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Validation of an analytical method for a potent antitumor agent, TZT-1027, in plasma using liquid chromatography–mass spectrometry

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Abstract

A sensitive and specific analytical method for a potent antitumor agent, TZT-1027, in plasma has been developed using liquid chromatography–mass spectrometry (LC–MS) with [$^2H_4$]TZT-1027 as an internal standard (I.S.). A plasma sample was purified by solid-phase extraction on a C_{18} cartridge, followed by solvent extraction with diethyl ether. The extract was then injected into the LC–MS system. Chromatography was carried out on a C_{18} reversed-phase column using acetonitrile–0.05% trifluoroacetic acid (TFA) (55:45) as a mobile phase. Mass spectrometric analysis was performed in atmospheric pressure chemical ionization (APCI) mode with positive ion detection, and the protonated molecular ions ($[M+H]^+$) of TZT-1027 and I.S. were monitored to allow quantitation. The method was applied to the determination of TZT-1027 in human, monkey, dog, rat and mouse plasma. As far as the sample preparation was concerned, good recoveries (73.5–99.1%) were obtained. The calibration curves were linear over the range of 0.25–100 ng per 1 ml of human, dog and rat plasma, per 0.5 ml of monkey plasma, and per 0.1 ml of mouse plasma. From the intra- and inter-day accuracy and precision, the present method satisfies the accepted criteria for bioanalytical method validation. TZT-1027 was stable when stored below −15°C for 6 months in human plasma and for 3 weeks in plasma from other species. TZT-1027 was also stable in plasma through at least three freeze–thaw cycles.

Keywords: Antitumour agent; TZT-1027

1. Introduction

Compound TZT-1027, Dov-Val-Dil-Dap-*N*-CH_2CH_2-Ph (Fig. 1), is a dolastatin 10 derivative developed in our laboratories [1]. It was found to possess potent antitumor activity due to its ability to bind to tublin and inhibit microtubule polymerization [2]. TZT-1027 is now undergoing clinical trials as an antitumor agent. Because of the potent activity, it was presumed that its plasma concentrations would be correspondingly low at effective dosage levels. Therefore, a highly sensitive and specific determination method was required for pharmacokinetic studies of TZT-1027.

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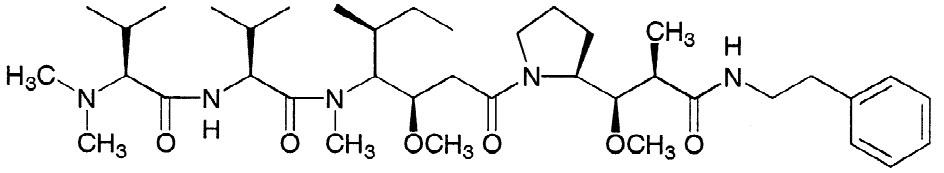

Fig. 1. Chemical structure of TZT-1027.

Progress in liquid chromatography–mass spectrometry (LC–MS) has enabled us to assay a variety of compounds in biological fluids. The technique can be used for the analysis of compounds with low volatility and/or thermally labile compounds such as peptides [3,4]. Therefore, we planned to develop a LC–MS method for the determination of TZT-1027 in human plasma using [$^2H_4$]TZT-1027 as an I.S. The method was also intended for application to monkey, dog, rat and mouse plasma. The present paper describes a sensitive and specific analytical method that meets the accepted criteria for bioanalytical method validation [5].

2. Experimental

2.1. Materials

TZT-1027 was synthesized in our laboratory. Phenylethyl-[1,1,2,2-$^2H_4$]-amine was obtained from C/D/N Isotope (Montreal, Canada). Sephadex LH-20 was obtained from Pharmacia Fine Chemical (Uppsala, Sweden). Preparative thin-layer chromatography (TLC) used Whatman silica-gel 60A plates (Clifton, NJ, USA). Bond Elut C_{18} cartridges (200 mg/3 ml) were obtained from Varian (Harbor City, CA, USA). HPLC grade acetonitrile was obtained from Nakalai Tesque (Kyoto, Japan). Water was used following treatment with a Milli-Q labo system (Nihon Millipore, Tokyo, Japan). All other chemicals were of analytical or special grade obtained from commercial sources.

