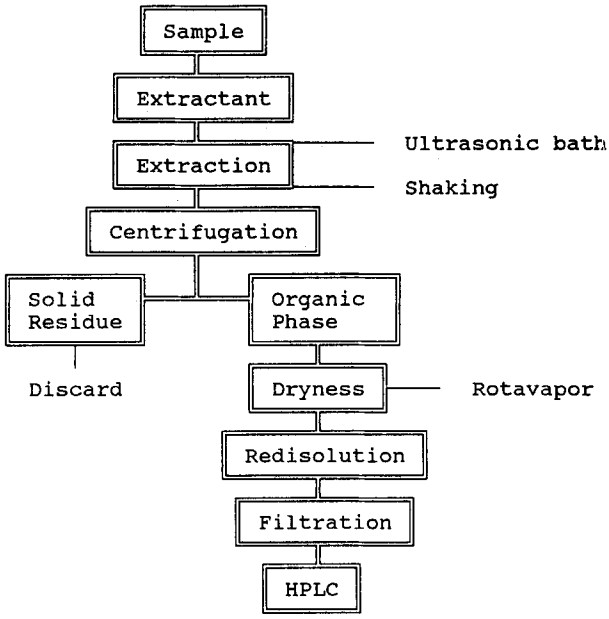

Figure 1 – Scheme of the overall procedure.

to separate the two phases. The organic phase was then evaporated to dryness in a rotavapor and the solid residue dissolved in 1 ml of 0.05 M ammonium phosphate. The resulting solution was used to inject 20-μl aliquots into the chromatograph. The process is schematically depicted in Fig. 1.

After several preliminary experiments, the best results were found to be provided by an *n*-propanol/ether mixture, consistent with previous findings in dealing with vitreous material [12]. However, the previously developed procedure was inapplicable to all the sample types studied in this work as it resulted in the simultaneous extraction of compounds that coeluted with 5-Fu or in very low extraction yields. Consequently, the influence of parameters potentially affecting the extraction process for each type of sample was studied in order to establish the optimal conditions for maximal recovery of 5-Fu with minimal extraction of potential interferents.

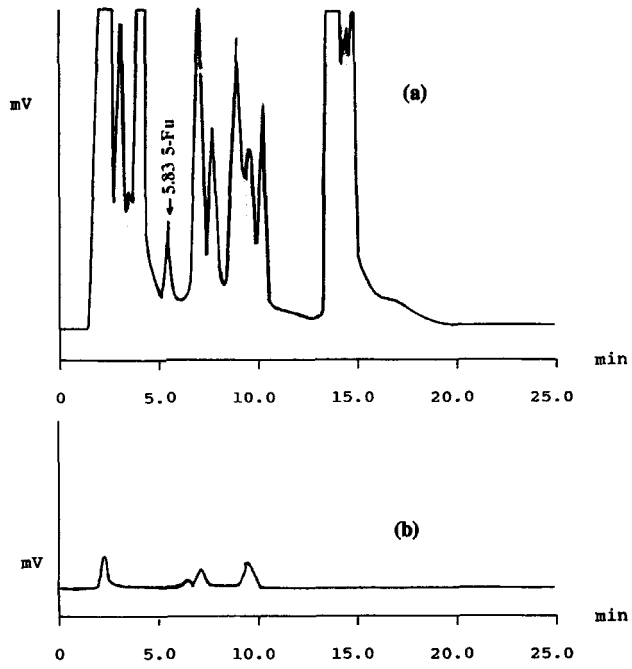

Figure 2 – Chromatograms obtained for a plasma sample on treatment with *n*-propanol-/ether mixtures of two different compositions: (*a*) 95:5, and (*b*) 5:95.

RESULTS AND DISCUSSION

The experimental variables whose effect on the extraction yield was investigated included the composition of the *n*-propanol/ether mixture, extractant volume, protein precipitant, matrix pH and ionic strength, shaking time, and pH of the redissolving solution.

Composition of the *n*-propanol/ether mixture

The various types of matrix studied were subjected to extraction with *n*-propanol-/ether mixtures of different composition, *viz.* from 5:95 to 95:5. Increased proportions of ether in the mixture resulted in neater chromatograms for all the samples, but also

TABLE 1. Optimal extractant composition.

