ELSEVIER

Journal of Chromatography B, 731 (1999) 383–387

JOURNAL OF CHROMATOGRAPHY B

Short communication

High-performance liquid chromatographic assay of formycin A in plasma after solid-phase extraction

Genowefa Misztal[a,*], Beata Paw[a], Janusz S. Skierski[b], Ewa Jagiełło-Wójtowicz[c]

[a]*Department of Medicinal Chemistry, Medical Academy, 6 Chodźki Str., 20-093 Lublin, Poland*
[b]*Independent Laboratory Flow-Cytometry, Drug Institute, 30/34 Chełmska Str., 00-725 Warsaw, Poland*
[c]*Department of Pharmacology, Medical Academy, 8 Jaczewskiego Str. 20-090 Lublin, Poland*

Received 29 October 1998; received in revised form 29 April 1999; accepted 3 May 1999

Abstract

A new, simple and accurate high-performance liquid chromatography (HPLC) method for the determination of formycin A in plasma is presented. The samples were chromatographed on a LiChrosorb RP-18 column after purification using a Bakerbond SPE column. The mobile phase was methanol–0.067 *M* phosphate buffer, pH 4.20 (1:4, v/v) containing 0.005 *M* sodium hexanesulfonate. Azathioprine was applied as an internal standard. UV detection was carried out at 293 nm. The method was tested for linearity (over the range 0.1–9.0 μg/ml). The recovery was 91.89% (mean). The described method has been successfully applied to the quantitative determination of formycin A in plasma and should be useful for clinical and bioavailability investigations.

Keywords: Formycin A

1. Introduction

Formycin A (7-amino-3-β-D-ribofuranosyl-1*H*-pyrazolo[4,3-*d*]-pyrimidine), an adenosine analog, is an antiviral and antitumor agent [1–3]. Formycin A inhibits the growth of Ehrlich cancer, Yoshida rat sarcoma, Mycobacterium 607 and *Xanthomonas oryzae* [1].

The biosynthesis of purine is blocked by formycin A through inhibition of phosphoribosyl-pyrophosphate synthesis. This analog inhibits the synthesis of these nucleotides from hypoxanthine and guanine. Formycin A inhibits the incorporation of lysine and methionine into protein [1].

Literature about the quantitative determination of formycin A is poor.

There are only two papers [4,5] that elaborate on the high-performance liquid chromatography (HPLC) methods. One of these was used to identify and quantitative five purine-metabolizing enzymes from a partially purified subcellular fraction of the eucaryotic microorganism *Dictyostelium discoideum*. Formycin A was used as the substrate. The mobile phase consisted of 65 m*M* phosphate buffer, pH 3.6, in 4% methanol and 1 m*M* tetra-*n*-butyl-ammonium phosphate. UV-detection was carried at 295 nm.

Spremulli et al. [5] used an anion-exchange HPLC method with a gradient mobile phase (0.5 *M* potassium dihydrogen, 1 *M* kalium chloride) for determin-

*Corresponding author. Tel.: +48-81-741-0321; fax: +48-81-742-5165.

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00210-8

ing formycin nucleotides in the livers of mice treated with formycin, either alone or in combination with 2′-deoxycoformycin.

This report describes a new procedure for the analysis of formycin A in plasma using reversed-phase HPLC with sodium hexanesulfonate, and is selective, sensitive and easy to perform.

2. Experimental

2.1. Reagents and materials

Formycin A and azathioprine (the internal standard) were obtained from Sigma (St. Louis MO, USA). Phosphate buffer (0.067 *M* potassium dihydrogen phosphate adjusted to pH 4.20 with phosphoric acid, pH tolerance ±0.05), methanol (LiChrosolv® for chromatography) and sodium hexanesulfonate (E. Merck, Darmstadt, Germany) were also used. Water was purified by double distillation. Heparinized human whole blood was provided by the District Blood Centre (Lublin, Poland). Blood samples were centrifuged and the plasma thus obtained was stored at −18°C. Albino–Swiss mice from the Department of Pharmacology of the Medical Academy (Lublin, Poland) were used in the experiments.

Stock solutions (1.0 mg/ml) of formycin A and azathioprine (I.S.) were prepared by dissolving appropriate amounts of these substances in methanol. Working methanolic dilutions of 0.01 and 0.001 mg/ml for formycin A and 0.2 mg/ml for azathioprine were prepared from stock solutions.

