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Journal of Chromatography B, 803 (2004) 367–370
JOURNAL OF CHROMATOGRAPHY B
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Short communication

Determination of 1-(3-fluoro-4-hydroxy-5-mercaptomethyltetrahydrofuran-2-yl)-5-methyl-1*H*-pyrimidine-2,4-dione in rat plasma and urine by high-performance liquid chromatography

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Received 13 October 2003; received in revised form 16 December 2003; accepted 7 January 2004

Abstract

A high-performance liquid chromatographic method using liquid–liquid extraction was developed for the determination of 1-(3-fluoro-4-hydroxy-5-mercaptomethyl-tetrahydrofuran-2-yl)-5-methyl-1*H*-pyrimidine-2,4-dione (L-FMAUS; I) in rat plasma and urine. A 100 μl aliquot of distilled water containing L-cysteine (100 mg/ml) was added to a 100 μl aliquot of biological sample. L-Cysteine was employed to protect binding between the 5′-thiol of I and protein in the biological sample. After vortex-mixing for 30 s and adding a 50 μl aliquot of the mobile phase containing the internal standard (10 μg/ml of 3-aminophenyl sulfone), 1 ml of ethyl acetate was used for extraction. After vortex-mixing, centrifugation, and evaporating the ethyl acetate, the residue was reconstituted with a 100 μl aliquot of the mobile phase. A 50 μl aliquot was injected onto a C_{18} reversed-phase column. The mobile phases, 50 mM KH_2PO_4 (pH = 2.5):acetonitrile (85:15, v/v) for rat plasma and 50 mM KH_2PO_4 (pH 2.5):acetonitrile:methanol (85:10:5, v/v/v) for urine samples, were run at a flow-rate of 1.2 ml/min. The column effluent was monitored by an ultraviolet detector set at 265 nm. The retention times for I and the internal standard were approximately 9.7 and 12.5 min, respectively, in plasma samples and the corresponding values in urine samples were 16.8 and 14.9 min. The quantitation limits of I in rat plasma and urine were 0.1 and 0.5 μg/ml, respectively.

Keyword: 1-(3-Fluoro-4-hydroxy-5-mercaptomethyl-tetrahydrofuran-2-yl)-5-methyl-1*H*-pyrimidine-2,4-dione

1. Introduction

1-(2-Deoxy-2-fluoro-β-L-arabinofuranosyl)-5-methyluracil (L-FMAU), the β-L isomer of the nucleoside analogue FMAU, has been shown to have antiviral activity in vitro against the hepatitis B and the Epstein-Barr viruses [1]. Recently, 1-(3-fluoro-4-hydroxy-5-mercaptomethyl-tetrahydrofuran-2-yl)-5-methyl-1*H*-pyrimidine-2,4-dione (L-FMAUS; I, Fig. 1), a new L-FMAU derivative, has been synthesized (Central Research Institute, Bukwang Pharmaceutical Company, Seoul, South Korea). Compound I is being evaluated in preclinical studies as an antiviral agent. This paper describes an HPLC method using liquid–liquid extraction for the determination of I in rat plasma and urine. L-Cysteine was employed to protect binding between the 5′-thiol of I and protein in the biological sample. The application of the present HPLC method to the pharmacokinetics of I in a male Sprague–Dawley rat is also reported.

2. Experimental

2.1. Chemicals

Compound I was supplied from Central Research Institute of Bukwang Pharmaceutical Company. 3-Aminophenyl sulfone (an internal standard, IS, for the HPLC assay, Fig. 1), L-cysteine, and dimethylacetamide (DMA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was a product of Kanto Chemical Company (Tokyo, Japan). Ethyl acetate and KH_2PO_4 were purchased from Yakuri Pure Chemical (Kyoto, Japan). Acetonitrile was a product from Burdick