Matrix	Composition	Recovery %
Liver	16:84	31.10 ± 0.40
Kidney	20:80	34.40 ± 0.52
Lung	88:12	45.32 ± 0.38
Heart	40:60	43.75 ± 0.47
Plasma	88:12	49.00 ± 0.32

in decreased 5-Fu recoveries, as can be seen in the chromatograms for plasma (in Fig. 2), which were similar to those obtained for the other samples.

Also, each matrix was found to give rise to different coextracted interferents, so the mixture composition must be adjusted accordingly. Table 1 gives the 5-Fu recoveries obtained by using the optimal extractant composition for each type of matrix.

Extractant volume

After the most suitable extractant composition for each type of sample was established, the effect of using different extractant volumes between 5 and 30 ml on 5-Fu recovery was studied. The results were similar for all the samples. As can be seen in Fig. 3 for heart tissue, increasing extractant volumes resulted in predictably increasing recoveries for both 5-Fu and coextracted substances, so 15 ml (*viz.* the lowest volume that resulted in an optimal 5-Fu/interferent signal ratio) was chosen.

Attempts at raising 5-Fu recovery by using successive extractions with 15 ml of mixture provided no significantly improved results in terms of analyte extraction yield; on the other hand, they gave rise to considerably increased extraction of interferents and lengthened the procedure exceedingly. Hence, a single extraction with the optimal volume was performed in all subsequent experiments.

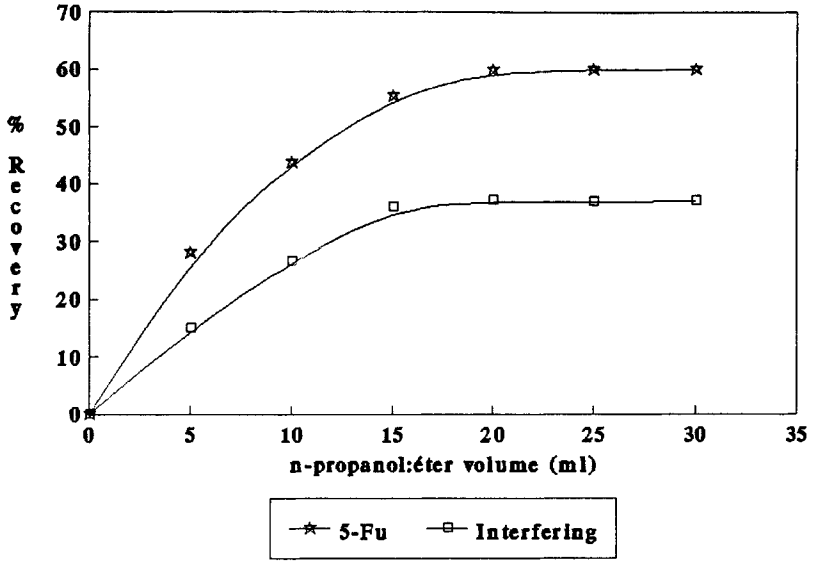

Figure 3 – Variation of 5-Fu recovery from a heart sample as a function of the extractant volume.

Protein precipitant

In order to avoid extraction of interferents giving rise to disturbing peaks in the chromatograms, samples were treated with various reagents typically used as protein precipitants, the most effective of which proved to be trichloroacetic acid and sodium acetate.

Trichloroacetic acid was tested at various concentrations (1 – 10% w/v) and in different volumes (< 1 ml). The results obtained showed its presence to be beneficial for the lung and heart samples and indifferent for the other types of sample. Figure 4 shows the variation of the analyte recovery from the lung and heart samples with the precipitant concentration and volume used. The highest recoveries were obtained by using 100 μl of 2% trichloroacetic acid.

