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Determination of a new thymidine phosphorylase inhibitor, TPI, in dog and rat plasma by reversed-phase ion-pair high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the determination of a new thymidine phosphorylase inhibitor, TPI, in dog and rat plasma is described. TPI was isolated from biological samples by solid-phase extraction on Bond Elut PRS columns. Chromatographic separation was achieved on a C_{18} column using a mobile phase consisting of acetonitrile–10 m*M* acetate buffer (pH 4.3) including hexanesulfonate, with UV detection at 276 nm. This method has been validated across the range of 50–50 000 ng/ml using a 0.1-ml plasma volume. The mean recoveries from spiked plasma were 93% for dog and 94% for rat, respectively. The accuracy, precision and specificity of the method were demonstrated to be acceptable, and it was applied to the toxicokinetic study of TPI in rats.

Keywords: Thymidine phosphorylase inhibitor; 5-Chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1*H*,3*H*)-pyrimidinedione hydrochloride

1. Introduction

Thymidine phosphorylase (TP) is an enzyme that catalyzes the conversion of thymidine to thymine, and is identical to an angiogenic factor, platelet-derived endothelial cell growth factor [1,2]. The increased expression of TP in gastric carcinomas is related to angiogenesis, tumor growth, invasiveness and ability to metastasize, and the prognosis of patients [3]. On the other hand, an antitumor nucleoside, 5-trifluorothymidine (F_3dThd) was found to be effective against 5-fluorouracil-resistant human tumors, but F_3dThd was rapidly degraded to inactive 5-trifluorothymine by TP. 5-chloro-6-(2-iminopyrrolidin - 1 - yl)methyl - 2, 4(1*H*, 3*H*) - pyrimidine - dione hydrochloride (TPI, Fig. 1) was found to be a novel TP inhibitor [4]. A combined form of F_3dThd and TPI (1:0.5 molar ratio) is considered to be a good treatment approach for human cancers with intrinsic and acquired resistance to 5-fluorouracil [5]. In addition, TPI alone or in combination with other anticancer agents may suppress the locoregional recurrence and metastasis of TP-expressing tumors [6].

We developed a method for the determination of

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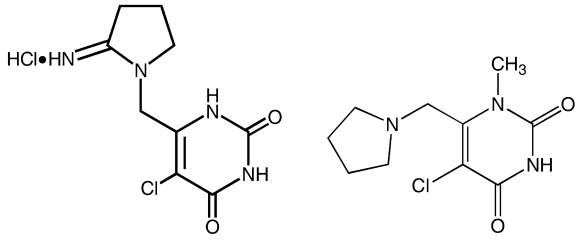

TPI I.S.

Fig. 1. Structures of TPI and I.S.

TPI in dog and rat plasma for toxicokinetic/pharmacokinetic study of TPI. In this paper, we describe the methodology and validation for the determination. TPI is a water-soluble basic compound and the retention time on a reversed-phase column is very short. By use of an ion pair reagent, an appropriate retention time for TPI on the column was attained, and accurate and rapid sample preparation using an ion-exchange solid-phase extraction column was also achieved.

2. Experimental

2.1. Chemicals and reagents

TPI: 5-chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1*H*,3*H*)-pyrimidinedione hydrochloride and the internal standard (I.S.): 5-chloro-1-methyl-6-(pyrrolidin-1-yl)methyl-2,4(1*H*,3*H*)-pyrimidinedione (Fig. 1) were synthesized by Taiho Pharmaceuticals (Tokyo, Japan).

Acetonitrile of HPLC grade, methanol of HPLC grade, 0.1 *M* hydrochloric acid of volumetric standard grade, and acetic acid, sodium acetate trihydrate and ammonia solution (25~28% NH_3 in water) of guaranteed reagent grades were obtained from Wako Pure Chemical Industries (Osaka, Japan). Sodium 1-hexanesulfonate of specially prepared analytical reagents grade was obtained from Tokyo Chemical Industry (Tokyo, Japan). Purified water from a MILLI-Q system (Nihon Millipore Ltd., Tokyo, Japan) was used.

2.2. Preparation of standard solution

A TPI standard solution (100 μg/ml) was prepared by dissolving 5 mg of TPI in 50 ml of water. TPI standard solutions (16, 2.5, 0.5, 0.1 μg/ml) were prepared by diluting the TPI standard solution (100 μg/ml) with water. An I.S. solution (10 μg/ml) was prepared by dissolving 1 mg of I.S. in 100 ml of water. All solutions were kept refrigerated.

2.3. HPLC conditions

The HPLC system consisted of a 616 pump, a 600S system controller, a 717 plus autosampler, a CHT column oven, a 486 UV detector and a Millenium 2010J Version 2.18 data system (Waters, Milford, MA, USA). A Mightysil RP-18 (150×4.6 mm I.D., 5 μm) from Kanto Chemical Co., Inc. (Tokyo, Japan) was used as the analytical column at 40°C, also, a Mightysil RP-18 (5×4.6 mm I.D., 5 μm) was used as the guard column.

