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Simultaneous gas chromatographic–mass spectrophotometric determination of α-fluoro-β-alanine and 5-fluorouracil in plasma

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Abstract

A gas chromatographic–mass spectrometric (GC–MS) method is reported for simultaneous determination of α-fluoro-β-alanine (FBA), the major end metabolite of 5-fluorouracil (5-FU), and 5-FU in plasma samples isolated from cancer patients. 5-Chlorouracil (5-ClU, 1 μg/ml) is added to samples as an internal standard. The method relies on protein precipitation of the plasma sample followed by derivatisation with pentafluorobenzyl bromide. Following sample purification with Sep-pak C_{18} columns the derivatives are analysed by GC–MS, with FBA, 5-FU and 5-ClU being determined at 36.96–37.03, 46.91–46.98 and 51.99–52.13 min, respectively. The ions measured in each case had *m/z* of 390, 490 and 506, respectively. The method showed good reproducibility with coefficients of variation between 3 and 10%, with a detection limit of <1 ng/ml for 5-FU and <5 ng FBA/ml plasma. The possibility of sensitive determination of FBA without the use of radioisotopes should permit routine estimation of rates of 5-FU metabolism in individual patients.

Keywords: α-Fluoro-β-alanine; 5-Fluorouracil

1. Introduction

5-Fluorouracil (5-FU) is a well-established phase-specific antimetabolite which is metabolised within cells to the active principle, fluorodeoxyuridine monophosphate, an inhibitor of thymidylate synthase. It is widely used in the treatment of cancer and is normally administered parenterally due to wide inter-patient variation in oral absorption. 5-FU is not a substrate for P-glycoprotein-mediated multi-drug resistance and it is the major drug currently used in the treatment of colorectal cancer in combination with folinic acid [1]. Its phase-specificity means that improved responses may be obtained by sustained or locoregional infusion and a range of clinical approaches is under investigation [2]. Infusional 5-FU is also of use in the treatment of other malignancies and its role is currently being tested in the treatment of ovarian, breast and gastric cancers where it is administered together with 3-weekly doses of cisplatin and epirubicin.

The plasma kinetics of 5-FU are well described and the plasma drug concentration can be determined using a range of conventional HPLC and GC assays [3]. It has a short plasma half-life (10–15 min) due to rapid metabolic inactivation and a large volume of distribution, and systemic clearance shows significant inter-patient variation [4,5]. Side effects associated with 5-FU are mainly gastrointestinal and their severity varies considerably between patients [6], possibly due to varied levels of expression of metabolising enzymes such as dihydropyrimidine

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dehydrogenase [7]. In addition 5-FU has non-linear kinetics which means that there can be a relatively large increase in plasma concentration for a given increase in dose. Determination of plasma concentration of 5-FU and its metabolites could therefore be predictive of 5-FU-related toxicity and could even be used for dose optimisation, since rapid metabolism is implicated in poor response and invites the use of increased drug doses [7].

Recently more comprehensive analysis of the organ distribution of 5-FU has been performed using positron emission tomography (PET) scanning [8,9]. This technique relies on determination of ^{18}F and cannot distinguish the active drug from any of its fluorine-containing metabolites. Analysis of tissue-specific drug distribution monitored by PET would be facilitated by knowledge of the relative pharmacokinetics and metabolism of 5-FU and its metabolites, permitting development of comprehensive compartmental models for drug metabolism, distribution and excretion.

The major end metabolite of 5-FU is known to be α-fluoro-β-alanine (FBA). FBA is hard to measure using conventional spectrophotometric absorption or fluorescence techniques because of similar physicochemical properties to other plasma components, such as free amino acids. In some studies the kinetics of FBA distribution have been examined using ^{3}H-labelled 5-FU coupled with extensive HPLC resolution and scintillation counting of fractions. This technique is labour-intensive, however, and the application of radioisotopes renders it unsuitable for routine determination of plasma kinetics in cancer patients.