The liver and kidney samples were treated with sodium acetate, on which they exerted a greater clearance effect than did trichloroacetic acid. The best results were

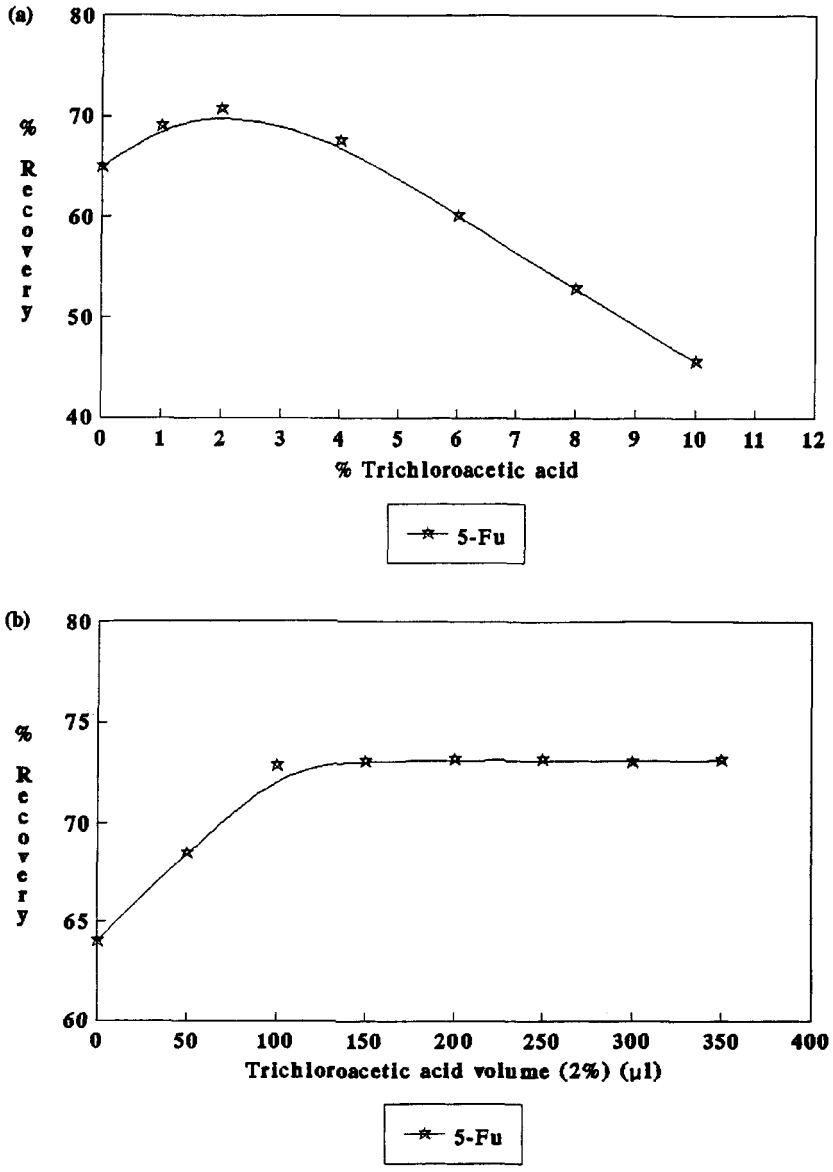

Figure 4 – Variation of 5-Fu recovery from a lung sample as a function of (*a*) the concentration and (*b*) volume of protein precipitant used.

provided by a volume of 50 μl of 1 M sodium acetate at pH 6 for liver and pH 5 for kidney, as can be seen from Fig. 5, which shows the chromatograms obtained for kidney tissue with and without the precipitant added.

Neither precipitant increased the 5-Fu recovery from plasma or resulted in a cleaner sample, though.

Matrix pH and ionic strength

The two groups of samples were added various amounts of acids, bases and salts prior to extraction with a view to improving the results. The solutions used included 2 N NaOH (V < 10 μl), 2 N H_2SO_4 (V < 10 μl), 1 M tris-hydroxymethylaminomethane (TRIS) buffer (V < 2 ml), K_2S (V < 10 μl) and saturated (4 g/20 ml) Na_2SO_4 (V < 2 ml).

The results obtained revealed the plasma, lung and heart samples to benefit from the addition of 2 N H_2SO_4, which increased 5-Fu recoveries without significantly raising coextraction of interferents (Table 2). The optimum acid concentrations for this purpose were found to be 2 μl for plasma and 5 μl for the lung and heart samples.

Also, addition of sodium sulphate —but none of the other solutions— to the liver and kidney samples was found to favour passage of the analyte into the organic phase, the effect being a function of the volume of salt solution added (Fig. 6). In fact, increasing volumes up to 0.5 ml gave rise to markedly increasing 5-Fu recoveries with no increased coextraction of interferents; however, volumes higher than 0.5 ml detracted from the extraction yield. A volume of 0.5 ml was thus chosen as optimal for the extraction of 5-Fu from liver and kidney tissues.

