Journal of Chromatography, 491 (1989) 129–138
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4731

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 5-BROMODEOXYURIDINE IN HUMAN PLASMA

PAOLO G. MONTALDO and MAURIZIO D'INCALCI*

Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milan (Italy)

(First received October 21st, 1988; revised manuscript received February 13th, 1989)

SUMMARY

A new method is described for the determination of submicromolar concentrations of 5-bromodeoxyuridine in human plasma. Sample pretreatment involves cold methanol deproteinization, freezing–thawing and lyophilization. The sample is then analysed by reversed-phase high-performance liquid chromatography. This method is very reproducible and has a detection limit of 0.1 μg/ml ($0.32 \cdot 10^{-6} M$). Comparison with other procedures indicates that the method is advantageous as regards sensitivity and specificity and can be readily applied in clinical pharmacological investigations.

INTRODUCTION

The halogenated nucleoside 5-bromodeoxyuridine (BUdR) is a thymidine analogue that is incorporated into the DNA of mammalian cells, where it takes the place of its natural counterpart thymidine when exogenously supplied to the cells. The main biological property of BUdR exploited so far for clinical purposes is its ability to increase the susceptibility of mammalian cells to the lethal effects of X-rays [1,2]. BUdR has been administered by intravenous injection and its pharmacokinetic properties have been studied by determining levels in patients' plasma.

BUdR is currently used to investigate the rate of DNA synthesis of tumour cells, using monoclonal antibodies that specifically react with BUdR incorporated into DNA. For these studies BUdR is given to patients in relatively low doses, and therefore monitoring of the plasma levels requires a sensitive assay.

0378-4347/89/$03.50 © 1989 Elsevier Science Publishers B.V.

Different sample pretreatment procedures and chromatographic methods have been described for analysis of BUdR in animal and human plasma, based on liquid–liquid [1–4] or liquid–solid [5] extraction followed by reversed-phase high-performance liquid chromatography (HPLC). However, our attempts to reproduce in human plasma the results reported by these authors have been fruitless, mainly because of the presence of endogenous peaks that interfere with the identification and quantification of BUdR and the most frequently used internal standard, 5-iododeoxyuridine (IUdR).

This paper describes a new quantitative HPLC assay for the determination of BUdR. It is sufficiently sensitive and specific for clinical pharmacological studies, and comparison with the previous methods shows it to offer clearly superior sensitivity with no interfering peaks.

EXPERIMENTAL

Chemicals and reagents

Methanol and acetonitrile, both HPLC grade, were purchased from Carlo Erba (Milan, Italy) and Omnia Res (Milan, Italy), respectively. Monobasic ammonium phosphate, potassium hydroxide, sodium hydroxide, ammonium sulphate, ammonium acetate, monobasic potassium phosphate, ethyl acetate, acetic acid, hydrochloric acid and acetone were from Merck (Darmstadt, F.R.G.). 5-Bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine were purchased from Sigma (St. Louis, MO, U.S.A.). 5-Iodouracil (IU) was from Aldrich-Chemie (Steinheim, F.R.G.). Water was obtained with a Millipore Milli-Q apparatus, and its absorbance at 254 nm was checked by HPLC.

Standard solutions

BUdR was dissolved in water at room temperature at a concentration of 100 μg/ml. IUdR was dissolved in 0.2 *M* sodium hydroxide at a concentration of 1 mg/ml. These solutions were protected from light and stored at +4°C. Their stability was checked daily and was longer than three weeks in these conditions. The stability was checked by calculating the mean height of BUdR and IUdR peaks after three or more consecutive 25-μl injections into the HPLC system of a mixture made up of 100 μl of BUdR stock solution, 20 μl of IUdR stock solution, 4 μl of 37% hydrochloric acid (to neutralize the alkaline IUdR solution) and water to a final volume of 1 ml. The mean height of peaks was compared with that on preceding days, and stability was assumed until a significant difference (i.e. >5%) from the initial value was observed. Addition of a few drops of acetic acid to 100 ml of BUdR stock solution prevented its degradation for longer periods (up to two months). In any case, new stock solutions of both BUdR and IUdR were prepared every two weeks.