We have developed a GC–MS method permitting simultaneous determination of FBA, together with 5-FU and 5-chlorouracil (5-ClU, used as an internal standard during sample analysis) in plasma samples from cancer patients. The method is based on derivatisation with pentafluorobenzylbromide and has a detection limit of <5 ng/ml for FBA in plasma samples.

2. Experimental

2.1. Chemical and solvents

5-ClU and pentafluorylbenzylbromide were purchased from Sigma (Poole, UK) and FBA was from TCI (via Fluorochemm, Old Glossop, UK). 5-FU for clinical studies was from David Bull Laboratories (Warwick, UK), and for in vitro studies from Sigma. Sep-Pak cartridges were from Waters (Watford, UK). Plastic ware was from Sarstedt (Leicester, UK) and glassware and all solvents were purchased from Fisher (Loughborough, UK).

2.2. Gas chromatography–mass spectrometry (GC–MS)

Analysis was performed using a Hewlett–Packard GC–MS 5902 (Stockport, UK) fitted with a J&W DB23 column (Fisher Scientific), 30 m×0.25 mm (I.D.) with film thickness 0.25 μm. The carrier gas was helium at 12 p.s.i., 0.6 ml/min (linear velocity 30.6 cm/s) and samples (1 μl) were injected by splitless injection. The total run time was 60.33 min with the initial oven temperature 150°C (0–10 min), rising at a rate of 6°C/min to 210°C which was maintained for 12 min. Temperature then rose further at a rate of 6°C/min to 260°C and was maintained for a further 20 min before resetting. The temperature of the injection port was 260°C and the interface between the GC and the MS was maintained at 280°C.

Following method development (vide infra) ions derived from FBA, 5-FU and 5-ClU were determined at 36.96–37.03, 46.91–46.98 and 51.99–52.13 min, respectively. The ions measured in each case had m/z of 390, 490 and 506, respectively. The 490 and 506 ions were the molecular ions of the di-derivatised respective compounds, while the 390 ion was the base peak from the di-derivatised FBA, representing the di-N-derivatised methylamine ion produced following loss of the fluoroacetic acid moiety. Peak areas were integrated using Hewlett–Packard Chemstation software.

2.3. Sample preparation

Plasma samples (1.0 ml) for analysis were placed into 15-ml polypropylene tubes and 5-ClU was added to all tubes (1 μg/50 μl) as an internal standard. Protein was precipitated by the addition of acetonitrile (1.0 ml) and vigorous vortexing (10 min) prior to centrifugation (3000 *g*, 15 min, 4°C). The supernatants were carefully removed and introduced

into 4-ml glass screw top vials. K_2HPO_4 (1 *M*, 100 μl, pH 11.0) was added to each, followed by liquid pentafluorobenzyl bromide (20 μl). The tubes were then sealed and heated to 100°C (60 min) with occasional shaking. After cooling to room temperature samples were centrifuged (3000 *g*, 15 min) and the supernatants applied to Sep-pak C_{18} columns which had been pretreated sequentially with methanol (3 ml) and water (3×3 ml). Columns were then washed three times with water (3 ml) and adhered sample components were eluted with ethanol (2×2.0 ml) into 15-ml polypropylene tubes. Samples were then evaporated to dryness using a vortex evaporator. Dried samples were resuspended in acetone (50 μl) and hexane (100 μl), centrifuged to remove undissolved solids (3000 *g*, 10 min, room temperature) and analysed by GC–MS. Quantities of 5-FU and FBA were calculated as an area ratio to the molecular ion for the 5-ClU internal standard. This procedure routinely gave recovery of 75–85% for both 5-FU and 5-ClU in spiked plasma samples. Recovery of FBA could not be determined precisely, we are not able to measure this substance by HPLC, although comparison of signals suggested recovery was of similar efficiency as 5-FU and 5-ClU.