Shaking time

Figure 7 shows the 5-Fu recoveries obtained by using different shaking times (the results for the other four types of sample were quite similar). As can be seen, recoveries increased substantially with increase in the extraction time up to 10 min; longer times resulted in very small variations or even no changes after 20 min. A shaking time of 15 min (*viz.* the shortest providing the highest possible yield) was thus selected.

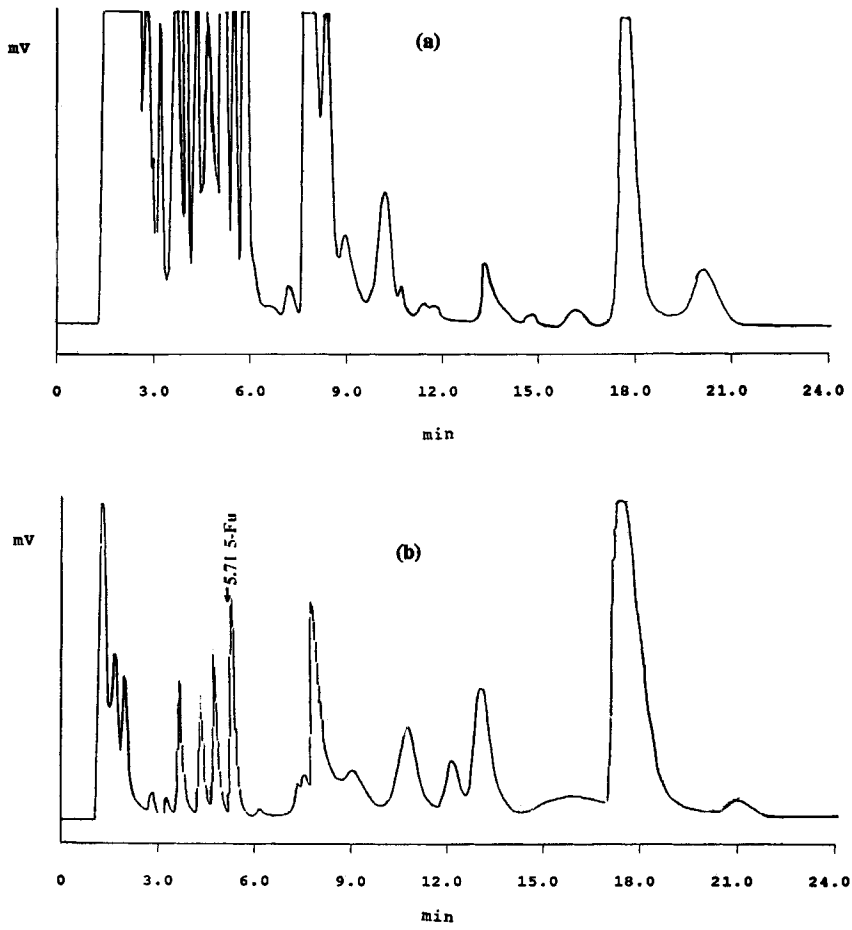

Figure 5 – Chromatograms obtained for kidney samples with (*a*) no acetate added, (*b*) 50 μl of 1 M sodium acetate (pH 6) added.

TABLE 2. Influence of H_2SO_4 volume on 5-Fu recovery %.

Matrix	0 μl	2 μl	4 μl	6 μl	8 μl	10 μl
Plasma	49.0	70.2	67.8	62.4	50.2	47.1
Lung	62.0	71.2	80.5	80.5	73.2	66.2
Heart	60.5	68.9	79.4	79.4	70.3	65.6

