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Determination of leucovorin and 5-fluorouracil in plasma by high-performance liquid chromatography

C. Vandenbosch, S. Van Belle and M. De Smet

Department of Pharmaceutical and Biomedical Analysis and Department of Oncology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels (Belgium)

G. Taton

Department of Gastroenterology, Université Libre de Bruxelles, Route de Lennik 808, B-1070 Brussels (Belgium)

V. Bruynseels and G. Vandenhoven

Medical Department, Cyanamid–Lederle, Rue de Bosquet 15, B-1348 Louvain-la-Neuve (Belgium)

D. L. Massart

Department of Pharmaceutical and Biomedical Analysis and Department of Oncology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels (Belgium)

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ABSTRACT

A method was developed for the determination of (6*R*)- and (6*S*)-leucovorin and 5-fluorouracil in plasma. As leucovorin diastereoisomers cannot be separated on a classical reversed-phase column, it was necessary to use a chiral stationary phase. The method presented is based on the same principle as the method described by Wainer and Stiffin [*J. Chromatogr.*, 424 (1988) 158], *i.e.*, coupling of a bovine serum albumin phase to an achiral stationary phase. Before the chromatography, the drug was isolated from the plasma matrix by solid-phase extraction. For 5-fluorouracil, chromatography was performed on a classical RP-18 column after extraction from the plasma by liquid–liquid extraction. Both methods were validated and applied to the analysis of patients' samples.

INTRODUCTION

Leucovorin is a reduced folate that can be administered in high doses as co-medication with the chemotherapeutic agent 5-fluorouracil (5FU) in the treatment of colorectal and gastric carcinoma [1,2]. A stable complex is formed between fluorodeoxyuridine monophosphate (a nucleotide with 5FU incorporated as base), a reduced folate (5,10-methylenetetrahydrofolate) and the enzyme thymidylate synthetase. This prevents the formation of deoxythymidine monophosphate, which is necessary for the synthesis of DNA. It is necessary that high doses of folate are present in the cells to achieve potent inhibition of the enzyme. Therefore, leucovorin (5-formyltetrahydrofolate) is administered as co-medication.

Correspondence to: D. L. Massart, Department of Pharmaceutical and Biomedical Analysis and Department of Oncology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium.

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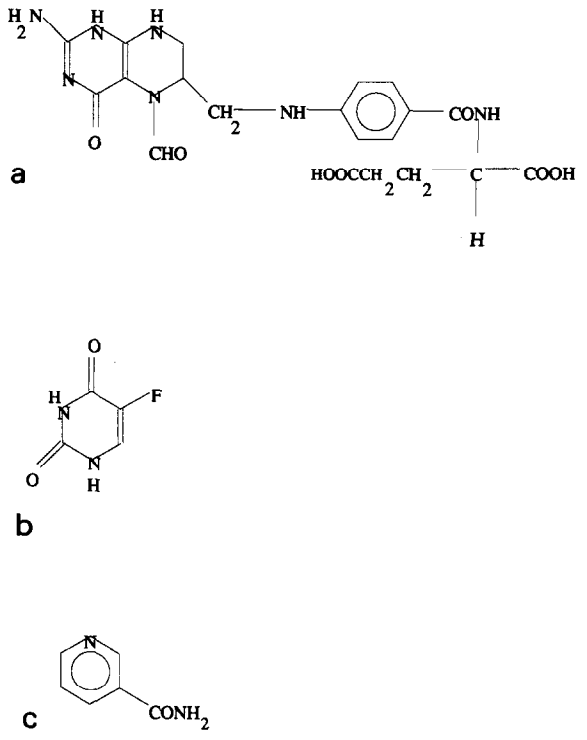

Fig. 1. Structural formulae of (a) leucovorin, (b) 5-fluorouracil and (c) nicotinamide.

Leucovorin contains two asymmetric carbon atoms (Fig. 1): as L-glutamic acid was used in the synthesis, leucovorin consists of two diastereoisomers. It has been shown that the 6*S* form is the most active [2]. Moreover, the pharmacokinetic profiles of the two diastereoisomers are different: (6*S*)-leucovorin disappears more rapidly from plasma [2]. A study was initiated to correlate the pharmacokinetic profile of leucovorin (after administration of a mixture of leucovorin diastereoisomers) and 5FU in patients with the clinical results. This required us to develop a method for the determination of both drugs in plasma.