Dissolution of BUdR and IUdR in methanol, as indicated by Stetson et al. [4], at the above concentrations was very slow and incomplete in our hands,

even with a moderately heated (37°C) magnetic stirrer. Furthermore, the methanolic solution was unstable and gave rise to broad, flattened peaks in HPLC analysis only few days after preparation (data not shown).

Plasma pretreatment

Blood samples from four normal donors were collected in heparinized plastic tubes and centrifuged at 400 *g* for 10 min. Plasma was immediately transferred to clean glass tubes to prevent the release of UV-interfering substances from platelets and red blood cells [6].

A constant amount of IUdR (2.5 μg) or IU (1 μg) as internal standards and increasing amounts of BUdR (from 0.05 μg to 1 μg) were added to 0.5 ml of plasma to obtain the following plasma concentrations: 0.1, 0.2, 0.4, 0.8, 1 and 2 μg/ml. For each experiment two blank plasma samples (i.e. without addition of drugs) were processed in the same manner as the treated samples.

Four different plasma pretreatment procedures were followed.

Method I. The liquid–liquid extraction method described by Stetson et al. [4] for BUdR assay in dog plasma. Briefly, this method involves protein precipitation with saturated ammonium sulphate, plasma salting with ammonium phosphate buffer (pH 6.7) and liquid extraction with ethyl acetate. The organic phase is then back-extracted with 0.5 *M* potassium hydroxide, and the resulting alkaline aqueous phase is injected for HPLC analysis. IU is used in this method as internal standard.

Method II. The method described by Russo et al. [2], in which plasma is salted with 1 *M* monobasic potassium phosphate (pH 4.6) and extracted with ethyl acetate. The organic phase is then evaporated under nitrogen, and the sample residue is dissolved in the HPLC mobile phase (see below) and analyzed.

Method III. The liquid–solid extraction method reported by Klecker et al. [5] in which samples are cleaned using Sep-Pak C_{18} cartridges. BUdR and IUdR are then eluted by washing the cartridge with methanol, concentrated by evaporation, redissolved in HPLC mobile phase and analysed.

Method IV. A new method developed by us. To plasma samples (blank or with BUdR and IUdR added), as described above, an equal volume (0.5 ml) of cold methanol (−20°C) was added in 1.5-ml plastic conical-bottomed Eppendorf tubes. The tubes were then tightly capped and plunged into a cold acetone–dry ice bath for 5 min. The tubes were then centrifuged in an Eppendorf Microfuge 5415 for 5 min at 12 900 *g* at room temperature. The supernatants were transferred into new Eppendorf tubes, frozen in the acetone–dry ice bath and again centrifuged. This procedure was repeated until no precipitated material was present on the tube bottom (usually three or four times). After the last centrifugation the samples were frozen and lyophilized. The resulting material was dissolved in 100 μl of HPLC mobile phase (see below) and filtered through a 0.45-μm HPLC certified Acro LC3A filter (Gelman Sciences, Ann Arbor, MI, U.S.A.); 50 μl were injected for HPLC analysis.

Chromatography

The HPLC equipment included a Waters Assoc. Model 6000 A pump, a Waters Assoc. Model 440 absorbance detector with a 254-nm filter and a Perkin-Elmer Model 561 recorder. A Waters Assoc. μBondapak C_{18} column (5 μm particle size, 30 cm × 3.9 mm I.D.) was used in the majority of experiments.

In earlier experiments for analysis of samples extracted with method I [4], a Supelcosil LC-8 column (5 μm particle size, 25 cm × 4.6 mm I.D., Supelco, PA, U.S.A.) was used. BUdR and IUdR peaks were identified by retention times compared with external standards and, in early experiments, by UV absorbance spectrum analysis using a rapid spectral detector Model 2140 (LKB, Bromma, Sweden). Once the two criteria of identification were ascertained to be equivalent, only the retention time was considered.