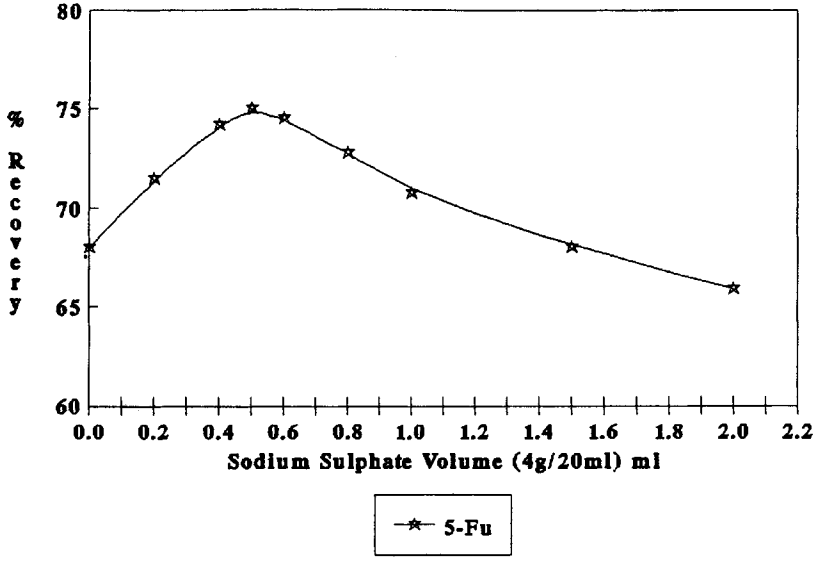

Figure 6 – Variation of 5-Fu recovery from a liver sample as a function of the volume of sodium sulphate used.

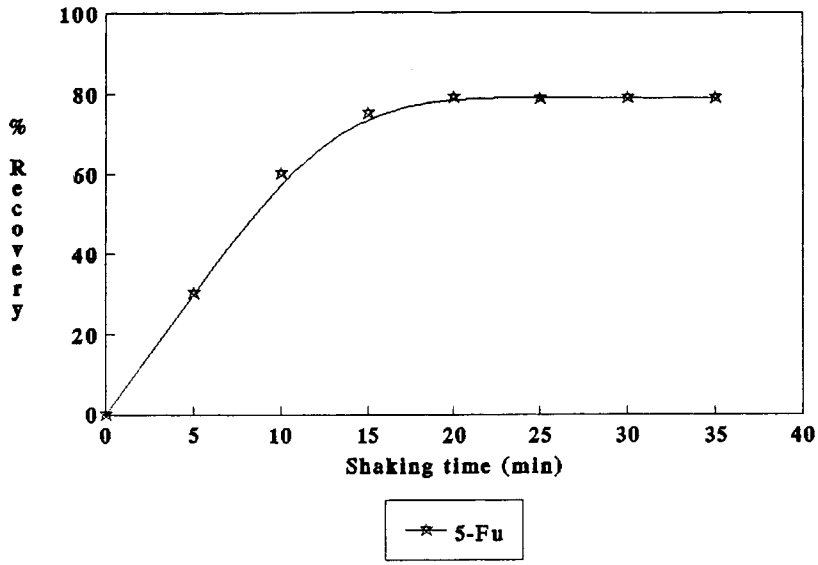

Figure 7 – Variation of 5-Fu recovery from a kidney sample as a function of the extraction time.

pH of the redissolving solution

The influence of the pH of the 0.05 M ammonium dihydrogen phosphate solution used to dissolve the analyte after evaporation of the organic phase on the analyte recovery was studied by adjusting it to values between 2 and 11 with a strong acid or base. Figure 8 shows the results obtained for the different types of matrix. Based on them, the most suitable pH for the 0.05 M ammonium dihydrogen phosphate solution, of which a volume of 1 ml was used in every case, was 2.5 for plasma and lung, 3 for kidney, 5 for heart and 11 for liver samples.

Table 3 summarizes the most suitable working conditions for each type of sample and the final 5-Fu recoveries obtained under them. As can be seen from the chromatograms for the liver sample in Fig. 9 (similar to those obtained for the other types of sample), the proposed method allows the accurate quantitation of 5-Fluorouracil.