Wainer and Stiffin [3] described a method for the determination of leucovorin diastereoisomers: they used a bovine serum albumin (BSA) chiral stationary phase (CSP) coupled to an achiral phenyl column by means of a switching valve. The phenyl column separated the analyte from remaining plasma interferences. The BSA protein-type stationary phase was used for the separation of (6*R*)- and (6*S*)-leucovorin. As the efficiency of BSA is low, broad peaks were obtained with this phase, which causes difficulties when leucovorin concentrations in the ppb range have to be determined. A chiral–achiral coupled system was therefore described by Silan *et al.* [4] (two C_{18} columns were coupled to the BSA phase): peak compression is obtained which allows smaller amounts of the drug to be determined.

Reversed- and normal-phase HPLC methods have been described for the determination of 5FU (*e.g.* refs. 5–7). As 5FU is a polar compound with a small number of carbon atoms (Fig. 1), it is difficult to obtain sufficient retention of the analyte. For the same reason, this drug is mostly administered by means of a continuous infusion because of its rapid disappearance from plasma. It was the aim of this work to develop a rapid method that allows the analyses of large numbers of patients' samples and to perform a validation of the developed method.

EXPERIMENTAL

Apparatus

A Varian (Walnut Creek, CA, USA) Model 5000 liquid chromatograph with a variable-wavelength UV detector and an injection loop of 100 μl was used. For the determination of leucovorin, the detection wavelength was set to 290 nm. For the determination of the diastereoisomers, a Gilson (Middleton, WI, USA) pump and additional UV detector were coupled to the system. Chromatograms were recorded and integrated with a Vista CDS or a Shimadzu (Kyoto, Japan) C-R6A Chromatopac integrator.

For the determination of 5FU, the detection wavelength was set to 265 nm. A Gilson Model 231 autosampler was used in this application.

The column used for the determination of total leucovorin was a Novapack RP-18 (300 mm × 4 mm I.D.; particle size 5 μm), obtained from Waters (Division of Millipore, Milford, MA, USA). The BSA chiral column (Resolvosil, 150 mm × 4 mm I.D.; particle size 7 μm) was obtained from Macherey–Nagel (Düren, Germany). This col-

umn was coupled to the C_{18} column by means of a switching valve, with a loop of 250 μl. The mobile phase fraction from the C_{18} column, which contained total leucovorin, was switched to the BSA chiral stationary phase for the determination of (6*R*)- and (6*S*)-leucovorin. The concentration of the diastereomeric mixture (total concentration) was determined by using calibration samples, obtained after adding different concentrations to blank plasma. From the total concentration and the ratio of the diastereoisomers, one can calculate the concentration of (6*S*)- and (6*R*)-leucovorin.

For the determination of 5FU, a LiChrosorb RP-18 column (250 mm × 4 mm I.D.; particle size 5 μm) from Merck (Darmstadt, Germany) was used. Precolumns, packed with the same material as the analytical columns, were used in combination with the different columns.

Chemicals

Sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate dihydrate, used for the preparation of buffer solutions, were of analytical-reagent grade (Merck) and were dissolved in water obtained from a Milli-Q purification system (Millipore). Acetonitrile was of HPLC grade from Merck.

Stock solutions of leucovorin and 5FU were prepared in water freshly every week. Stock solutions of nicotinamide were prepared in methanol.

The mobile phase used for the determination of total leucovorin was the following gradient elution system: time 0 min = 100% phosphate buffer (pH 7.0), ionic strength (μ) = 0.1 (A); time 10 min = A–acetonitrile (70:30); time 18 min = 100% A. The flow-rate was 1 ml min^{-1}. A mobile phase consisting of phosphate buffer (pH 7.0, μ = 0.1) was pumped over the BSA CSP at a flow-rate of 0.5 ml min^{-1}. For the determination of 5FU, phosphate buffer (pH 7.0, μ = 0.05) was used as the mobile phase at a flow-rate of 1 ml min^{-1}.

Sample preparation

Blood samples were centrifuged at 3000 *g* for 15 min and 100 μl of a solution of ascorbic acid (10 mg ml^{-1}) were added per millilitre of plasma. The plasma was stored at −20°C.