2.4. Isolation of plasma samples

Plasma samples for method development were obtained from the National Blood Transfusion Service. Experimental samples of blood (10 ml) were taken from a cancer patient before (0) and 17, 24, 27.5, 32.7, 45, 62.3, 75.4, 90.5, 104.5 and 119.5 min following the start of a 15.5-min infusion of 5-FU (400 mg/m^2). Blood was collected into lithium–heparin tubes and immediately centrifuged at 4°C (2000 rpm, 15 min) to isolate plasma. Plasma samples were stored frozen at −20°C for up to 4 h prior to storage at −80°C until analysis.

3. Results and discussion

3.1. Development of GC–MS methodology

Total ion current analysis was performed from *m/z* 50–550 using the GC–MS detection protocol described above. Blank plasma samples were compared with samples spiked with FBA, 5-FU and 5-ClU individually and together to identify retention times (Fig. 1). Full mass spectrometric analysis was then performed at the retention time appropriate for FBA and two major ions were determined (with *m/z* values 181 and 390; Fig. 2). The molecular ion (*m/z* 467) was present only in tiny quantities, so the more abundant di-N-derivatised methylamine ion (*m/z* 390) was used for routine quantification instead. 5-FU and 5-ClU were determined using molecular ion species (*m/z* 490 and 506, respectively) as described by Anderson et al. [10].

The length of this assay can be shortened for routine use by increasing the temperature ramp to 40°C/min. This decreases total analysis time to about 30 min with no effects observed on sensitivity and resolution. The longer analysis was used here to maximise information stored for future retrospective analysis of other, as yet unidentified, metabolites.

3.2. Validation of GC–MS methodology

Human plasma was spiked with 5-FU and FBA at various concentrations, together with 5-ClU at a final concentration of 1 μg/ml. After 15 min at room temperature samples were processed as above. A limit of quantification (LOQ) of 5 ng FBA/ml plasma was determined at a signal-to-noise ratio of 4, shown in detail in Fig. 3. The coefficients of variation are summarised in Table 1 and the linearity of the method (R=0.999) is illustrated in Fig. 4.

3.3. Assessment of sample stability

Human plasma was spiked with 5-FU and FBA at various concentrations and stored at −70°C before spiking with 5-ClU and GC–MS analysis. Both 5-FU and FBA signals showed deterioration at a rate of less than 1%/week for at least three months when stored at −70°C, and no changes were observed in sealed samples stored ready for injection for 24 h at room temperature in acetone–hexane.

3.4. Measurement of 5-FU and FBA in experimental plasma samples

Human plasma samples were isolated from a patient receiving 5-FU treatment as described above. Levels of