HPLC mobile phases used were as indicated in the published methods. For the extraction method I [4], the mobile phase was 12% methanol (v/v) in 0.05 *M* ammonium phosphate buffer (pH 7.3); the flow-rate was 1.0 ml/min. For the extraction method II [2], it was 9% methanol (v/v) in 0.01 *M* monobasic potassium phosphate (pH 3.5); the flow-rate was initially 3.0 ml/min (as indicated in the original method, which does not specify the column size), then lowered to 1.7 ml/min because of the high back-pressure generated even with new columns. For samples extracted according to method III [5], the mobile phase was 6% acetonitrile in 25 m*M* acetic acid; the flow-rate was 2.0 ml/min. For method IV the same mobile phase was used but the percentage of acetonitrile was lowered to 5% and the flow-rate to 1.0 ml/min. Between each injection the column was washed for 10 min with acetonitrile–water (70:30, v/v) and allowed to equilibrate for 20 min before the following sample injection.

Prior to use all mobile phases were filtered through a 0.45-μm filter and degassed by ultrasonication for at least 15 min.

Calculations

The linearity of the dose-to-signal ratio was established by plotting the ratio of the peak height of BUdR to that of the internal standard (IUdR) as a function of the plasma BUdR concentration. The best-fit straight line was determined by the least-squares method; graphic elaboration was done with a Macintosh Cricket Graph computer program.

RESULTS

Method I

Fig. 1 shows a representative chromatographic profile of standards (BUdR and IU, 0.25 μg of each injected) (A), a blank plasma sample (B), a plasma sample from the same donor containing 1 μg/ml BUdR and 2 μg/ml IU (C) and plasma sample spiked with 2 μg/ml BUdR and 2 μg/ml IU (D) extracted

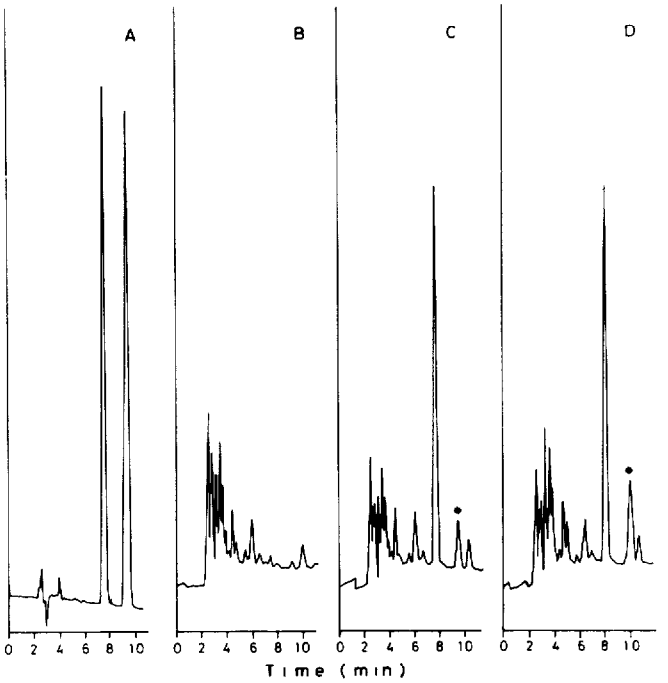

Fig. 1. Chromatographic profiles of (A) IU (first peak) and BUdR (second peak), 0.25 μg each, (B) blank plasma, (C) plasma containing 2 μg/ml IU and 1 μg/ml BUdR and (D) plasma containing 2 μg/ml IU and 2 μg/ml BUdR. Samples were treated as described under *Method I* in Experimental, and 100 μl of the final alkaline phase were injected. Column, Supelcosil LC-8, 5 μm particle size (25 cm×4.6 mm I.D.); mobile phase, 12% (v/v) methanol in 0.05 *M* ammonium phosphate (pH 7.3); flow-rate, 1.0 ml/min; detection, 254 nm at sensitivity 0.01 a.u.f.s. Asterisk = BUdR.

and chromatographed according to method I. The BUdR peak is very low even at relatively high plasma concentrations (Fig. 1C and D).