Extraction of leucovorin. Bakerbond C_{18} solid-phase extraction (SPE) cartridges were obtained from Baker. They were conditioned with 1 ml of methanol and 1 ml of acetic acid (1%, v/v). To 1 ml of plasma, 1 ml of acetic acid (5%, v/v) was added. This mixture was then applied to the SPE cartridge. As the elution solvent, 500 μl of phosphate buffer (pH 7.0, μ = 0.1)–acetonitrile (80:20) were used. A 100-μl volume of the extract obtained was injected into the chromatographic system.

Extraction of 5-fluorouracil. To 1 ml of plasma, 100 μl of a solution of nicotinamide (100 μg ml^{-1}) were added as an internal standard. Acetonitrile (2 ml) was added dropwise for deproteinization. After centrifugation for 30 min at 3000 *g*, the liquid phase was separated. After evaporation of the acetonitrile, 1 ml of saturated ammonium sulphate solution was added, followed by 5 ml of diethyl ether–2-propanol (80:20). The tubes were placed in a shaking bath for 15 min. After centrifugation at 3000 *g* for 15 min, the organic layer was discarded and another 5 ml of the extraction mixture were added to the aqueous phase. After shaking and centrifugation, the organic phase was added to the previously collected phase and was evaporated to dryness. The residue was reconstituted in 1 ml of the mobile phase.

RESULTS AND DISCUSSION

It was our initial purpose to develop a method for the simultaneous determination of 5FU and leucovorin. As the numbers of carbon atoms in the two drugs are very different, it is not surprising that this could not be achieved and two separate methods were developed.

Determination of leucovorin diastereoisomers

First, a method was developed for the determination of the mixture of leucovorin diastereoisomers, using a C_{18} column. It was our purpose to use a precolumn chiral derivatization technique for the separation of the leucovorin diaste-

reoisomers, in order to overcome the problem of low stability and high costs that often occur with CSPs. However, no suitable agent could, be found for this purpose. Therefore, a BSA CSP was also used for the separation of (6*R*)- and (6*S*)-leucovorin. The column was coupled to the C_{18} column, based on the technique described by Wainer and Stiffin [3], who coupled the BSA phase to an achiral phenyl column. Owing to the fairly low stability of the BSA CSP, the use of a column-switching technique and a precolumn, filled with BSA material, is necessary to protect the chiral column.

In the sample pretreatment step, Wainer and Stiffin [3] added acetonitrile or methanol to the plasma; after centrifugation and evaporation of the supernatant, the residue obtained was reconstituted in the mobile phase. In our experiments, leucovorin was extracted from the plasma matrix in order to minimize the matrix interferences loaded on to the analytical column. An SPE procedure was developed. Surprisingly, however, when acetonitrile or methanol was added to the samples for deproteinization, leucovorin could not be found in the extract. Therefore, acetic acid (5%, v/v) was added to the plasma samples to obtain a denaturation of the proteins. As leucovorin contains two carboxylic acid functions and thus has a polar character, a C_{18} SPE cartridge was used in order to obtain sufficient adsorption. It was not possible, however, to find a wash solvent that did not cause co-elution of the analyte and the wash step therefore could thus not be executed. The optimum elution solvent consisted of phosphate buffer (pH 7.0, μ = 0.1)–acetonitrile (80:20). The extraction recovery was determined at a plasma concentration level of 0.5 μg ml^{-1} and was found to be 88.5% with a relative standard deviation (R.S.D.) of 5.9% (n = 6); at a plasma concentration level of 0.2 μg ml^{-1}, the recovery was 90% (R.S.D. = 8%).

After extraction from the plasma samples, leucovorin was chromatographed by means of the two systems described above. Wainer and Stiffin [3] first determined the total concentration on the phenyl column, using extracts of spiked blank plasma as calibration samples. Second, they used the column-switching technique for the determination of the diastereoisomers, using aqueous solutions of (6*R*)- and (6*S*)-leucovorin as calibration samples. A chromatographic run with the first system took about 40 min, and with the second system about 30 min. To minimize the total analysis time, we determined the concentration of the leucovorin diastereoisomers from the concentration of total leucovorin (determined on the C_{18} column) and from the ratio of the two diastereoisomers. As this ratio is independent of the total concentration, possible losses caused by the column-switching technique are corrected for in this way.