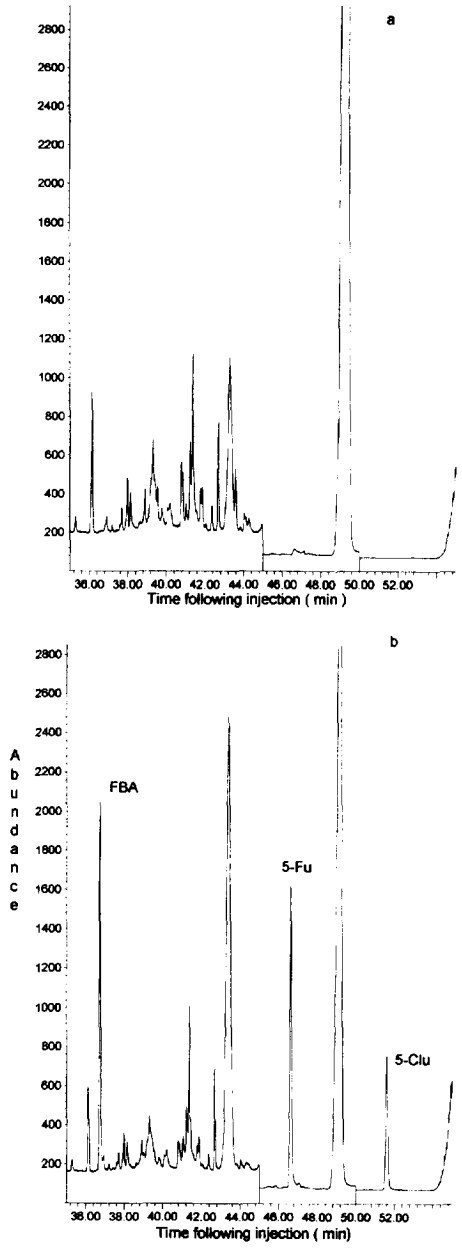

Fig. 1. Comparison of (a) blank plasma and (b) plasma spiked with 5-FU (200 ng/ml), 5-ClU (1 μg/ml) and FBA (200 ng/ml) prepared and analysed by GC–MS as described above. Drugs were monitored by selected-ion monitoring as follows: 0–35 min, determination of m/z 530 (irrelevant ion); 35–45 min, m/z 390 (characteristic of FBA); 45–50 min, m/z 490 (characteristic of 5-FU); 50–53 min, m/z 506 (characteristic of 5-ClU); 53–60.3 min, m/z 530 (irrelevant ion). Positions for the individual peaks within these time windows are described in Section 2.2.

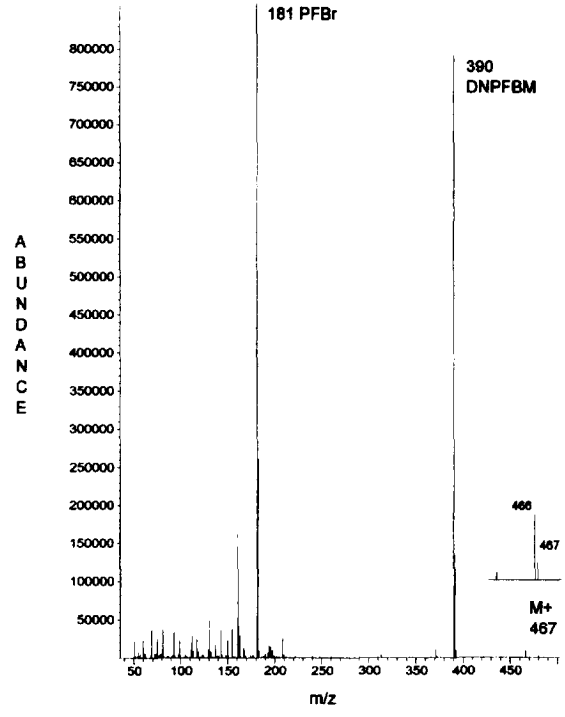

Fig. 2. Mass spectrum of di-pentafluorobenzyl derivative of FBA determined in a plasma sample. The two major ions detected have m/z 181 (corresponding to the free pentafluorobenzyl reagent; PFBr) and 390 (thought to represent the di-N-derivatised methylamine ion, produced following fission of the FBA adduct; DNPFBM). The molecular ion (M^+) of the FBA adduct (m/z 467) was present in very small quantities (shown in the insert) and was not used for quantification.

FBA and 5-FU were determined simultaneously by GC–MS and calculated against the internal standard. 5-FU showed an early rise to a peak (94.8 μM) at the first sample following the end of infusion, followed by rapid clearance with a half-life of 11 min, approximately (Fig. 5). FBA conversely showed initial low levels, rising fairly steadily to reach a peak after 90.5 min (76.3 μM) and then falling back towards baseline. These data suggest that the metabolic pathway leading from 5-FU to FBA can be completed in 60–80 min at these drug concentrations.