The experiment was repeated twice with the plasma of each donor, giving very similar results, suggesting that this method, although apparently sensitive and efficient on dog's plasma, is not suitable for assays with human plasma.

Method II

Fig. 2 illustrates the results of extracting plasma according to method II [2] and chromatographing in the conditions indicated by these authors. Blank plasma contains an unidentified peak that almost completely overlaps the internal standard (IUdR) (Fig. 2B). In this case too the efficiency of extraction is low, giving rise to a barely evident BUdR peak for a plasma concentration less than 0.5 μg/ml. This was confirmed using plasma from all four donors; the

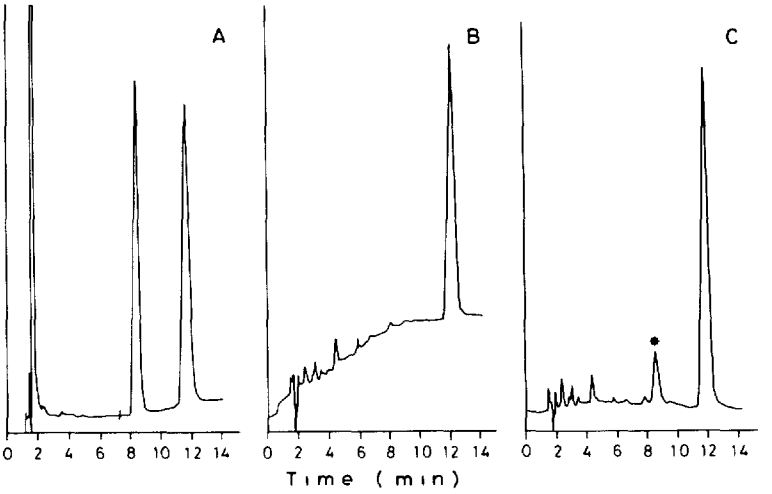

Fig. 2. Chromatographic profiles of (A) BUdR (0.25 μg, first peak) and IUdR (0.5 μg, second peak), (B) blank plasma and (C) plasma containing 1 μg/ml BUdR and 2 μg/ml IUdR. Samples were treated as described under *Method II* in Experimental, and 50 μl of the total 150 μl were injected. Column, Waters μBondapak C_{18}, 5 μm particle size (30 cm × 3.9 mm I.D.); mobile phase, 9% (v/v) methanol in 0.01 *M* potassium phosphate (pH 3.5); flow-rate, 1.7 ml/min; detection, 254 nm at sensitivity 0.01 a.u.f.s. Asterisk = BUdR.

analytical result was not improved by slightly modifying the flow-rate, the organic solvent composition, the pH or the ionic strength of the mobile phase (data not shown).

Method III

Fig. 3 shows the chromatographic profile obtained by the liquid–solid extraction method described by Klecker et al. [5]. The chromatogram of the blank plasma (Fig. 3B) is crowded and shows a compound with retention behaviour very similar to that of BUdR. This peak is low and partially separated from BUdR (Fig. 3C), but at a low BUdR concentration it does not allow reliable quantitative determination of this drug. Most important, however, is the presence of a very high, broad endogenous peak that totally hides the internal standard (IUdR), preventing quantitative assessment of BUdR.

Chromatographic performance was not improved by minor modifications to the extraction and chromatographic conditions.