Fig. 2 illustrates the chromatograms of an extract of drug-free plasma and an extract of a spiked plasma sample, obtained on the C_{18} column.

The mobile phase composition used with the BSA phase that leads to optimum separation of the isomers has been determined previously [8].

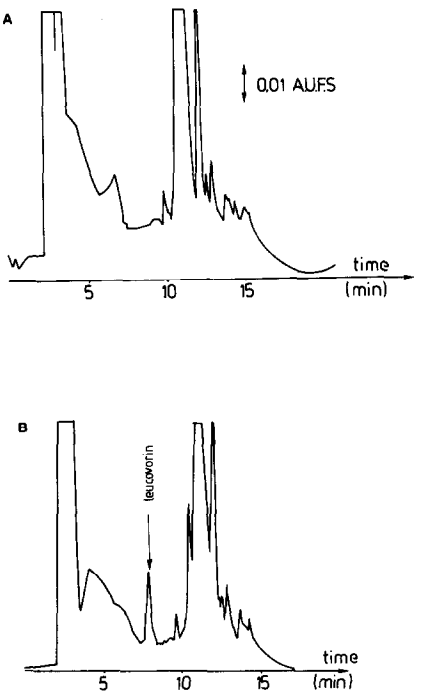

Fig. 2. (A) Chromatogram of extracted blank plasma (system 1; sensitivity 0.01 a.u.f.s.). (B) Chromatogram of extracted plasma spiked with leucovorin (plasma concentration of leucovorin, 500 ng ml^{-1}); the capacity factor of leucovorin in this system is 2.5.

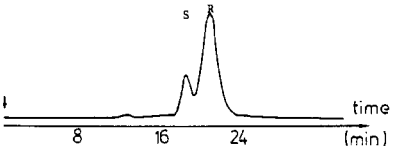

Fig. 3. Chromatogram of the analysis of a patient's sample, obtained 1 h after intravenous administration of (6*S*)-leucovorin. The first-eluting peak represents (6*S*)-leucovorin. The sample was diluted ten-fold (system 2; sensitivity 0.005 a.u.f.s.).

In this study, it was the purpose to develop a method that can be used for the routine analysis of patients' samples. We therefore modified the optimum mobile phase composition in order to shorten the retention times. Fig. 3 illustrates the separation of leucovorin diastereoisomers in a patient's sample obtained 1 h after administration of the mixture of leucovorin diastereoisomers.

To be able to decide whether this method can be used correctly for the analysis of plasma samples, validation of the method was performed. The validation results of the technique described by Wainer and Stiffin [3] have not been published, to our knowledge. The candidate method was evaluated for the analysis of a patient's plasma sample. It was found that 24 h after administration of leucovorin, plasma concentrations of at least 2 $\mu g\ ml^{-1}$ were obtained. Therefore, the samples could be diluted at least ten-fold. This facilitated the SPE procedure, as the flow of undiluted patient sample through the SPE cartridge was difficult. Also, less impurities were loaded on the C_{18} column. Therefore, the validation was performed on drug-free plasma that was diluted ten-fold and that was obtained from different sources.

Validation of the determination of total leucovorin (system 1)

The precision of the method was determined by replicate analyses of six spiked plasma samples on one day (repeatability) and on six different days (within-laboratory reproducibility) (for results, see Table I). The samples were analysed by comparison with a calibration line obtained by spiking drug-free plasma with several known concentrations. The standards of the calibration line were extracted and chromatographed in the same way as the samples. Linear regression analysis was applied to the data. Two replicate measurements were carried out at each concentration level. Analysis of variance showed that there was no lack of fit.

TABLE I

RESULTS OF THE PRECISION MEASUREMENTS FOR THE MIXTURE OF LEUCOVORIN DIASTEREOISOMERS

Concentration added ($\mu g\ ml^{-1}$)	Concentration found (mean) ($\mu g\ ml^{-1}$)	*n*	R.S.D. (%)
Repeatability			
0.500	0.497	8	8.7
2.50	2.42	8	6.3
Reproducibility			
0.500	0.558	5	8.9
2.50	2.68	6	5.9

In Table I, the mean values obtained from the reproducibility study are higher than the expected values at both concentration levels. Therefore, the accuracy of the method was studied more closely.