2.2. Synthesis of the internal standard [$^2H_4$]TZT-1027

Stable isotope labeled TZT-1027 was synthesized from a tetrapeptide (Dov-Val-Dil-Dap-OH) and commercially available phenylethyl-[1,1,2,2-$^2H_4$]-amine according to the literature [1]. To the solution of tetrapeptide (253 mg, 0.422 mmol) and phenylethyl-[1,1,2,2-$^2H_4$]-amine (58 mg, 0.463 mmol) in *N*,*N*-dimethylformamide (2.5 ml), diethyl phosphorocyanidate (87 mg, 0.533 mmol) and triethylamine (71 μl, 0.533 mmol) were added under ice-cooling with stirring. Stirring was continued overnight under ice-cooling, then the solution was evaporated in vacuo. The residue was dissolved in ethyl acetate–benzene (4:1) (50 ml) and washed successively with saturated aqueous sodium hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous sodium sulfate and evaporated in vacuo. The residue was purified by preparative TLC using dichloromethane–methanol (10:1), followed by column chromatography on Sephadex LH-20 with *n*-hexane–dichloromethane–methanol (4:15:5) to give [$^2H_4$]TZT-1027 as an amorphous powder. Crystallization from diethyl ether gave [$^2H_4$]TZT-1027 (226.7 mg) as colorless crystals. The tetradeuterio isotopic purity of [$^2H_4$]TZT-1027 estimated from the mass spectrum was 92.5%.

2.3. Apparatus and conditions

The apparatus used was a Hitachi L-6000 HPLC pump connected to a Hitachi M-2000 mass spectrometer via an APCI interface (Tokyo, Japan). Chromatography was performed using an Inertsil ODS-2 column (250×4.6 mm I.D., GL Sciences, Tokyo, Japan) fitted with a guard column of the same packing material (Inertsil ODS-2, 10×4.0 mm I.D.). The mobile phase consisted of acetonitrile–0.05% TFA (55:45). The flow-rate was 1 ml/min at ambient temperature. The mass spectrometer was operated in positive ion mode. The nebulizer and desolvator were operated at 260 and 400°C, respectively. The needle current was fixed at 5 μA and the drift voltage was set at 198 V. In the selected ion

monitoring (SIM) mode, the protonated molecular ions ($[M+H]^+$) of TZT-1027 (m/z 702.5) and I.S. (m/z 706.5) were monitored.

2.4. Standard solutions

Stock solutions of TZT-1027 and I.S. were prepared at concentrations of 100 μg/ml solutions by dissolving them in methanol. These two solutions were further diluted with the same solvent to obtain working solutions at a concentration of 100 ng/ml.

2.5. Calibration standards

Calibration standards containing 0.25, 0.5, 1, 5, 20 and 100 ng of TZT-1027 were prepared using 1 ml of blank plasma, except for monkey (0.5 ml) and mouse (0.1 ml), as follows. To the test tubes containing 100 μl of I.S. working solution, 2.5, 5, 10, 50, 200 and 1000 μl of TZT-1027 working solution were added, respectively ($n=1$ at each level). The solvent was evaporated to adjust the volume consistently, and the residue was dissolved in blank plasma by briefly vortex-mixing.

2.6. Quality and stability control samples

Quality control samples at the levels of 0.25, 0.5, 5, 20 and 100 ng were prepared in the same way as the calibration standards. To prepare the stability control samples at the concentrations of 5 and 80 ng per 1 ml of human, dog and rat plasma, per 0.5 ml of monkey plasma, and per 0.1 ml of mouse plasma, the working solution of TZT-1027 was evaporated and dissolved in each pooled plasma. Then they were divided into multiple tubes and stored below −15°C. Immediately before sample preparation, I.S. was added to the stability control samples.

2.7. Sample preparation and analytical procedures

The plasma sample (1 ml each of human, dog and rat plasma, 0.5 ml of monkey plasma, and 0.1 ml of mouse plasma) was diluted with 0.5 ml water and vortex-mixed. The resulting solution was passed through a Bond Elut C_{18} cartridge, conditioned with 3 ml acetonitrile and 6 ml water. The cartridge was rinsed successively with 1 ml test tube washing water, 3 ml water and 3 ml acetonitrile–0.05% TFA (20:80). Then, TZT-1027 and I.S. were eluted from the cartridge with 3 ml acetonitrile–0.05% TFA (60:40). The eluate was concentrated to dryness using a centrifugal evaporator (Yamato Scientific, Tokyo, Japan) at 50°C. To the residue, 0.1 ml water and 0.1 ml saturated aqueous sodium hydrogen carbonate were added. The solution was extracted with 5 ml diethyl ether by vortex-mixing for 15 s and centrifuged at 1500 *g* for 5 min. The aqueous layer was frozen in a dry ice–acetone cold bath. The organic layer was transferred to another test tube and evaporated to dryness under a stream of nitrogen gas at 50°C. The residue was then dissolved in 100 μl mobile phase and 50 μl of this solution was injected into the LC–MS system. The recorded peak areas were used to calculate the ratio of TZT-1027 to I.S.