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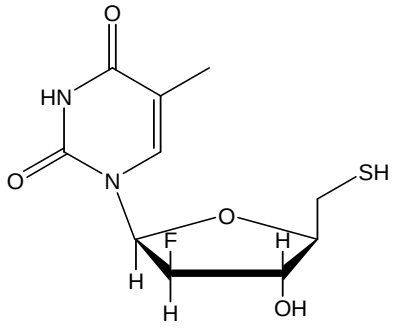
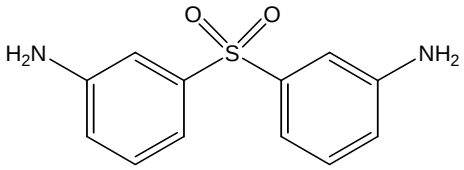
doi:10.1016/j.jchromb.2004.01.007



L-FMAUS (compound I)



3-Aminophenyl sulfone (IS)

Fig. 1. Chemical structures of I and 3-aminophenyl sulfone (IS).

& Jackson (Muskegon, MI, USA). Various buffer solutions having pHs ranging from 1 to 13 were products of Shinyo Pure Chemicals (Osaka, Japan).

2.2. HPLC apparatus

The HPLC system consisted of a GINA 50 autosampler (Gynkotek HPLC, Münich, Germany), a model PU-980 pump (Jasco, Tokyo, Japan), a Hypersil ODS (C_{18}) column (150 mm length × 4.6 mm i.d.; particle size, 5 μm; Alltech Associates, Deerfield, IL, USA), a model UV-975 UV/Vis detector (Jasco), and a model chromatocorder 21 integrator (SIC, Tokyo, Japan). The mobile phases, 50 mM KH_2PO_4 (pH = 2.5):acetonitrile (85:15, v/v) for plasma and 50 mM KH_2PO_4 (pH = 2.5):acetonitrile:methanol (85:10:5, v/v/v) for urine, were run at a flow-rate of 1.2 ml/min at room temperature, and the column effluent was monitored at 265 nm absorbance. Methanol was added to the mobile phase for the urine analysis to improve selectivity.

2.3. Preparation of stock and standard solutions

A stock solution (10 mg/ml) of I was prepared in DMSO. Appropriate dilutions of the stock solution were made with DMSO (0.1, 0.2, 0.5, 1 and 5 mg/ml). Standard solutions of I in rat plasma and urine were prepared by spiking with an appropriate volume (10 μl/ml of biological sample) of the diluted stock solutions, giving final concentrations of 0.1, 0.2, 0.5, 1, 5, 10, and 100 μg/ml for plasma and 0.5, 1, 5, and 10 μg/ml for urine. The IS working solution was prepared by dissolving 3-aminophenyl sulfone in the mobile phase to give a final concentration of 10 μg/ml.

2.4. Sample preparation

A 100 μl aliquot of distilled water containing L-cysteine (100 mg/ml) was added to a 100 μl aliquot of biological sample. After vortex-mixing for 30 s, a 50 μl aliquot of the mobile phase containing the IS (10 μg/ml) and a 1 ml aliquot of ethyl acetate were added. After vortex-mixing and centrifugation at 9000 × *g* for 4 min, the ethyl acetate layer was transferred into a clean plastic tube, and evaporated under a gentle stream of nitrogen gas. The residue was reconstituted with a 100 μl aliquot of the mobile phase, the tube was centrifuged, and the sample was transferred to an autosampler vial and a 50 μl aliquot of the sample was injected directly onto the HPLC column.

2.5. Stability of I in various pH solutions and rat whole blood, plasma, and urine

A stock solution of I in DMSO was spiked (less than 10 μl/ml to minimize dilution of buffer of biological solutions) into glass test tubes containing 10 ml of varying buffer solutions or rat whole blood, plasma, and urine to final concentrations of 10 μg/ml of I. After vortex-mixing, each test tube was placed in a water-bath shaker kept at 37 °C and at a rate of 50 oscillations per min. At 0, 1, 2, 4, 6, 8, 12, 24, and 48 h, a 50 μl aliquot was sampled from each test tube. The concentrations of I in the above samples were analyzed as soon as the sample was collected. For blood samples, two volumes of distilled water were added. A 50 μl aliquot of distilled water containing the L-cysteine (100 mg/ml) was added to a 50 μl aliquot of sample and immediately injected (without liquid–liquid extraction for the buffer solutions) onto the HPLC column. The same sample preparation described in 2.4 was employed for rat whole blood, plasma, and urine.