Method IV

The results of this method are shown in Fig. 4. By simple plasma deproteinization with cold methanol we obtained a chromatographic profile from blank plasma which is highly populated in its initial portion, where the bulk of hy-

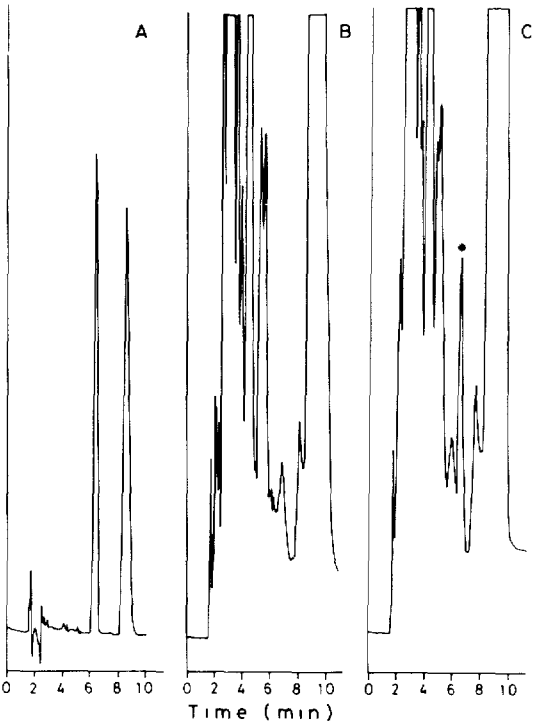

Fig. 3. Chromatographic profiles of (A) BUdR (0.25 μg, first peak) and IUdR (0.5 μg, second peak), (B) blank plasma and (C) plasma containing 0.8 μg/ml BUdR and 2 μg/ml IUdR. Samples were treated as described under *Method III* in Experimental, and 100 μl of the total 150 μl were injected. Column and detection conditions, as in Fig. 2; mobile phase, 6% (v/v) acetonitrile in 25 m*M* acetic acid; flow-rate, 2.0 ml/min. Asterisk = BUdR.

drophilic endogenous compounds is eluted, but is clear enough in the remainder to allow baseline separation of BUdR and IUdR peaks and their distinction from other concomitant endogenous compounds. The very bulky peak that masked IUdR in methods II and III is still present, but is well separated and does not interfere with IUdR peak measurement.

Table I shows the recovery of BUdR at different plasma concentrations calculated in four experiments done on four different days. BUdR was recovered with an efficiency of ca. 50%, regardless of its plasma concentration. In spite of this incomplete recovery, very low concentrations of the drug (0.1 μg/ml or $0.32 \cdot 10^{-6}$ *M*) can still be reliably detected. With this clean-up and chromatographic procedure, the extrapolation of BUdR concentration to unknown plasma samples appears very reliable.

The computer-extrapolated best-fit straight line obtained by plotting the ratio of BUdR peak heights to that of the internal standard (y) as a function