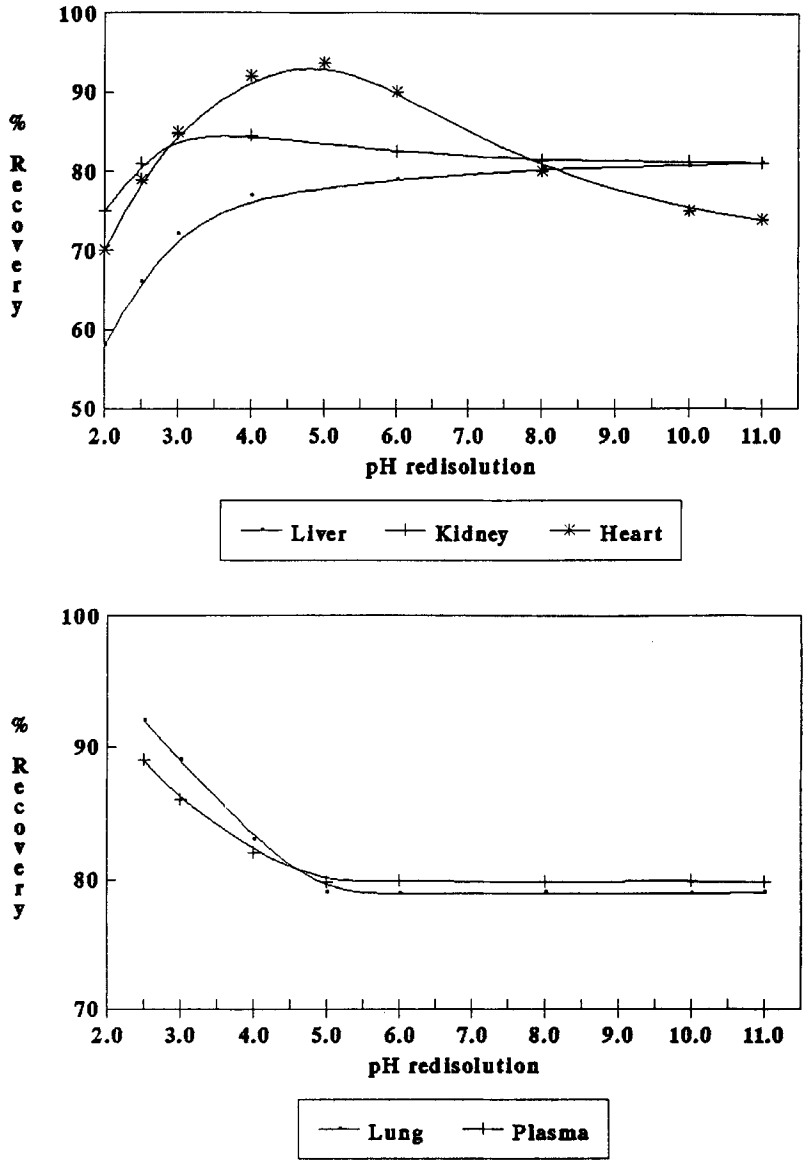

Figure 8 – Variation of 5-Fu recovery as a function of the redissolving solution pH.