2.6. Pharmacokinetics in rats after intravenous administration of I

Compound I dissolved in DMA to make a concentration of 10 mg/ml, was infused for 1 min via the jugular vein to a male Sprague–Dawley rat at a dose of 20 mg/kg. Blood samples (0.25 ml) were collected via the carotid artery at 0 (to serve as a control), 1 (at the end of the infusion), 5, 15, 30, 45, 60, 90, 120, 240, 360, and 480 min after the start of infusion. Blood was centrifuged immediately to collect plasma and the plasma was stored in a −70 °C freezer (Revco ULT 1490 D-N-S, Western Mednics, Asheville, NC, USA) until analysis. Urine was collected between 0 and 24 h. After measuring the exact volume of the urine sample, a 100 μl aliquot of the urine sample was similarly stored.

3. Results and discussion

Compound I was relatively stable in all buffer solutions having pHs ranging from 1 to 11 and in rat whole blood,

plasma, and urine for up to 48 h at 37 °C; more than 95% of the spiked amount of I was recovered after 48 h of incubation. However, compound I was not stable at pH 12 or 13; The disappearance half-lives were 173 and 54.1 h, respectively.

When extracted plasma was injected without having added L-cysteine, L-FMAUS was not detected (Fig. 2C). We decided to employ cysteine, a natural thiol source, which might saturate potential protein binding sites. Fig. 2 shows typical chromatograms of the extracted drug-free rat plasma, drug standards in rat plasma spiked with 0.5 μg/ml of I, drug standards in rat plasma spiked with 0.5 μg/ml of I (without adding L-cysteine), and plasma collected at 90 min after the start of 1 min intravenous infusion of 20 mg/kg of I to a male Sprague–Dawley rat. Fig. 3 shows typical chromatograms of the extracted drug-free rat urine, drug standards in rat urine spiked with 1.0 μg/ml of I, and urine collected between 0 and 24 h after the start of 1 min intravenous infusion of 20 mg/kg of I to a male Sprague–Dawley rat. No interferences from endogenous substances were observed in either of the biological samples (Figs. 2 and 3). The retention times of I and the internal standard were approximately 9.7 and 12.5 min, respectively, in rat plasma samples (Fig. 2), and the corresponding values in rat urine were approximately 16.8 and 14.9 min (Fig. 3). After adding L-cysteine, I was essentially completely recovered compared to those in water; the values ranged from 98.3 to 128%.