The mobile phase was acetonitrile–7 m*M* sodium 1-hexanesulfonate in 10 m*M* acetate buffer (pH 4.3) [9:91, v/v], at a flow-rate of 1.0 ml/min. The autosampler temperature was 20°C. The detection of TPI and I.S. occurred at a UV wavelength of 276 nm. The autosampler rinse solution (Injector wash) was acetonitrile/water [9:91, v/v].

2.4. Preparation of reagents

The 10 m*M* acetate buffer (pH 4.3) was made by adding 10 m*M* acetic acid solution to 10 m*M* sodium acetate solution to adjust the pH to 4.3 exactly, and was monitored using a pH meter. 7 m*M* sodium 1-hexanesulfonate in 10 m*M* acetate buffer (pH 4.3) was made by adding sodium 1-hexanesulfonate to 10 m*M* acetate buffer (pH 4.3) to a final concentration of 7 m*M*. This mobile phase was de-gassed. A 2% ammonia solution-methanol was made by mixing ammonia solution and methanol [2:98, v/v].

2.5. Sample preparation

A total of 0.1 ml of plasma was transferred to a glass test tube and 50 μl of I.S. solution, 70 μl of 0.1

M HCl and 0.65 ml of water were added to the plasma and mixed. The mixed sample was loaded onto a Bond Elut PRS column (1 cc/100 mg, Varian, Harbor City, CA, USA) set up at a Vac Elut SPS 24 (Varian) that prior to the sample loading had been conditioned with 1 ml of methanol, and then with 1 ml of water. After passing the sample, the column was washed with 1 ml of water and then with 1 ml of methanol. The eluate was collected with 2 ml of 2% ammonia solution–methanol in a glass test tube, and dried under a stream of N_2 gas at 37°C on a water-bath. The residue was reconstituted in 0.2 ml of 10 m*M* acetate buffer (pH 4.3), and a 60 μl aliquot was injected into the HPLC.

2.6. Calibration standard preparation

A total of 50 μl of each TPI standard solution, 50 μl of I.S. solution, 70 μl of 0.1 *M* HCl and 0.6 ml of water were added to 0.1 ml of blank plasma and mixed. After that, the calibration standards for TPI were prepared in a similar treatment as the determination samples.

The range of calibration curves were from 50 to 50 000 ng/ml of plasma. The calibration curve was made by the internal standard method using $1/x^2$ weighing regression ($y=a+bx$). Y is the peak area ratio of TPI to I.S. and x is the concentration of TPI.

2.7. Precision and accuracy

Intra-assays ($n=5$) and inter-assays ($n=1$/day, 5 days) were evaluated by spiked plasma at three TPI concentrations (QC samples). The lower limit of quantitation (LOQ) assays ($n=5$) were evaluated by spiked plasma at a TPI concentration of 50 ng/ml. For each concentration level, mean and standard deviation were calculated. The accuracy was expressed as relative error (RE). The precision was expressed as relative standard deviation (RSD).

$$\text{RE (\%)} = \frac{\text{mean calculated concentration} - \text{nominal concentration}}{\text{nominal concentration}} \times 100$$

$$\text{RSD (\%)} = (\text{standard deviation}/\text{mean calculated concentration}) \times 100$$

The acceptance criteria were as follows: RE must be within ±15% (±20% at LOQ) for accuracy, and RSD must be within 15% (20% at LOQ) for precision.

3. Results and discussion

3.1. Extraction

TPI was water-soluble and not extracted with organic solvents. An initial trial for sample preparation was plasma-deproteinization using trichloroacetic acid and perchloric acid, but it was not useful for sample preparation because of the appearance of a lot of interfering peaks.

Secondly, we investigated cation-exchange using solid-phase extraction columns. TPI in standard solution was retained by a Bond Elut CBA column (Varian), but TPI in a plasma mixture was not retained by the CBA column. Therefore, two strong cation-exchange columns (Bond Elut SCX, Bond Elut PRS, Varian) were tested. TPI in plasma mixture was retained by both columns but more interfering peaks appeared in the eluate from SCX than that from PRS. That phenomenon may have come from non-polar interactions of benzene moieties on SCX sorbent surface [7]. Therefore, Bond Elut PRS was selected as the sample preparation column.

The pK_a of TPI was estimated to around pH 6.0 by comparison of UV absorbency, and the pK_a of I.S. was estimated to pH 5~5.5. When the plasma mixture was adjusted to pH 3~4, TPI and I.S. existed as cationic species. A total of 70 μl of 0.1 *M* HCl for 0.1 ml plasma was an appropriate volume to retain TPI and I.S. on the PRS column, and, using 2 ml of 2% ammonia solution-methanol as the eluent, TPI and I.S. were eluted sufficiently from the PRS column.

