Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 4/5, pp. 379–384, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Simultaneous determination of a new anticancer drug galocitabine and its metabolites in blood by high-performance liquid chromatography

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Abstract: A relatively simple and sensitive high-performance liquid chromatographic (HPLC) method is described for measuring galocitabine (Ro 09-1390) and its meatbolites, i.e. 5′-deoxy-fluorocytidine (5′-DFCR), 5′-deoxy-fluorouridine (5′-DFUR) and 5-fluorouracil (5-FU), in blood for the purpose of studying pharmacokinetics and toxicokinetics in small animals. The procedure for blood includes deproteinization with acetonitrile. Blood components were separated on a reversed-phase C_{18} column with a linear gradient of acetonitrile and water and detected at a wavelength of 270 nm. The between-day relative standard deviation (RSD) was less than 10% for all compounds at concentrations of 10–100 μg ml^{-1}. The calibration curves obtained from the analysis of blood samples were linear and the correlation coefficients ranged from 0.997 to 0.999. The calculated determination limits were 6.9 μg ml^{-1} for galocitabine, 3.0 μg ml^{-1} for 5′-DFCR, 4.0 μg ml^{-1} for 5′-DFUR and 3.7 μg ml^{-1} for 5-FU.

Keywords: *Galocitabine; 5′-deoxy-fluorocytidine; 5′-deoxy-fluorouridine; 5-fluorouracil; reversed-phase high-performance liquid chromatography; blood.*

Introduction

5′-Deoxy-5-fluorouridine (5′-DFUR) is currently used as an oral anticancer agent in Japan. 5′-DFUR is a prodrug that is converted to 5-fluorouracil (5-FU) by pyrimidine nucleoside phosphorylase and the efficiency of conversion from 5′-DFUR to 5-FU in tumour tissue accounts for its patent antitumour activity [1]. The enzyme activity of pyrimidine nucleoside phosphorylase is predominantly localized in the tumours. It is also found in the intestine to some extent, but is not found in other normal tissues [1]. Even though 5′-DFUR is tumour selective it also has intestinal toxicity, which can be ascribed to the conversion to 5-FU in the intestinal tract.

Recently galocitabine (N^4-trimethoxybenzoyl-5′-deoxy-5-fluorocytidine, Ro 09-1390) was synthesized to avoid the intestinal toxicity of 5′-DFUR [2]. Galocitabine is a prodrug that is converted to 5′-deoxy-fluorocytidine (5′-DFCR) by acylamidase in the liver and is then converted to 5′-DFUR by cytidine deaminase. Therefore, the efficient conversion of galocitabine to 5-FU through the metabolic pathway shown in Fig. 1 is necessary for galocitabine to have a therapeutic effect.

Consequently, the simultaneous determination of galocitabine and its metabolites is useful for the calculation of the conversion ratio of galocitabine to its active metabolite. Although the high-performance liquid chromatography (HPLC) methods for the simultaneous determination of 5′-DFUR and 5-FU with the use of an internal standard has been reported [3, 4], the simultaneous determination of galocitabine and its metabolites, i.e. 5′-DFCR, 5′-DFUR and 5-FU, has not been reported. Only separate HPLC analysis of the parent drug and its metabolites without the use of an internal standard was reported [5].

This paper describes a simple and rapid simultaneous HPLC determination for galocitabine, 5′-DFCR, 5′-DFUR and 5-FU in whole blood samples (100 μl) for pharmacokinetic and toxicokinetic studies in small animals.

Experimental

Chemicals and reagents

Galocitabine (N^4-trimethoxybenzoyl-5′-deoxy-5-fluorocytidine) and 5′-deoxy-5-fluorocytidine (5′-DFCR) were synthesized by