2.8. Method validation

2.8.1. Specificity

Three different blank plasma samples were extracted and analyzed for potential interference from endogenous substances which could interfere significantly with the determination of TZT-1027.

2.8.2. Recovery

The recovery was evaluated from the peak area ratios of plasma samples by comparison with those from standard mixtures as follows. Plasma samples spiked with TZT-1027 at levels of 0.5, 1 and 20 ng were extracted without addition of I.S., according to the method described above ($n=3$ at each level). Then, 100 μl I.S. working solution was added and assayed. Standard mixtures of TZT-1027 and I.S., equal to the plasma samples, were also assayed without extraction ($n=2$ at each level).

2.8.3. Calibration curve

Calibration standards at levels of 0.25, 0.5, 1, 5, 20 and 100 ng ($n=1$ at each level) were extracted and assayed as described above. The calibration curve was prepared by plotting the peak area ratio

(TZT-1027/I.S.) against the amount of TZT-1027 added, using linear least-squares regression.

2.8.4. Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of quality control samples at levels of 0.25, 0.5, 5, 20 and 100 ng ($n=5$ at each level) on the same day. These levels were chosen to demonstrate the accuracy and precision of the method at or near the lower limit of quantitation (LOQ) as well as at low, moderate and high concentrations of the calibration curve. To assess the inter-day accuracy and precision, analysis of quality control samples at the same levels ($n=1$ at each level per day) was performed on three different days. Accuracy and precision were expressed as bias and relative standard deviation (RSD), respectively.

2.8.5. Stability

The effect of three freeze–thaw cycles on TZT-1027 in plasma was evaluated by analysis of stability control samples. Also the stability of TZT-1027 stored below −15°C in plasma at the same levels was assessed. Portions of the above-mentioned stability control samples were assayed after three freeze–thaw cycles prior to sample preparation. The remainder were removed and assayed periodically. The mean values of TZT-1027 were compared with the initial values, which were assayed immediately after preparation of stability control samples. The stability was expressed as a percentage of the initial value.

3. Results and discussion

3.1. Mass spectrum

The base peak at m/z 702 corresponded to the protonated molecular ion ($[M+H]^+$) of TZT-1027 and no significant fragmentation occurred. Because I.S. is a tetra-deuterated analog of TZT-1027, the mass spectrum of I.S. was similar to TZT-1027, except for the base peak at m/z 706 which was four mass units higher than that of TZT-1027. Thus, the protonated molecular ions ($[M+H]^+$) of TZT-1027 and I.S. were selected to monitor quantitation (Fig. 2).

3.2. Specificity

The retention times of both TZT-1027 and I.S. were 2.3 min and no endogenous peaks that would interfere with the detection of TZT-1027 and I.S. were observed (Fig. 3). These results suggested that each selected ion was specific for the corresponding analyte. Similar results were obtained from rat, dog, monkey and mouse plasma (data not shown).

3.3. Recovery

The recovery for the sample preparation, which involved solid-phase extraction on a C_{18} cartridge from plasma followed by solvent extraction with diethyl ether, was estimated at three levels of TZT-1027. For human plasma, the extraction recoveries were 73.5, 78.6 and 83.8% at levels of 0.5, 1 and 20 ng (Table 1). These results suggested that there was no difference in extraction recovery at different levels of TZT-1027. Good recoveries of TZT-1027 from dog, rat, monkey and mouse plasma were also obtained and almost equal to those from human plasma (79.0–99.1%, Table 1).

3.4. Calibration curve

Each calibration curve, which was prepared by plotting the peak area ratio versus the amount of TZT-1027 added, was linear over the range 0.25–100 ng with the correlation coefficient better than 0.9998. The parameters of the calibration curves obtained in this study are shown in Table 2. The slope and intercept of the calibration curves were similar in all species examined.