The recoveries of TPI and I.S. were determined by comparing the peak areas of extracted sample with those of unextracted standards. The recoveries

(mean±SD) from dog plasma ($n=15$) were 93.2±4.2% and 91.0±2.2%, and from rat plasma ($n=15$) were 94.0±5.0% and 91.8±3.8% for TPI and I.S. respectively.

3.2. Chromatography and specificity

The λ_{max} of the UV spectra of TPI were 276~279 nm (wavelength) at pH 2.5~5.5 in phosphate buffer and at 301 nm in pH 7 phosphate buffer. This suggested that TPI existed in a neutral form under neutral pH conditions. However, the retention time of TPI was only 4.1 min even under the mobile phase condition of acetonitrile–10 m*M* phosphate buffer (pH 7.2) [5:95, v/v]. Therefore, we tried the addition of ion pair reagents to the mobile phase to ensure an appropriate retention time for TPI.

Various alkylsulfonates were examined as ion pair reagents. The retention of TPI using pentanesulfonate was weak, whilst retention using heptanesulfonate and octanesulfonate was strong, but the retention of some endogenous peaks became strong to interfere with TPI. Eventually, a combination of hexanesulfonate and phosphate buffer (pH 2.3) led to an appropriate retention time.

Moreover, we tried to achieve better separation of TPI from interference peaks by changing the pH of the mobile phase. The chromatographic separations at pH 2.3~3.8 where both TPI and I.S. existed as cations were not good enough. Therefore, it was examined at pH 4~5 using acetate buffer. The pH of 10 m*M* acetate buffer was evaluated to optimize the resolution of TPI and endogenous interference, and the optimum was found to be pH 4.3. Furthermore, the concentration of sodium 1-hexanesulfonate in acetate buffer was optimized to 7 m*M*.

Typical chromatograms of dog plasma extract are shown in Fig. 2. The retention times for TPI and I.S. were 6.4 and 10.5 min, respectively. Dog blank plasma from four separate batches and rat blank plasma from six separate batches were tested for

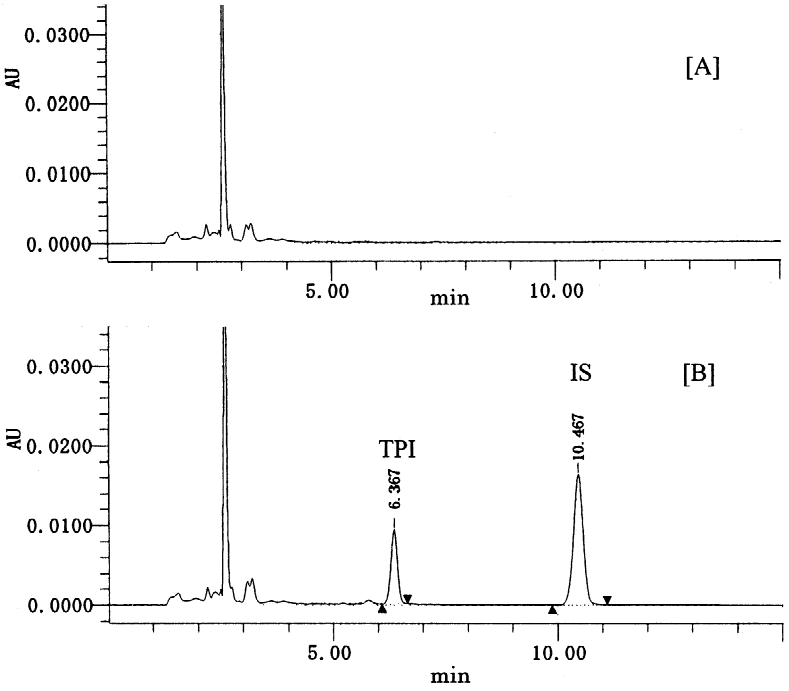

Fig. 2. Typical chromatograms of dog blank plasma [A], and dog plasma spiked with TPI (2000 ng/ml) and I.S. (5000 ng/ml) [B].

endogenous interferences. All batches were free of interferences in the TPI and I.S. regions.

3.3. Validation

The calibration curves were linear from 50 to 50 000 ng/ml. The weighted least-squares linear regression equation from the curves ($n=6$) for dog plasma was $y=(0.001353\pm0.001285)+(0.000199\pm0.000005)x$, and the correlation coefficients were greater than 0.9997. The equation from the curves ($n=6$) for rat plasma was $y=(0.001206\pm0.002438)+(0.000197\pm0.000001)x$, and the correlation coefficients were greater than 0.9998.