The accuracy was determined in the following way. Drug-free plasma was spiked at several concentration levels and analysed and the amount found was plotted against the amount added. Each value was the mean of four results, measured on four different days. Linear regression was applied to these data and the following equation was obtained: $y = 1.0246x - 0.0031$, where x is the amount added to the plasma ($\mu g\ ml^{-1}$) and y is the amount found after analysis ($\mu g\ ml^{-1}$). The confidence limits on the slope and intercept were [0.9037,1.1455] and [−0.0705,0.06432], respectively. As the confidence limits on the slope include the value 1 and the confidence limits on the intercept include the value 0, one can conclude that the amount obtained does not differ significantly (at the 5% lev-

el of significance) from the added amount at all concentration levels investigated and that the method is accurate.

The limit of reliable measurement of total leucovorin was determined taking account of the uncertainty of the calibration line [9].

The upper confidence limit for the signal observed when the concentration of the analyte is zero, Y_C is given by

$$Y_C = b_0 + t^{\alpha}_{n-2}\, s_{yx} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\bar{X}^2}{\Sigma(X_i - \bar{X})^2}}$$

where b_0 is the intercept of the calibration line, t^{α}_{n-2} the value of the Student's t-table at significance level α and $n - 2$ degrees of freedom, s_{yx} the residual standard deviation, m the number of replicate measurements, n the number of standard solutions, $\bar{X}$ the mean of the analysed concentrations and X_i each concentration value. The analyte concentration, X_C, corresponding to this signal, is determined as $(Y_C - b_0)/b_1$, where b_1 is the slope of the calibration line. X_C is called the decision limit.

The limit of reliable measurement, X_D, is calculated from the equation

$$X_D = X_C + t^{\beta}_{n-2}\, s_{yx}/b_1 \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(2X_C - \bar{X})^2}{\Sigma(X_i - \bar{X})^2}}$$

where t^{β}_{n-2} is the value in the Student's t-table at significance level β and $n - 2$ degrees of freedom. The corresponding instrumental signal Y_D is calculated as $b_1 X_D + b_0$. In this application, the limit of reliable measurement was found to be 0.050 μg ml^{-1}. As a leucovorin concentration of 2 μg ml^{-1} was determined in a patient's plasma sample 24 h after administration, one can conclude that the detection limit of the method is acceptable in the present application.

Validation of the column-switching technique (system 2)

We validated the column-switching technique by adding several ratios of (6*S*)- to (6*R*)-leucovorin to drug-free plasma at two concentration levels, 500 and 2500 ng ml^{-1}. The results are summarized in Table II.

Table II shows that the repeatability and the

TABLE II

REPEATABILITY AND REPRODUCIBILITY OF THE COLUMN-SWITCHING TECHNIQUE

Concentration level (ng/ml^{-1})	Ratio (6*S*/6*R*)	(6*S*)-Leucovorin			(6*R*)-Leucovorin		
		Theoretical value (ng ml^{-1})	Mean result obtained (ng ml^{-1})	R.S.D. (n=6) (%)	Theoretical value (ng ml^{-1})	Mean result obtained (ng ml^{-1})	R.S.D. (n=6) (%)
Repeatability							
500	25:75	130	119	2.6	397	408	0.77
	50:50	227	242	2.6	227	212	3.0
	75:25	407	425	1.6	124	106	6.4
2500	25:75	651	658	1	1986	1979	0.34
	50:50	1134	1194	2.4	1134	1074	2.7
	75:25	2035	1970	0.81	621	686	2.3
Reproducibility							
500	25:75	130	146	9.3	397	382	3.5
	50:50	227	245	3.1	227	209	3.6
	75:25	407	418	1.7	124	114	6.1
2500	25:75	651	774	7.4	1986	1863	3.1
	50:50	1134	1215	3.3	1134	1054	3.8
	75:25	2035	2097	0.98	621	559	3.7

reproducibility of the method are good, as small relative standard deviations are obtained. The accuracy was determined in the same manner as described above, but here each data point represents the mean of six measurements obtained from the repeatability study. The confidence limits on the slope and the intercept are [0.9031, 1.0437] and [−46.45,95.10], respectively, for (6*S*)-leucovorin and [0.9102,1.0698] and [−75.21,82.22], respectively, for (6*R*)-leucovorin. The confidence limits on the slopes include 1 and those on the intercepts include 0. From these data, we can conclude that the method for the determination of the diastereoisomers is accurate and precise.