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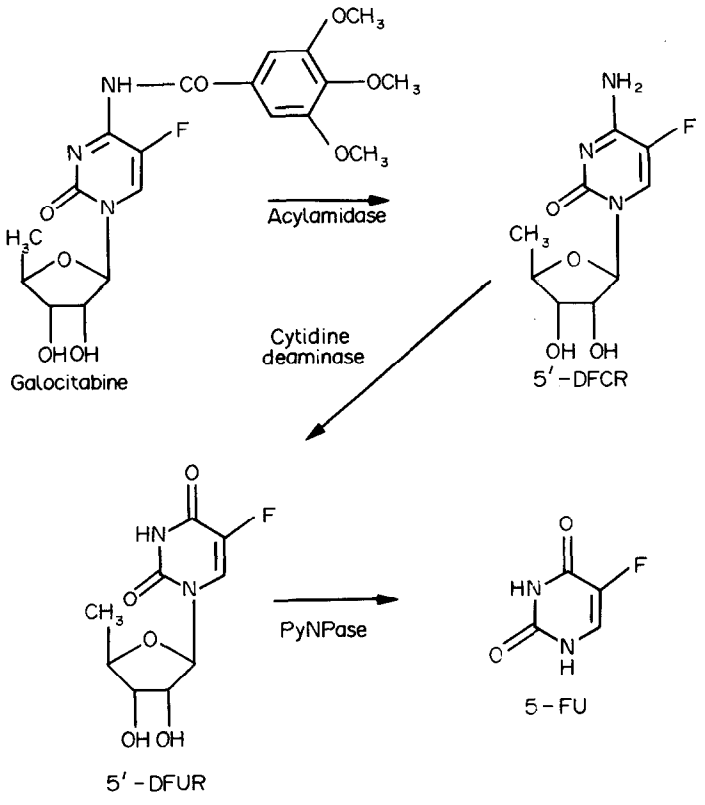

Figure 1
Structures of galocitabine (N^4-trimethoxybenzoyl-5′-deoxy-5-fluorocytidine, Ro 09-1390), 5′-deoxy-fluorocytidine (5′-DFCR), 5′-deoxy-fluorouridine (5′-DFUR) and 5-fluorouracil (5-FU), and metabolic pathway of galocitabine.

Nippon Roche K.K. (Tokyo, Japan). 5′-Deoxy-5-fluorouridine (5′-DFUR) was synthesized by Hoffman–La Roche (Basel, Switzerland). 5-Fluorouracil (5-FU) and 5-bromouracil (I.S.) were obtained from Sigma Chemical Co. (St Louis, MO, USA). These compounds were used without further purification. Water used was purified through a Milli-Q system (Millipore, Bedford, MA, USA) and filtered through a 0.22-μm filter. All other reagents used were analytical grade.

HPLC instrumentation

The HPLC system consisted of two computer controlled pumps (Model CCPE, Toyo Soda, Tokyo, Japan), a syringe loading sample injector (Model 7125, Rheodyne, Berkeley, CA, USA), a spectrophotometric detector (Model SPD-2A, Shimadzu, Kyoto, Japan) operated at 270 nm, and a Nova-Pak C_{18} Radial-Pak (10 cm × 5 mm i.d., particle size 4 μm, Millipore–Waters, Milford, MA, USA) equipped in a compression module (Model RCM 8 × 10, Millipore–Waters). For protection of the analytical column, a Guard-Pak Nova-Pak C_{18} column (particle size 4 μm) equipped with a Guard-Pak precolumn module (Millipore–Waters) was used. Detector output (0.8 V/AU) was processed with a Chromatocorder 12 reporting integrator (System Instrument, Tokyo, Japan) in a peak height mode. The mobile phases, i.e. acetonitrile–water (2:98, v/v) and acetonitrile–water (50:50, v/v), were pumped at 2 ml min^{-1} and mixed by a dynamic mixer (Toyo Soda, Tokyo, Japan).

5-FU, 5′-DFCR, 5′-DFUR and galocitabine were resolved using the following mobile phases in succession: acetonitrile–water (2:98, v/v) for 1.3 min, a linear gradient to acetonitrile–water (5.4:94.6, v/v) for 3.7 min, a linear gradient to acetonitrile–water (50:50, v/v) for 1 min, and maintained for 2.5 min. The next sample was injected following a 6.5 min isocratic flow of acetonitrile–water (2:98, v/v). All chromatography was performed at ambient temperature.

Analytical procedures

To 100 μl of blood, 8 μg of I.S. (0.4 mg

ml^{-1} in methanol), 0.1 ml of distilled water and 1 ml of acetonitrile were added, then the tubes were vortex mixed for 30 s. After centrifugation for 10 min at 1500*g* the upper layer was transferred to another tube and evaporated to dryness at 30–35°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of acetonitrile–water (2:98, v/v) and 20 μl injected into the HPLC.