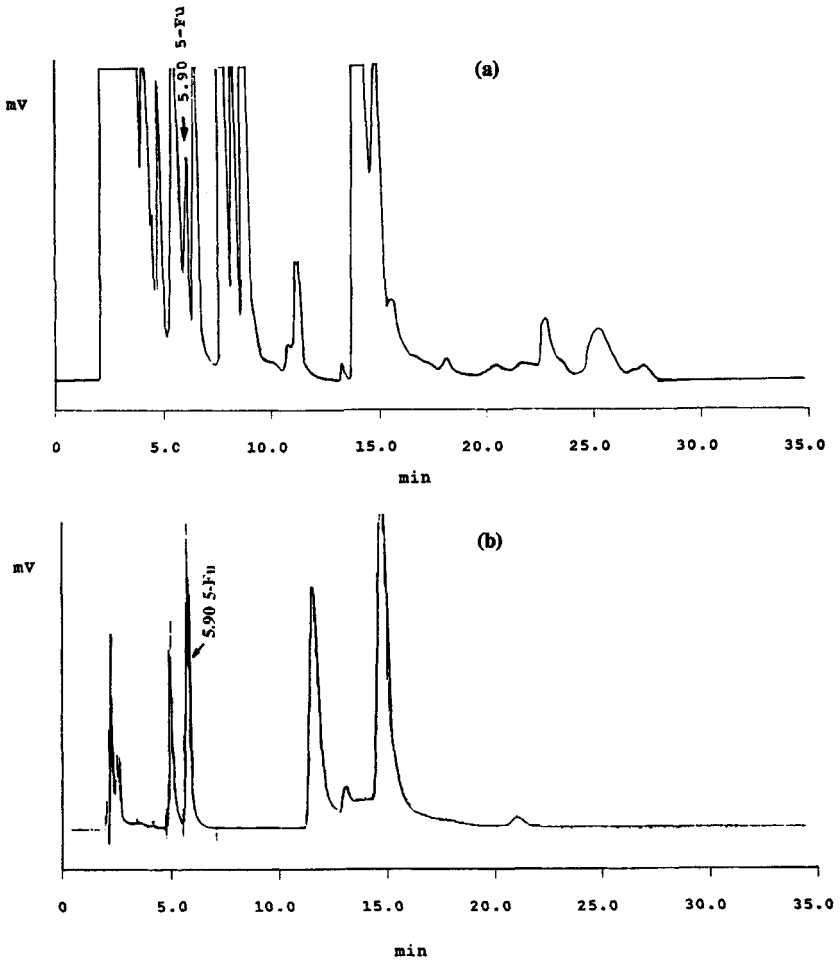

Figure 9 – Chromatograms obtained for a liver sample subjected to extraction with 15 ml of extractant (*a*) and the proposed procedure as shown in Table 3 (*b*).

TABLE 3. General procedure for sample preparation.

Matrix	Liver	Kidney	Lung	Heart	Plasma
Sample	0.5 g	0.5 g	0.5 g	0.5 g	1 ml
H_2SO_4 2 N	-	-	5 μl	5 μl	2 μl
Na_2SO_4 (4g/20ml)	0.5 ml	0.5ml	-	-	-
NaAcO 1 M	50 μl	50 μl	-	-	-
	pH 6	pH 5	-	-	-
Cl_3AcOH	-	-	2 %	2 %	-
	-	-	100 μl	100 μl	-
n-propanol/ether (15 ml)	16:84	20:80	88:12	40:60	88:12
Ultrasonic bath	30 s	30 s	30 s	30 s	30 s
Shaking	15 min	15 min	15 min	15 min	15 min
Centrifugation	15 min	15 min	15 min	15 min	15 min
pH phosphate (1 ml)	11	3	2.5	5	2.5
Recovery %	81.20	84.75	95.04	93.89	89.00

CONCLUSIONS

The results obtained in this work clearly reveal that type of matrix involved influences the final recovery of 5-Fu. Thus, plasma samples have a different effect from those of the four tissues assayed, of which liver and kidney on the one hand, and lung and heart on the other, bear some similarities.

The results also show that *n*-propanol and ether in the extracting mixture have opposing effects and that the most suitable composition for the mixture varies from sample to sample.

In contrast with the widespread use of a single extraction procedure, we believe each type of matrix should be extracted under specific, optimized conditions.

REFERENCES

1.- J.L. Cohen and P.B. Brennan. *J. Chromatogr.*, 311, 125 (1984).

2.- R. Kar, A. Cohen, M. Terem, M. Nahabedian and A. Wile. *Cancer Research*, 46, 4491 (1986).

3.- H. Odagiri, S. Ichihara, E. Semura, M. Utoh, M. Tateishi and I. Kuruma. *J. Pharmacobio-Dyn*, 11, 234 (1988).

4.- J.L.S. Au, M.H. Su and M.G. Wientjes. *Clin. Chem.*, 35, 48 (1989).

5.- A.A. Miller, J.A. Benvenuto and T.L. Loo. *J. Chromatogr.*, 228, 165 (1982).

6.- F. La Greta and W. Williams. *J. Chromatogr.*, 414, 197 (1987).

7.- R.S. Benjamin, P.H. Wiernik and N.R. Bachur. *Clinical Pharmacologic Correlation* (1974).

8.- N. Christophidis, G. Mihaly, F. Vadja and W. Louis. *Clin. Chem.*, 25, 83 (1979).

9.- J. Cummings, J. Stuart and K. Calman. *J. Chromatogr.*, 311, 125 (1984).

10.- P. Speth, P.C. Linssen, L. Beex, J. Boezeman and C. Haanen. *Cancer Chemother Pharmacol*, 18, 78 (1986).

11.- T.A. Stein, G.P. Burns, B. Bailey and L. Wise. *J. Chromatogr.*, 507, 259 (1990).

12.- M.J. del Nozal, J.L. Bernal and A. Pampliega. J.C. Pastor and M.I. López. *J. Chromatogr.*, 607, 183 (1992).

Received: October 9, 1993
Accepted: October 19, 1993