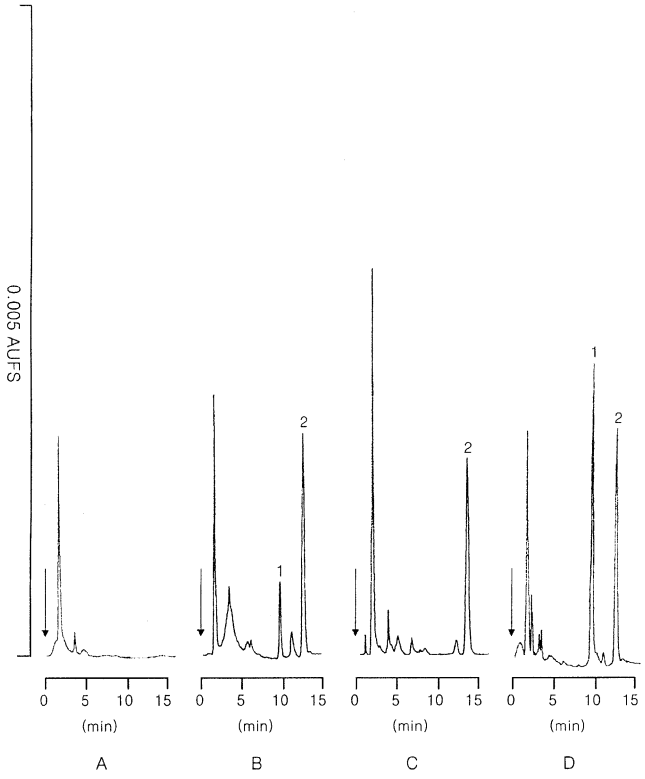


Fig. 2. Chromatograms after extraction of drug-free rat plasma (A), rat plasma spiked with 0.5 μg/ml of I and 10 μg/ml of an IS (B), rat plasma spiked with 0.5 μg/ml of I and 10 μg/ml of IS without adding L-cysteine (C), and plasma collected 90 min after a 1 min intravenous infusion of 20 mg/kg of I (detected concentration of 1.92 μg/ml) to a male Sprague–Dawley rat (D). Peaks: (1) I (9.7 min) and (2) IS (12.5 min). The arrows mark the point of injection. The detector's sensitivity was set at 0.005 AUFS and the recorder's sensitivity was set at 32 mV.

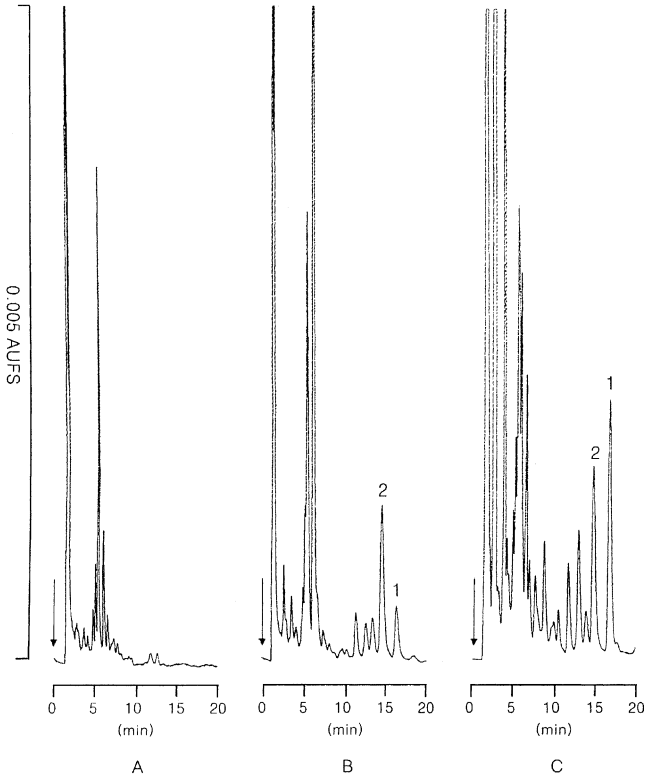


Fig. 3. Chromatograms after extraction of drug-free rat urine (A), rat urine spiked with 1.0 μg/ml of I and 10 μg/ml of an IS (B), and urine collected between 0 and 24 h after a 1 min intravenous infusion of 20 mg/kg of I (detected concentration of 3.15 μg/ml) to a male Sprague–Dawley rat (C). Peaks: (1) I (16.8 min) and (2) IS (14.9 min). The arrows mark the point of injection. The detector's sensitivity was set at 0.005 AUFS and the recorder's sensitivity was set at 32 mV.