On considering the data obtained from the reproducibility study, however, one observes that all the values analysed are larger than the theoretical values for (6*S*)-leucovorin and smaller than the theoretical values for (6*R*)-leucovorin. These experiments were performed *after* the repeatability studies and the stock solutions of (6*R*)- and (6*S*)-leucovorin were not freshly prepared because of the small amounts of the separate isomers available. The stock solutions were stored at 4°C. The chromatograms show that the peak areas obtained for (6*S*)-leucovorin were approximately the same for both the repeatability and reproducibility studies, whereas for (6*R*)-leucovorin a decrease in the peak areas occurred. This might indicate a degradation of (6*R*)-leucovorin in the stock solutions, which should be prepared freshly at least every week. These findings made it necessary to evaluate the stability of leucovorin in the stored frozen plasma samples. This was done for a period of two months. No degradation was observed during this period.

Determination of 5-fluorouracil in plasma

Sample pretreatment. The extraction recovery (for extraction procedure, see Experimental) was determined at a plasma concentration level of 2 $\mu g\ ml^{-1}$ and was found to be 75.9% with an R.S.D. of 4.8% ($n = 6$). In this application, an internal standard was used as possible losses of the substance might occur when applying liquid–liquid extraction. Nicotinamide (Fig. 1) was found to be a suitable internal standard owing to its structural similarity to 5FU. The extraction recovery of nicotinamide was found to be 67.5% (R.S.D. = 7.5%).

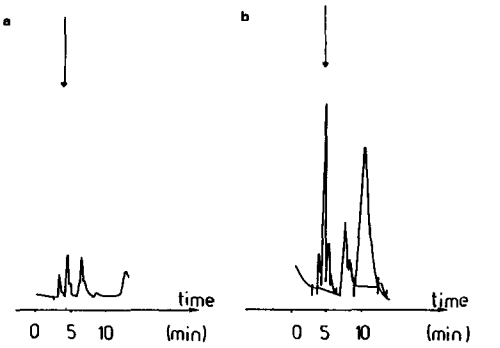

Fig. 4. (a) Chromatogram of extracted blank plasma. (b) Chromatogram of extracted plasma spiked with 5FU (plasma concentration 1 $\mu g\ ml^{-1}$, sensitivity 0.008 a.u.f.s.); the capacity factor of 5FU in this system is 1.0.

Fig. 4 illustrates the chromatograms of an extract of drug-free plasma and an extract of a plasma sample spiked with 5FU and nicotinamide.

Validation of the determination method for 5-fluorouracil. The repeatability and reproducibility of the method were determined at two concentration levels (2 and 20 $\mu g\ ml^{-1}$, concentrations determined from analysis of a patient's sample with the candidate method). The repeatability and reproducibility, expressed by means of the R.S.D. on the measurements, were found to be 5.3 and 7.7%, respectively, for a plasma concentration of 2 $\mu g\ ml^{-1}$ and 5.5 and 4.1%, respectively, for a plasma concentration of 20 $\mu g\ ml^{-1}$. The measurements of the 5FU concentrations in plasma were performed by comparison with a calibration line the standards for which were treated in the same way as the samples. The relationship between signal and concentration was linear for the studied concentration range. Two replicate measurements were performed at each concentration level of the calibration line. Analysis of variance showed that there was no lack of fit. The accuracy of the method was determined in the same way as described above. The confidence limits on slope, [0.9927,1.0436], and intercept, [−0.2666,0.1777], include 1 and 0 and one