2.2. Apparatus

For solid-phase extraction, octadecyl (C_{18}) minicolumns with a capacity of 3 ml and a vacuum manifold column processor (Baker SPE 12 G from J.T. Baker, Philipsburg, NJ, USA) were used.

A liquid chromatograph from Spectra Physics, with a UV-Spectra 100 variable wavelength detector and a Hewlett-Packard 3396 integrator (Avondale, PA, USA) were used. A stainless-steel column (250×4 mm) packed with LiChrosorb RP-18, dp=7 μm (E. Merck, Darmstadt, Germany) was also used.

2.3. Chromatographic conditions

Sample volumes of 20 μl were injected into the column. The mobile phase comprised methanol–phosphate buffer, pH 4.20 (1:4, v/v) containing 0.005 *M* sodium hexanesulfonate. The flow-rate was 1.5 ml/min. Detection was at 293 nm and the detector output range was 0.01 a.u.f.s.

2.4. Calibration procedure

Using the working solutions of formycin A and azathioprine (I.S.), samples were spiked with both compounds at concentrations ranging from 0.1 to 9.0 μg/ml for formycin A and with a fixed concentration of I.S. (20 μg/ml). A 20-μl volume of each sample was injected into the analytical column. All measurements were repeated five times at each concentration. A calibration curve based on the peak area ratios of formycin A to internal standard was constructed using ten different concentrations of formycin A. The data were subjected to linear-regression analysis in order obtain the appropriate calibration factors.

2.5. Sample preparation

To ten centrifuges tubes containing 1.0 ml of plasma, formycin A (from 0.2 to 18 μg) was added. Then, 40 μg of azathioprine was added to each sample and the volume was brought up to 4 ml using methanol. The mixtures were centrifuged for 15 min at approximately 1100 *g*.

Then, a 2.0-ml volume of the supernatant was injected onto a Bakerbond SPE C_{18} extraction column that had previously been rinsed with 2 ml of methanol followed by 2 ml of water. The analyzed compounds were eluted with two 1 ml volumes of methanol. The methanolic extracts were evaporated under a stream of nitrogen at room temperature. Each residue was dissolved in 1.0 ml of methanol and 20 μl volumes were injected into the analytical column. All measurements were repeated five times at each concentration.

Absolute recovery was determined by comparing the average peak area for ten extracted plasma samples at each standard concentration of formycin A and the I.S. with those for unextracted samples with an identical content of both substances.

2.6. Precision and reproducibility

Samples were prepared for inter- and intra-day validation. Five samples at each of the following concentrations (0.20, 0.80 and 3.00 μg/ml) were prepared for calculation of the coefficient of variation.

2.7. Accuracy

The accuracy of the method was determined by injecting samples containing theoretical amounts of formycin A at the same concentrations as those used for the calibration curve. Calculated values were compared with theoretical values and the percentage error was determined.

3. Results and discussion

A comparison between the results of the proposed assay and previous methods is difficult. One of the literature methods was used to determine the nucleotides of formycin A in the livers of mice but the second method described the determination of purine-metabolizing enzymes. This study uses a different mobile phase, an internal standard and reports the precision, reproducibility, accuracy and recovery of the method.

The method described in this work is rapid, simple and selective. The advantages associated with this procedure are extraction of the formycin A from plasma, a quantification limit of 20 ng/ml, linear calibration from 0.1–9.0 μg/ml with excellent correlation coefficients ($r=0.9996$), adequate inter- and intra-assay precision and the absence of interference from fludarabine, ftorafur. This method can be used in pharmacokinetic studies.

Over the concentration range 0.1–9.0 μg/ml, the relationship between the peak area ratios of formycin A to the I.S. and the concentration of the drug was linear. The regression equation for standard solutions was $y=0.1837(\pm0.0018)x+0.0174(\pm0.0074)$ (correlation coefficient, $r=0.999$), the regression equation for plasma samples was $y=0.1655(\pm0.0018)x+0.0187(\pm0.0076)$ ($r=0.999$), where y=peak area ratio of formycin A to that of the I.S. and x= concentration of formycin A, in μg/ml. The results indicate a good linear proportionality between the detector response and the concentration of formycin A in plasma.