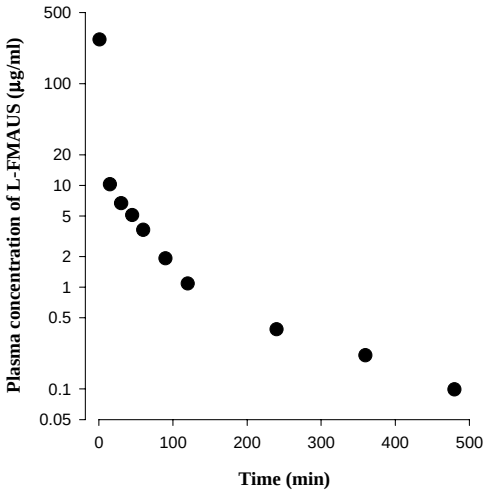


Fig. 4. Plasma concentration–time profile of I after a 1 min intravenous infusion at a dose of 20 mg/kg to a male Sprague–Dawley rat.

Table 1
Response factors and accuracies of I at various concentrations in rat plasma and urine

Theoretical concentration (μg/ml)	Response factor[a]		Accuracy (%)[b]	
	Within-day ($n = 4$)	Between-day ($n = 4$)	Within-day ($n = 4$)	Between-day ($n = 4$)
Rat plasma				
100	5.50 ± 0.0419 (0.761)	5.55 ± 0.316 (5.69)	95.8	96.5
0.2	5.92 ± 0.268 (4.53)	5.84 ± 0.208 (3.56)	104	101
0.1	5.56 ± 0.324 (5.83)	5.20 ± 0.447 (8.60)	97.4	90.3
Rat urine				
10	4.25 ± 0.0614 (1.44)	4.02 ± 0.217 (5.39)	105	102
1	3.94 ± 0.0805 (2.04)	3.97 ± 0.424 (1.07)	97.5	100
0.5	4.01 ± 0.0204 (0.569)	3.90 ± 0.346 (8.86)	99.1	98.8

Values in parentheses are coefficients of variation, CVs (%).
[a] [Drug peak height (in cm)/drug concentration (μg/ml)]/[internal standard peak height (in cm)/drug concentration (μg/ml)] (mean ± standard deviation).
[b] (Mean observed concentration/theoretical concentration) × 100 (mean).

The HPLC assay results on three concentrations (0.1, 0.2, and 100 μg/ml for rat plasma, and 0.5, 1, and 10 μg/ml for rat urine) are listed in Table 1. Concentrations of I were determined by dividing peak height of I by peak height of IS based on calibration curves of standard solutions. Response factors were calculated by the following: [(peak height (cm) of I/its concentration (μg/ml))/(peak height (cm) of the internal standard/its concentration (μg/ml))]. Accuracy was calculated by dividing mean observed concentration by theoretical concentration.

The quantitation limits of I in rat plasma and urine were 0.1 and 0.5 μg/ml, respectively (Table 1), based on a signal-to-noise ratio of 3.0. The sensitivity was sufficient to study pharmacokinetics of I in a rat (see below). Reproducibility of the present method was fairly good. The mean within-day CVs for response factor of I in rat plasma and urine were 2.4% (range 0.8–5.8%) and 1.4% (range 0.5–2.0%), respectively, within the concentration ranges of 0.1–100 μg/ml for plasma and 0.5–10 μg/ml for urine. The corresponding values for between-day (3 consecutive days) were 4.6% (range 2.4–8.6%) and 4.3% (range 1.1–8.9%). The within-day accuracies in the same concentration ranges ((mean observed concentration/theoretical concentration) × 100) of I were 95.8–104 and 97.5–105% for rat plasma and urine, respectively. The corresponding values for between-day were 90.3–104 and 98.8–101%.

The present HPLC method was successful for a pharmacokinetic study after an intravenous administration of I to a rat (Fig. 4). The plasma concentrations of I declined in a polyexponential manner with a terminal half-life of 123 min (Fig. 4). The estimated total body clearance, apparent volume of distribution at steady state (V_{SS}), and mean residence time of I were 16.3 ml/min kg, 878 ml/kg, and 54 min, respectively.

Acknowledgements

This work was supported by the contract, “Pharmacokinetics of L-FMAUS” from Central Research Institute, Bukwang Pharmaceutical Company, Seoul, South Korea.

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