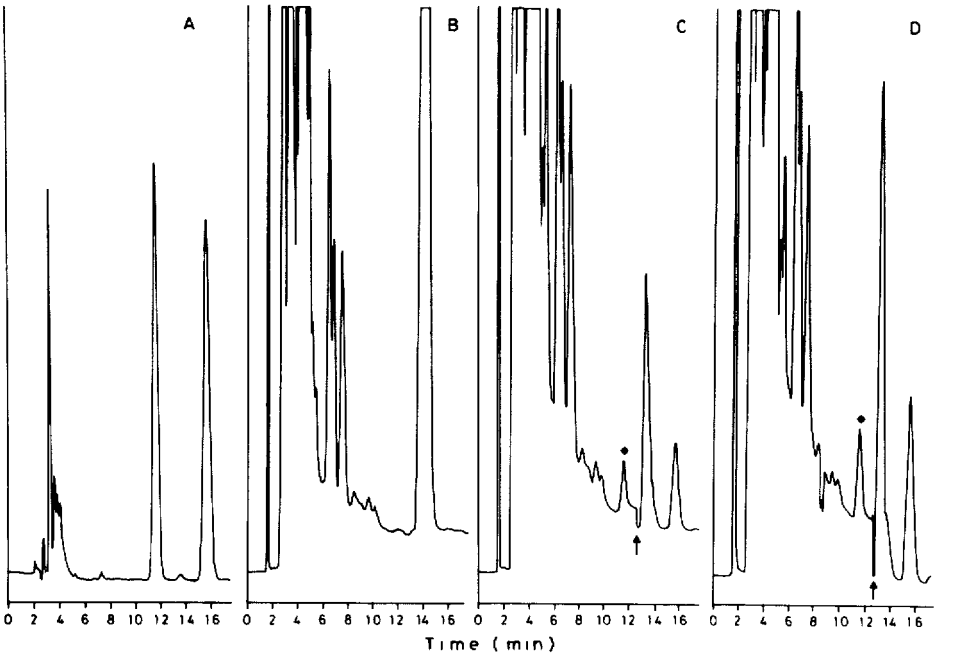

Fig. 4. Chromatographic profiles of (A) BUdR (0.25 μg, first peak after the solvent front) and IUdR (0.5 μg, second peak after the solvent front), (B) blank plasma, (C) plasma containing 0.2 μg/ml BUdR and 2 μg/ml IUdR and (D) plasma containing 0.4 μg/ml BUdR and 2 μg/ml IUdR. Samples were treated as described under *Method IV* in Experimental, and 50 μl of the total 100 μl were injected. Mobile phase, 5% (v/v) acetonitrile in 25 m*M* acetic acid; flow-rate, 1.0 ml/min; column, as in Fig. 2; detection, 254 nm with initial sensitivity at 0.01 a.u.f.s., switched to 0.1 a.u.f.s. (arrow) in C and to 0.05 a.u.f.s. (arrow) in D. Asterisk = BUdR.

TABLE I

ANALYTICAL RECOVERY OF BUdR EXTRACTED AND CHROMATOGRAPHED ACCORDING TO METHOD IV

Values are mean ± S.D. from four experiments done on different days.

Plasma concentration of added BUdR (μg/ml)	Concentration recovered (μg/ml)	Recovery (%)	Coefficient of variation (%)
0.2	0.094 ± 0.0055	47.8 ± 3.8	7.8
0.4	0.197 ± 0.0095	49.5 ± 2.5	5.05
0.8	0.385 ± 0.029	48.3 ± 3.7	7.79

of BUdR plasma concentration (x) (in the range 0.2–2.0 μg/ml) is described by the equation $y = 0.70x$, with $r = 0.99$.

DISCUSSION

The pharmacokinetic behavior of intravenously infused BUdR has been studied in various clinical trials [1,2,7]; given the high toxicity of this drug (mainly myelosuppression), only very low plasma concentrations can be safely achieved, and a sensitive assay is needed to monitor them. Furthermore, BUdR is structurally very similar to other endogenous pyrimidine nucleosides and deoxynucleosides, so a high degree of assay specificity is also needed.

Different methods have been described that reportedly meet these requirements. We tested three of these extractive and chromatographic procedures, two based on liquid–liquid and one on liquid–solid sample pretreatment. Unfortunately, in our hands none of them was entirely satisfactory, mainly because of low extraction recovery (and related low sensitivity), or the presence of interfering endogenous peaks (and the inherent low selectivity), or both.

It is not clear why all these trials failed in our laboratory; technical mistakes or oversights in sample clean-up and chromatographic process are unlikely, since we repeated the assays several times on the same donor's plasma and on plasma from different normal subjects, each time using new reagents, buffers and materials; nevertheless results were nearly identical in different experiments.

Method I, developed by Stetson et al. [5] for the assay of BUdR in dog plasma, was unsatisfactory in our setting because of too low extraction efficiency and subsequent low sensitivity (see Fig. 1). The most obvious explanation for this failure is that animal species differ in the serum enzymes that bring about the decomposition of halogenated pyrimidines. This has been observed comparing the capacity of serum from different species to convert in vitro IU or BUdR into their dehalogenated forms or halogenated bases [8].