4. Conclusions

The GC–MS method reported here enables simultaneous determination of 5-FU and FBA in

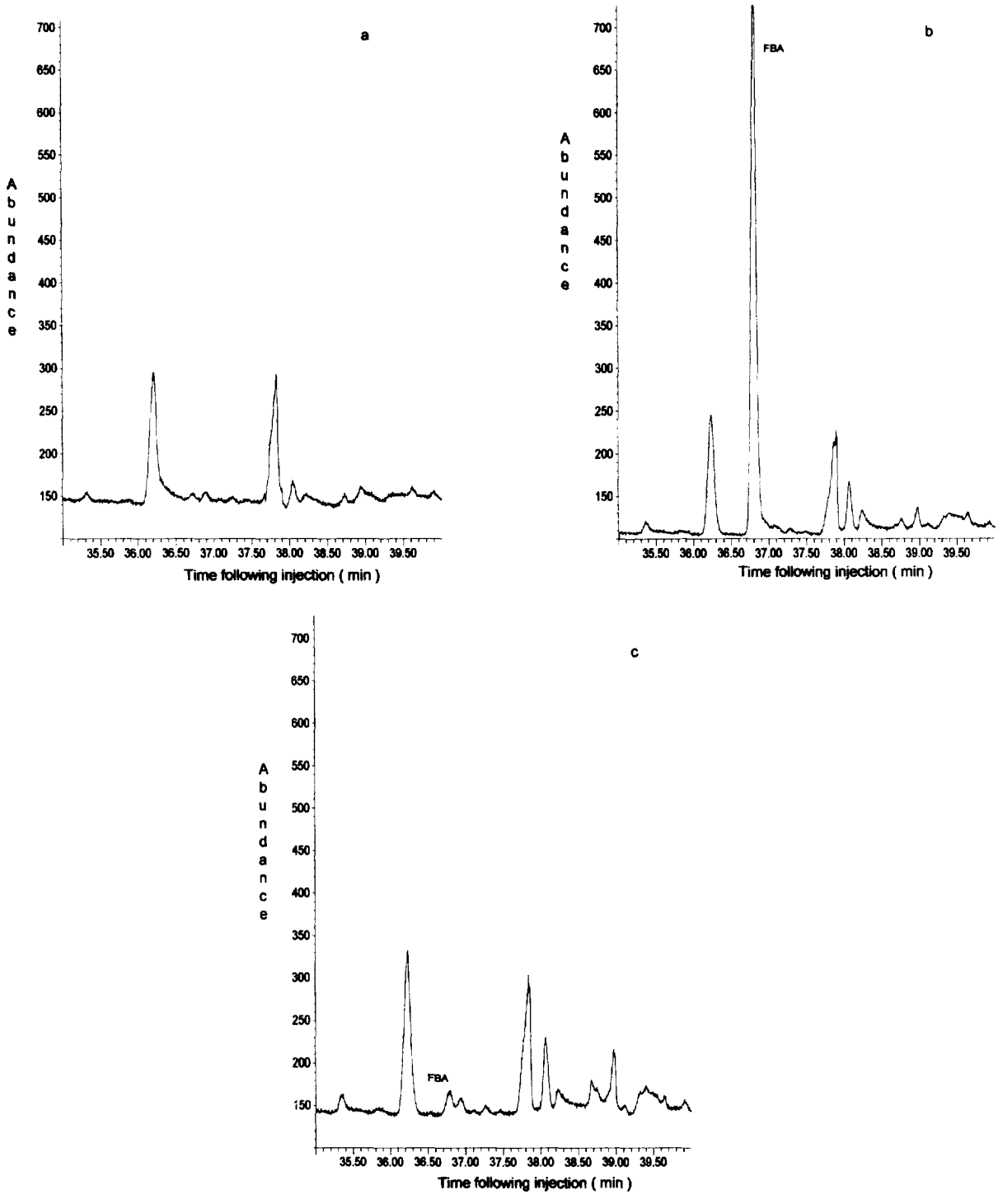

Fig. 3. Comparison of (a) blank plasma with (b) plasma spiked with 200 ng/ml FBA and (c) 5 ng/ml FBA, prepared and analysed by GC–MS as described above. The m/z 390 signal was monitored between 35–40 min, and at 5 ng/ml (c) the FBA signal was found to be 4× the background signal, defined as the LOQ.