Table 1
Intra- and inter-day validation of the method (precision and reproducibility)

Amount added (μg/ml)	Amount found (mean ±SD)[a] (μg/ml)	Coefficient of variation (%)	
		Intra-day validation	Inter-day validation
0.20	0.1915±0.0035	1.83	3.13
0.80	0.7480±0.0054	0.72	1.80
3.00	2.7345±0.0141	0.51	0.79

[a] $n=5$.

Bakerbond SPE C_{18} columns were successfully applied to the isolation of formycin A and I.S. from plasma. Liquid–solid extraction with C_{18} minicolumns is selective, efficient and involves minimal handling of the sample, therefore, it saves time, glassware and reagents, and was found to be faster and more reliable than previous methods [6]. It gave the best reproducibility and good recovery for both substances, i.e., formycin A and the I.S. The mean absolute recovery (±S.D.) over the tested range was 91.89±2.96% (C.V., 3.22%) for formycin A.

In our work, the formycin A and the I.S. were isolated from plasma by means of liquid–liquid extraction. Diethyl ether, dichloromethane and

Table 2
Accuracy of the determination of formycin A in plasma

Theoretical concentration (μg/ml)	Concentration found (μg/ml)	Error (%)
0.10	0.0970	3.0
0.20	0.1915	4.5
0.40	0.3615	9.6
0.60	0.5595	6.7
0.80	0.7480	6.5
1.00	0.8755	12.45
3.00	2.7345	8.85
5.00	4.4765	10.47
7.00	6.3865	8.76
9.00	8.0495	10.56

propan-2-ol were used. Recoveries of analyzed substances were about 30%. The intra- and inter-day reproducibility and precision are given in Table 1. The accuracy of the method is given in Table 2. The limit of detection was 20 ng/ml with signal-to-noise ratio of 3:1. The limit of quantitation was 0.1 μg/ml.

Fig. 1 shows examples of the chromatograms obtained from an extracted pooled plasma sample and a plasma sample containing formycin A and the internal standard added from standard solutions. As can be seen, the peaks representing both substances are symmetrical and are well removed from the solvent front. The retention times for formycin A and azathioprine were 3.20 and 8.10 min, respectively.

This method was tested in a pharmacokinetic study on mice. Animals used in this study were male mice (Albino–Swiss) weighing 20–28 g. The concentration–time data course of formycin A in plasma after a single i.p. administration of 3.2 mg/kg was determined. Blood samples were collected at 3, 6, 9, 15, 30, 45, 60, 120, 180 and 240 min after administration. The pharmacokinetics of formycin A are presented in Fig. 2. The half-live was $t_{1/2}=34.82$ min. The equation of elimination of formycin A was $C_{(t)}=44.26^{-0.0199t}$.

The advantages of the proposed method for the determination of formycin A are its short analysis time and the simple procedure used for sample preparation.

The described method of determination of formycin A in plasma is specific, precise, sensitive and accurate for pharmacokinetic studies.