Calibration and validation

For each assay a six-point calibration curve was prepared by spiking control samples with galocitabine, 5′-DFCR, 5′-DFUR and 5-FU in each concentration of 0–100 μg ml^{-1} to blank samples of blood. The ratios of the peak height of galocitabine, 5′-DFCR, 5′-DFUR and 5-FU to the peak height of the I.S. were calculated and calibration curves were constructed by linear regression analysis using SAS.

The extraction recovery of the compounds and I.S. were determined with an internal–external standard method [6, 7]. The inter-assay relative standard deviations were determined at concentration levels of 5–100 μg ml^{-1} for all compounds. Results are presented as mean and relative standard deviations.

Results and Discussion

Chromatograms

Typical chromatograms of the assay of blood samples from rats are shown in Fig. 2. Some endogenous peaks, indicating the presence of substances that could possibly interfere with the determination, appeared in the chromatogram of the blank sample of blood. However, interferences by these endogenous peaks was negligible when calibration curves were prepared. 5-Bromouracil was chosen as an internal standard because its structure is similar to 5-FU. In addition, it is not used as a therapeutic agent and is not a metabolite of either galocitabine, 5′-DFCR, 5′-DFUR or 5-FU. The retention times of 5-FU, I.S., 5′-DFCR, 5′-DFUR, and galocitabine were 1.0, 2.0, 4.3, 5.3 and 7.5 min, respectively.

Recovery

The recoveries of galocitabine, 5′-DFCR, 5′-DFUR, 5-FU and I.S. in the blood sample analysis were found to be better than 95%.

Linearity and precision

The results of the method validation pro-

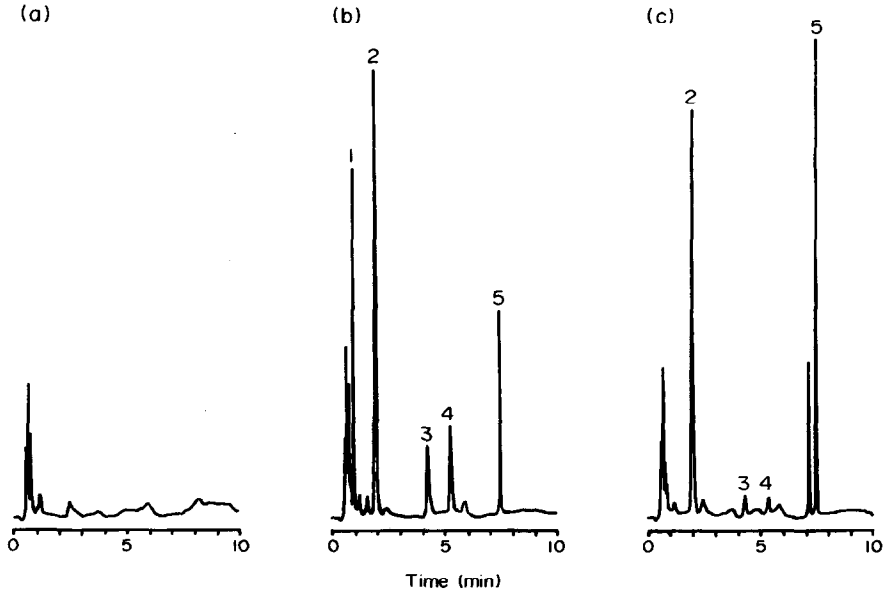

Figure 2
Representative chromatograms showing retention times (min) for galocitabine (peak 5), 5′-DFCR (peak 3), 5′-DFUR (peak 4), 5-FU (peak 1) and I.S. (peak 2). (a) Blood sample blank; (b) blood sample spiked with 25 μg ml^{-1} galocitabine, 5′-DFCR, 5′-DFUR and 5-FU; (c) 1-h post-dosed blood sample. The chromatograms were obtained at 0.32 a.u.f.s.

cedures in blood analysis are summarized in Table 1. The between-day relative standard deviation (RSD) was less than 10% for all compounds at concentrations of 10–100 μg ml^{-1}. The results of linear regression analysis are shown in Table 2. The calibration curves obtained from the analysis of blood samples were linear in the range studied, and the intercepts occasionally deviated from zero. The correlation coefficients and the RSDs ranged from 0.997 to 0.999 and from ±2.48 to ±5.72%, respectively.