The accuracy and precision of the assays for TPI in dog and rat plasma are shown in Table 1. The REs and RSDs of QC samples (200, 2000 and 40 000 ng/ml) were within ±2.9% and within 3.8%, respectively. The lowest concentration (50 ng/ml) meeting the acceptance criteria was established as the limit of quantitation. The RE and RSD in dog plasma were −0.7% and 7.8%, and in rat plasma were −5.8%, 4.4%, respectively.

3.4. Stability

Freeze/thaw stability was tested by subjecting QC samples (200 and 5000 ng/ml, $n=3$) to two freeze/thaw cycles. The REs in dog plasma were −0.4% and −5.6%, and in rat plasma were −2.1%, −2.2% for samples containing 200 and 5000 ng/ml TPI respectively.

Long term stability was tested by assaying QC samples (200 and 5000 ng/ml, $n=3$) that had been stored in the freezer at −80°C for 1 month. The REs in dog plasma were −1.7% and −2.9%, and in rat plasma were 1.7%, −2.2% for samples containing 200 and 5000 ng/ml TPI respectively. TPI in dog and rat plasma was stable at −80°C for at least 1 month.

The stability of the extracted sample from QC samples (2000 ng/ml, $n=5$) was evaluated by comparing of the extracted samples that had been left in the autosampler at 20°C for 24 and 48 h ($n=2$, each) to the initial values ($n=5$). The REs from dog initial values were 0.2% and 3.1%, and from rat initial values were −0.1%, −1.0% for samples after 24 and 48 h, respectively.

The standard solution stability was evaluated by comparing of peak areas of TPI and I.S. standard solutions that had been stored in the refrigerator at 5°C for 1 month to those of freshly prepared standard solutions. The assays at each solution occurred in triplicate. The REs from fresh TPI standard solutions were 0.3% and −0.6% for solutions of 100 and 0.5 μg/ml respectively, and the RE from fresh I.S.

Table 1
Accuracy and precision of the assays for TPI in dog and rat plasma

Nominal concentration (ng/ml)	Intra-assay ($n=5$)		Inter-assay ($n=5$)	
	RE[a] (%)	RSD[b] (%)	RE (%)	RSD(%)
Dog plasma				
50	−0.7	7.8		
200	0.0	2.1	1.2	2.0
2000	0.9	2.7	−2.0	1.2
40 000	0.6	1.4	−1.4	1.2
Rat plasma				
50	−5.8	4.4		
200	2.9	1.6	−0.1	3.8
2000	−1.3	0.8	−1.1	0.9
40 000	−1.2	0.4	−0.9	3.1

[a] RE = relative error.
[b] RSD = relative standard deviation.

standard solution was 0.5%. TPI and I.S. standard solution were stable at 5°C for 1 month.

3.5. Application to biological samples

The validated method was applied to the determination of TPI in rat plasma samples in a toxicokinetic study. Fig. 3 shows the mean plasma concentration of TPI after oral administration of 2000 mg/kg TPI to rats. The concentration range and sensitivity of the method were sufficient to follow the study. Also, no interfering peak was observed in the biological samples.

The method has been applied to the toxicokinetic/pharmacokinetic study on dogs and the sample preparation of this method has been applied to the determination of TPI in human plasma/urine samples for clinical study.

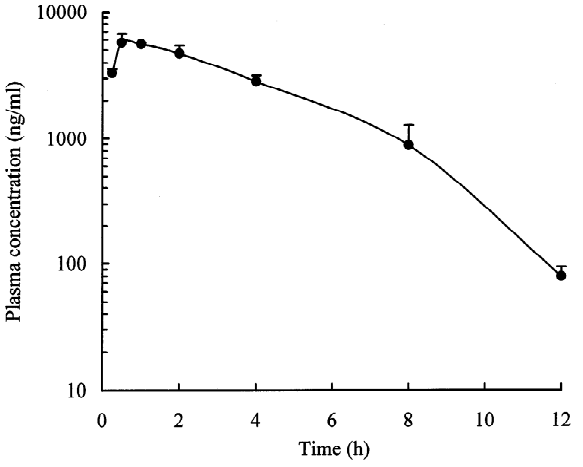

Fig. 3. Plasma concentration-time profile after oral administration of 2000 mg/kg of TPI to rats. Each point represents the mean+SD ($n=3$).

4. Conclusion

This report describes the determination of TPI using solid-phase extraction and reversed-phase ion-pair high-performance liquid chromatography. This method has been validated across the range of 50–50 000 ng/ml using a 0.1 ml sample volume. The accuracy, precision and specificity of the method were acceptable. The method was developed for high dose toxicokinetic studies of TPI in dog and rat. Therefore, we used 0.1 ml of plasma as the sample volume. Using a 0.5 ml plasma sample, the range of the method can be lowered to 10–10 000 ng/ml.

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