Table 1
Precision of α-fluoro-β-alanine determination in spiked human plasma

Concentration FBA standard (ng/ml plasma)	Concentration FBA determined by GC–MS (ng/ml)	Standard deviation (ng/ml)	Coefficient of variation (intra-day) (%)	Coefficient of variation (inter-day) (%)
5	4.7	0.24	5.1	N.D.
25	24.9	2.4	9.7	3.7
200	199.4	11.1	5.5	8.5

Values shown represent the mean of 6 determinations.

human plasma samples, with good precision and reproducibility. Measurement of both parent drug and its major metabolite in the same plasma sample should minimise experimental errors as well as accelerating the analysis. The method also has significant advantages over previously-published systems for determination of FBA since it does not involve the use of radioisotopes and can be used routinely on samples from patients receiving conventional 5-FU treatment. The results obtained are in agreement with the published metabolism and pharmacokinetic data obtained using [^{3}H]5-FU to determine drug levels with HPLC resolution of metabolites [11].

The possibility for routine measurement of FBA levels in plasma samples will permit evaluation of 5-FU metabolism in individual patients, enabling assessment of the relationship between metabolism, systemic toxicity and possible efficacy.

In addition, study of the pharmacokinetics of FBA in individual patients, and understanding its distribution and clearance, will permit development of compartmental models of central importance in decoding the ^{18}F-based measurements obtained by PET scanning.

Acknowledgments

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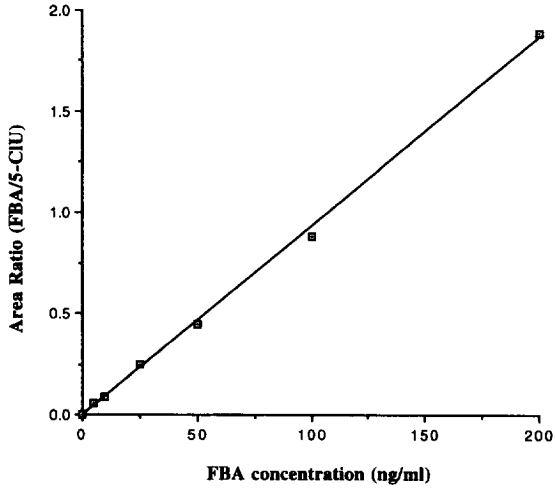

Fig. 4. Linearity of FBA determination. FBA was added to samples of plasma at final concentrations from 0–200 ng/ml, with 5-ClU (1 μg/ml) as internal standard. Following sample preparation and GC–MS analysis as described in the text, ratios of FBA to 5-ClU signal areas were graphed against FBA concentration. The graph shows a coefficient of linear regression of 0.999.

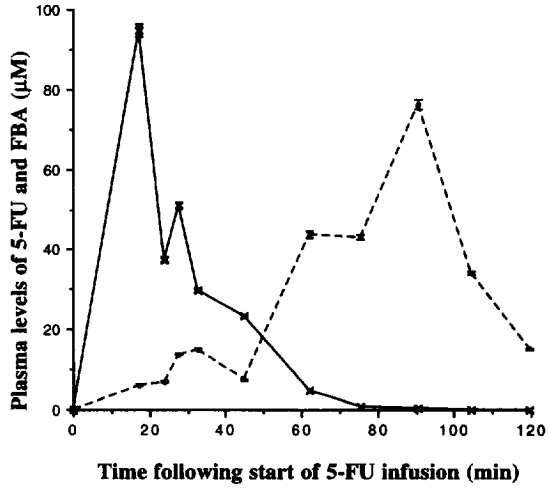

Fig. 5. GC–MS determination of 5-FU (solid line, symbol X) and FBA (dashed line, symbol ○) in plasma samples isolated from a cancer patient receiving a 15.5-min infusion of 5-FU, at a dose of 400 mg/m^2 (total 720 mg). Samples were prepared and analysed in the presence of a 5-ClU internal standard as described in the text, (n=6 for each determination) and the mean value±S.D. is represented in each case.

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