Bonate [8] reported the statistical method for the calculation of the limit of detection. In the case of a simple linear regression model, the limit of detection (LOD) can be estimated by

$$\text{LOD} = \frac{t \times \text{SE}_{(\text{Blank})}}{\text{slope of calibration curve}},$$

where t is the Student two-tailed t value associated with a particular confidence interval and $\text{SE}_{(\text{Blank})}$ is the standard error of the blank. The determination limits obtained, setting the confidence interval to 99.5% are 6.9 μg ml^{-1} for galocitabine, 3.0 μg ml^{-1} for 5′-DFCR, 4.0 μg ml^{-1} for 5′-DFUR and 3.7 μg ml^{-1} for 5-FU.

In the present analysis, the peak in the chromatogram could not be detected in the blood sample, when the concentration of the samples were low (<1 μg ml^{-1}). On the other hand, the peak could be detected at 1 μg ml^{-1} in the pure standard solution. In the gradient

Table 1
Between-day precision and accuracy of the analysis of rat blood samples

Compound	Spiked conc. (μg ml^{-1})	Measured mean conc. (μg ml^{-1})	n	Relative standard deviation (%)	Error (%)
Galocitabine					
	5.0	5.5	4	3.5	10.2
	10.0	10.1	4	5.9	1.2
	25.0	23.7	4	5.9	−5.2
	50.0	50.7	4	3.7	1.4
	100.0	100.0	4	3.6	0.0
5′-DFCR					
	5.0	4.7	4	2.7	−5.5
	10.0	9.8	4	2.4	−2.4
	25.0	25.0	4	3.2	0.0
	50.0	51.0	4	1.9	2.0
	100.0	99.6	4	1.1	−0.4
5′-DFUR					
	5.0	4.9	4	15.7	−2.9
	10.0	9.7	4	6.6	−2.6
	25.0	24.5	4	2.0	−2.0
	50.0	51.6	4	1.0	3.2
	100.0	99.5	4	1.5	−0.5
5-FU					
	5.0	4.9	4	1.7	−2.3
	10.0	9.9	4	0.8	−1.2
	25.0	24.5	4	1.6	−2.0
	50.0	51.2	4	0.9	2.5
	100.0	99.6	4	1.9	−0.4

Table 2
Standard curves in rat blood and its square root of mean square error, correlation coefficient, relative standard deviation and concentration of detection limit*

Compound	Calibration curve	Square root of mean square error	Correlation coefficient	Relative standard deviation (%)	Detection limit (μg ml^{-1})
Galocitabine	$y = -0.00966 + 0.01967x$	0.04219	0.997	5.72	6.9
5′-DFCR	$y = 0.00230 + 0.00662x$	0.00629	0.999	2.48	3.0
5′-DFUR	$y = 0.00570 + 0.00836x$	0.01035	0.999	3.20	4.0
5-FU	$y = 0.01880 + 0.03204x$	0.03667	0.999	2.97	3.7

*Data shown in Table 1 were used for a linear regression analysis.
†The confidence interval of 99.5% was used.

analysis, the interference peaks of the biological samples sometimes appear with an increase in the concentration of organic solvent in the mobile phase even though they do not appear in the isocratic analysis. In addition to this general defect of gradient analysis, the slight broadening of peaks occurs in the blood sample, but not in the pure standard solution, due to either insufficient equilibration of the column in the gradient system or the interaction between the packing material in the column and components of the blood sample. Therefore, the sensitivity in the gradient analysis is reduced in contrast to that of the isocratic analysis even though the analyte signal-to-background noise (*S/N*) ratio of 3 is used to define the detection limit [7]. Beside this defect, the simultaneous analysis in the gradient system reduces the analysis time and this is the great advantage of the present system. As mentioned above, 5′-DFUR is converted to 5-FU by pyrimidine nucleoside phosphorylase. However, 5′-DFUR is a relatively poor substrate for this enzyme, which probably reflects in the high doses of 5′-DFUR that are necessary to obtain a therapeutic effect [9] and it also demands the high dose of galocitabine. Toxicokinetics is defined as pharmacokinetic studies in animal during actual toxicity studies [10]. A major objective of the toxicology programme is the detection of potential unwanted drug effects at the higher dose level. Consequently, the low sensitivity of the present analytical method does not interfere with pharmacokinetic and toxicokinetic studies in small animals.