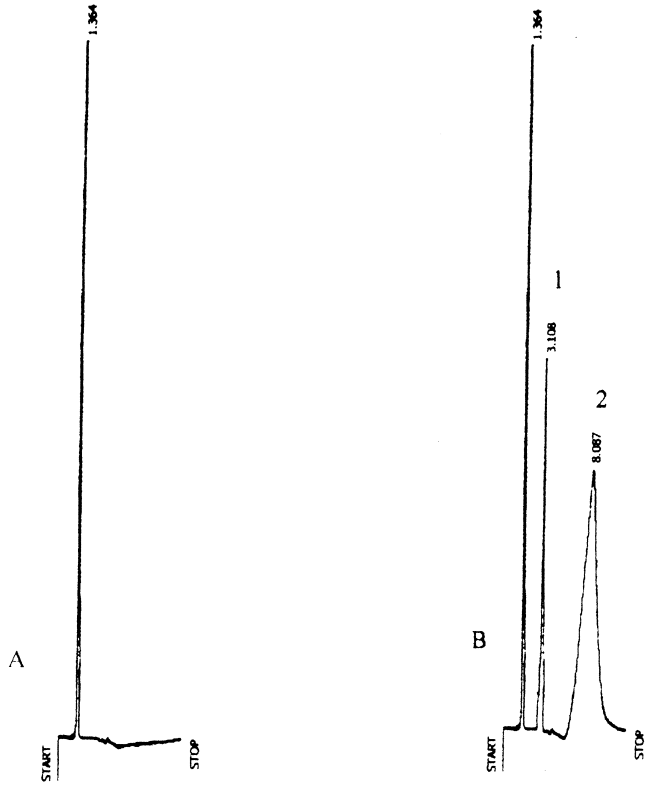

Fig. 1. Chromatograms of extracted plasma samples: (A) Blank plasma; (B) plasma sample containing 1.0 μg/ml formycin A. 1, Formycin A; 2, azathioprine (I.S.).

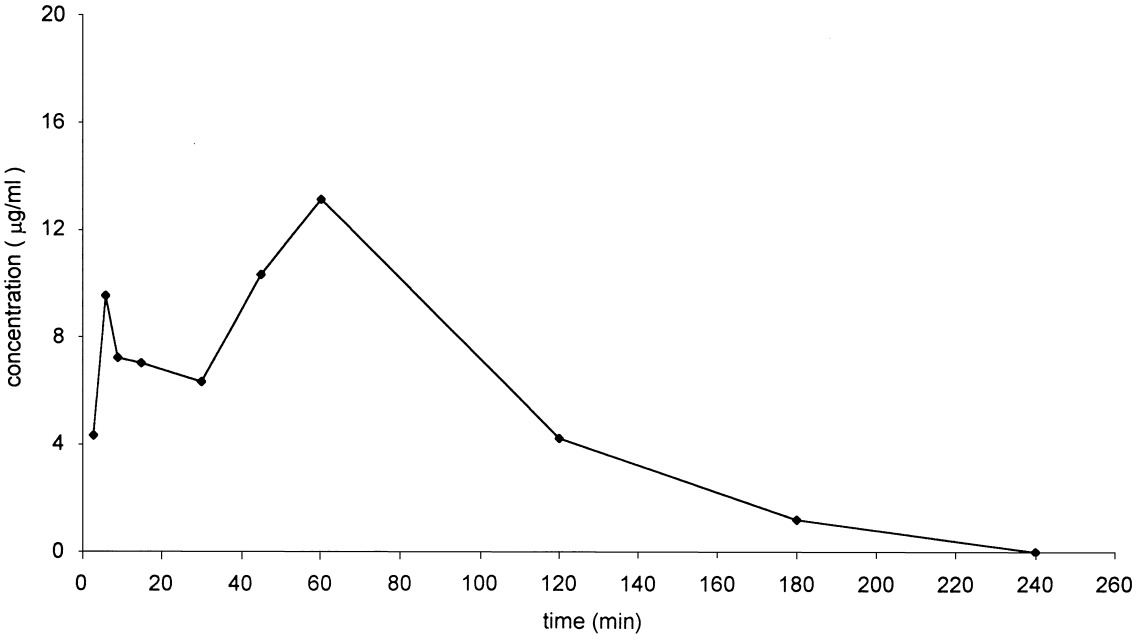

Fig. 2. Plasma concentration in mouse versus time curve of formycin A after administration of 3.2 mg/kg formycin A.

References

[1] I.F. Henderson, A.R.P. Paterson, I.C. Caldwell, M. Hori, Cancer Res. 27 (1967) 715.
[2] R. Bossa, F. Dubini, J. Galatulas, M. Taniguchi, Arch. Ital. Patol. Clin. Tumori 13 (1970) 135.
[3] Y. Sakaitomita, Y. Mitani, M. Tsuda, T. Tsuchiya, Chem. Pharm. Bull. 39 (1991) 1325.
[4] E.G. Jahngen, E.F. Rossomando, Anal. Biochem. 137 (1984) 493.
[5] E.N. Spremulli, G.W. Crabtree, D.L. Dexter, S.H. Chu, D.M. Farineau, L.Y. Ghoda, D.L. McGowan, J. Diamond, R.E. Parks, P. Calabresi, Cancer Treat. Rep. 67 (1983) 267.
[6] L.S. Yago, T.J. Good, in: P.M. Kabra, L.J. Marton (Eds.), Clinical Liquid Chromatography, Vol. 1, CRC Press, Boca Raton, FL, 1984, p. 200.