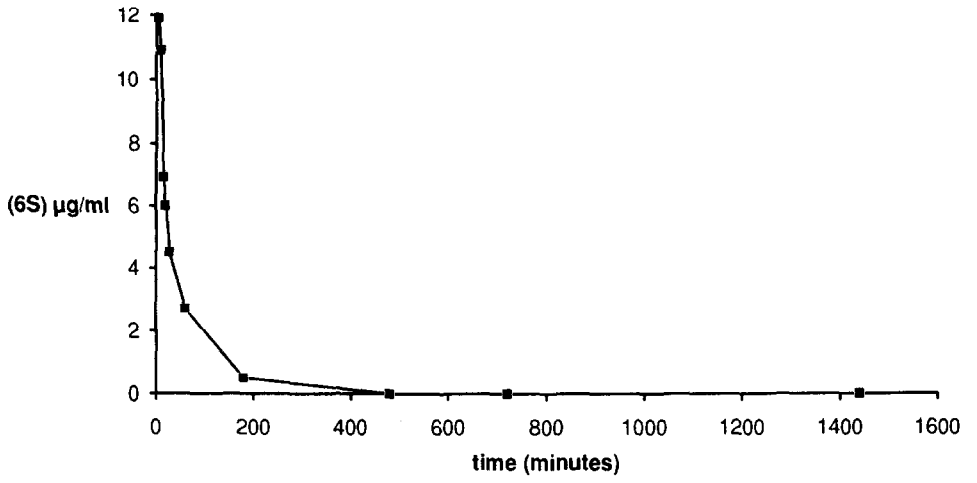

Fig. 5. Plasma concentration–time curve for (6*S*)-leucovorin in a patient's plasma sample.

can therefore conclude that the method is accurate.

The limit of reliable measurement for 5FU was calculated from the calibration data in the same way as described for leucovorin and was found to be 0.69 μg ml^{-1}. The concentration obtained is higher than that for the standards of the calibration line with the lowest concentration. A possible explanation might be the large concentration range of the calibration line, *i.e.* 0.250–20 μg ml^{-1}, which influences $\bar{X}$. It would be better to use calibration standards with concentrations close to zero.

The stability of 5FU in frozen plasma samples was evaluated for a period of two months. No degradation of the compound was observed.

CONCLUSION

Two methods were developed for the determi-

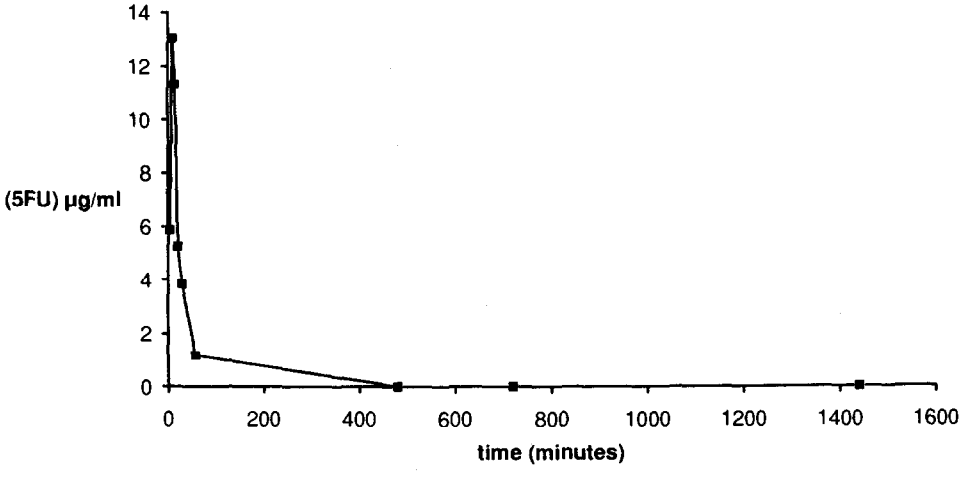

Fig. 6. Plasma concentration–time curve for 5FU in a patient's plasma sample.

nation of leucovorin and 5FU in plasma. Leucovorin was extracted from plasma by means of an SPE technique and 5FU by means of liquid–liquid extraction. The concentration of the leucovorin diastereoisomers was determined from the concentration of total leucovorin and from the ratio of (6*S*)- and (6*R*)-leucovorin, determined by coupling a BSA phase to the achiral column. The precision and accuracy of the developed techniques were good. The methods have been applied in our laboratories to the routine analysis of samples from patients treated with 5FU and leucovorin. A disadvantage is the relatively short lifetime of the BSA stationary phase. Figs. 5 and 6 illustrate plasma concentration–time curves for (6*S*)-leucovorin and 5FU, respectively, obtained after analysis of patients' samples.

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