The maximum UV absorbance of galocitabine, 5′-DFCR, 5′-DFUR, 5-FU and I.S. were 317, 275, 270, 275 and 275 nm, respectively. The wavelength of 270 nm was chosen for the detection to obtain a higher peak height for 5′-DFUR in the chromatogram because galocitabine is the prodrug of 5′-DFUR.

Pharmacokinetic application

The application of the present method for the determination of galocitabine, 5′-DFCR, and 5′-DFUR in blood is shown in Fig. 3. In this sampling schedule, the area under the blood concentration–time curve (AUC) of galocitabine was estimated >90% of the total AUC. However, the peaks of 5′-DFUR and 5-FU were difficult to identify from the endogenous peaks, and this may be explained by the slow elimination of 5′-DFCR and formation of 5′-DFUR in rats. The details will be published elsewhere.

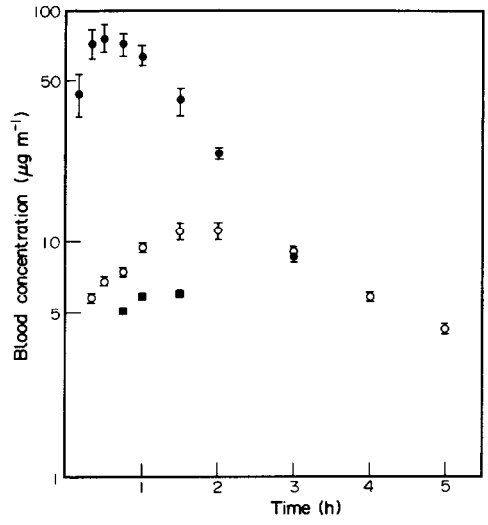

Figure 3
Blood concentration–time courses following oral administrations of 100 mg kg^{-1} galocitabine in rats. The symbols show galocitabine (●) and 5′-DFCR (○), and 5′-DFUR (■), respectively. Each point is the mean ±SE (n = 4).

Conclusions

The present method enables the simultaneous quantitation of galocitabine, 5′-DFCR, 5′-DFUR and 5-FU in blood. The present method is considered to be sufficiently sensitive when the defect of gradient separation is taken into consideration. It is a reliable method for detailed pharmacokinetic and toxicokinetic studies in small animals. The present method can be applied to a radioisotope-carrier method for the quantitation of the label compounds in blood.

Acknowledgements — We thank Miss N. Ushiyama for the expert technical assistance.

References

[1] H. Ishitsuka, M. Miwa, K. Takemoto, K. Fukuoka, A. Itoga and H.B. Maruyama, *Gann* **71**, 112–123 (1980).
[2] I. Umeda, M. Fujiu, K. Yokose, M. Miwa and H. Ishitsuka, European Patent No. 87.116926.4 (1989).
[3] L.J. Schaaf and D.G. Ferry, *J. Chromatogr.* **342**, 303–313 (1985).
[4] J.L.-S. Au and L.C.Q. Gunnarsson, *Pharm. Res.* **6**, 323–327 (1989).
[5] Y. Ninomiya, M. Miwa, H. Eda, H. Sahara and K. Fujimoto, *Jap. J. Cancer Res.* **81**, 188–195 (1990).

[6] M.C.M. Roosemalen, P.A. Soons, T. Funaki and D.D. Breimer, *J. Chromatogr.* **565**, 516–522 (1991).
[7] G.W. Peng and W.L. Chiou, *J. Chromatogr.* **531**, 3–50 (1990).
[8] P.L. Bonate, *J. Chromatogr. Sci.* **28**, 559–562 (1990).
[9] S. Ajmera, A.R. Bapat, E. Stephanian and P.V. Danenberg, *J. Med. Chem.* **31**, 1094–1098 (1988).
[10] D.A. Smith, M.J. Humphrey and C. Charuel, *Xenobiotica* **20**, 1187–1199 (1990).

[Received for review 12 May 1992; revised manuscript received 10 August 1992]