Although no data exist on this topic, it can be hypothesized that the dog's enzymes are much less active than their human equivalents, so allowing much more intact BUdR is recovered by the extraction process. If this is the case, however, two things must be considered: first, these enzymic activities are not immediately inhibited by the extraction process in humans; second, one or two major peaks corresponding to deoxyuridine and/or bromouracil would have appeared in the chromatogram from human plasma, unless neither of these two compounds is subsequently recovered during the organic–alkaline extraction. Given the good recovery of internal standard, IU, this appears unlikely.

Failure of the methods of Russo et al. [2] (method II) and Klecker et al. [5] (method III) is even more difficult to explain. The authors did not include a representative chromatogram in their reports, so we could not compare our results with theirs. In our hands, these methods lacked both sensitivity and selectivity. In particular, method II gave a very low BUdR recovery, and the blank plasma contained an endogenous peak totally overlapping that of the internal standard IUdR. The method of Klecker et al. [5], based on sample clean-up on solid C_{18} matrix, gave better recovery of BUdR but as the chro-

matogram was very crowded, the BUdR peak was superimposed on background noise that rendered identification and precise measurement problematic at low BUdR concentrations. Moreover, a bulky endogenous peak completely hid the internal standard; neither modifications in the flow-rate nor changes in the composition of the mobile phase were successful in resolving these two peaks.

We therefore developed a method based on cold methanol deproteinization, freezing–thawing and lyophilization of the sample. This resolved baseline BUdR and IUdR from other concomitant peaks. The resulting chromatograms are noisy in their initial and middle portions, where many hydrophilic compounds elute, and there is still a major unidentified peak (probably the same as whatever prevented IUdR detection in method II and III) between BUdR and the internal standard; however, this peak is well separated and does not interfere with IUdR measurement, provided that the column is carefully washed and allowed to reequilibrate (these two steps were mandatory to ensure reproducible chromatograms in all four methods, in spite of what Stetson et al. [4] claim).

With our method BUdR recovery was incomplete (ca. 50%), so the detection limit of the method is not lower than 0.1 μg/ml; however, it is well reproducible in different experiments and remains constant regardless of drug concentration in the range tested.

In conclusion, this new method developed for the quantitative determination of BUdR in human plasma is somewhat time-consuming (mainly because of lyophilization of the samples and column washing and reequilibrating times), but in our hands it offers much better sensitivity and selectivity than other methods described so far.

ACKNOWLEDGEMENT

The generous contribution of the Italian Association for Cancer Research (Milan, Italy) is gratefully acknowledged.

REFERENCES

1 S. Phuphanich, E.M. Levin and V.A. Levin, Int. J. Radiat. Oncol. Biol. Phys., 10 (1984) 1769.
2 A. Russo, L. Gianni, T.J. Kinsella, R.W. Klecker, Jr., J. Jenkins, J Rowland, E. Glatstein, J.B. Mitchell, J. Collins and C. Myers, Cancer Res., 44 (1984) 1702.
3 S. Phuphanich and V.A. Levin, Cancer Res., 45 (1985) 2387.
4 P.L. Stetson, U.A. Shukla, P.R. Amin and W.D. Ensminger, J. Chromatogr., 341 (1985) 217.
5 R.W. Klecker, Jr., J.F. Jenkins, T.J. Kinsella, R.L. Fine, J.M. Strong and J.M. Collins, Clin. Pharmacol. Ther., 38 (1985) 45.
6 M. Zakaria and P.R. Brown, Anal. Biochem., 120 (1982) 25.
7 T.J. Kinsella, A. Russo, J.B. Mitchell, J. Rowland, J. Jenkins, J. Schwade, C.E. Myers, J.M. Collins, J. Speyer, P. Kornblith, B. Smith, C. Kufta and E. Glatstein, Int. J. Radiat. Oncol Biol. Phys., 10 (1984) 69.
8 R. Saffhill and W.J. Hume, Chem. Biol. Interact., 57 (1986) 347.