3.5. Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of quality control samples at levels of 0.25, 0.5, 5, 20 and 100 ng. Intra-day bias was −16.4% at the LOQ (0.25 ng per 1 ml) in human plasma and ranged from −14.4 to −1.5% at higher concentrations (Table 3). Intra-day RSD ranged from 0.6 to 4.4%, except for the LOQ where the RSD was

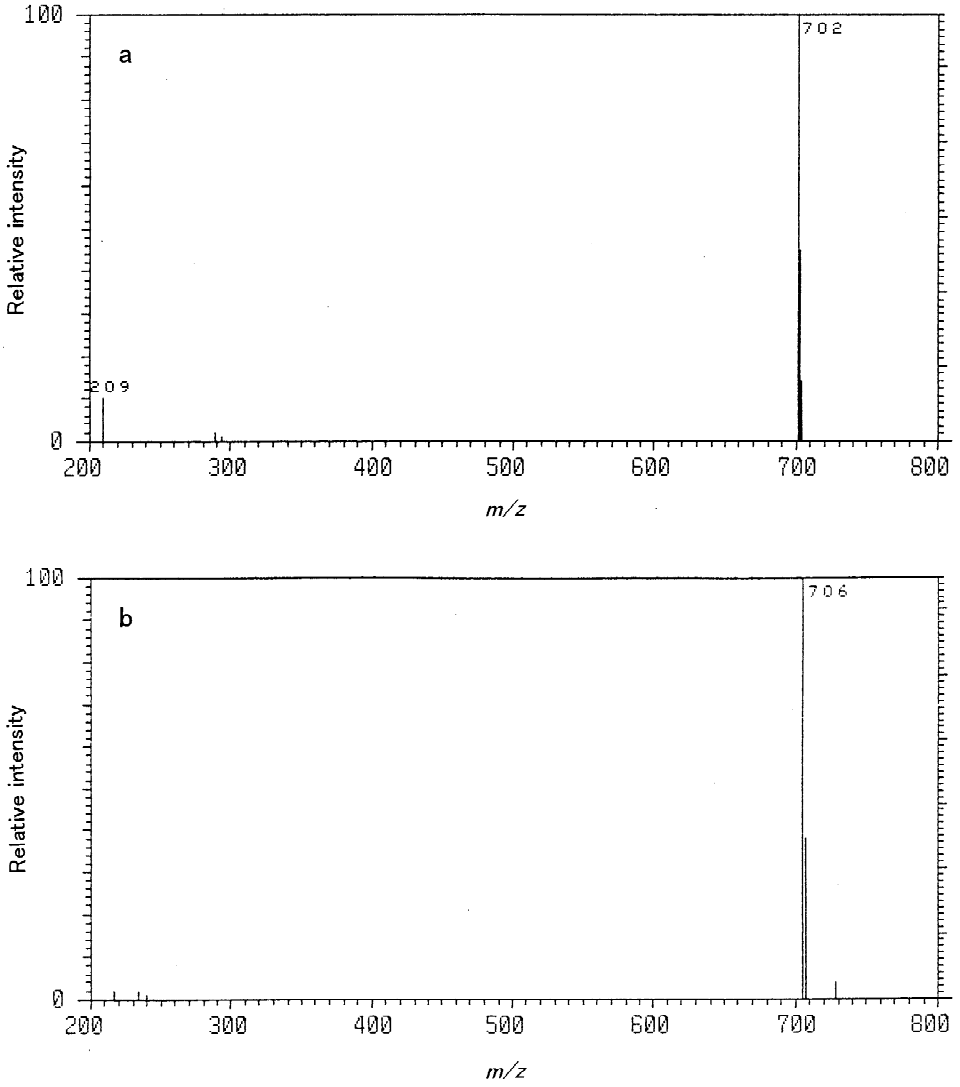

Fig. 2. APCI mass spectra of TZT-1027, (a), and the internal standard, (b).

13.4%. In dog, rat, monkey and mouse plasma, the intra-day bias and RSD were within 8.8% and 7.3%, respectively. Inter-day bias and RSD for human plasma were −4.0% and 13.8% at the LOQ (Table 4). At higher concentrations, the bias and RSD ranged from −3.0 to −1.0% and from 0.7 to 11.3%, respectively. These results satisfied the validation criteria [5], because the accuracy and precision were within±15% and 15%, except for the LOQ where the accuracy and precision were within±20% and 20%, respectively.

3.6. Stability

The effect of three freeze–thaw cycles on the stability of TZT-1027 in human plasma was evaluated by assaying stability control samples at concentrations of 5 and 80 ng per 1 ml. The mean values after three freeze–thaw cycles were within −4.7% of the initial values (Table 5). As far as the stability of TZT-1027 in human plasma when stored below −15°C for 3 weeks was concerned, the mean values were within −4.5% of the initial values. Similar

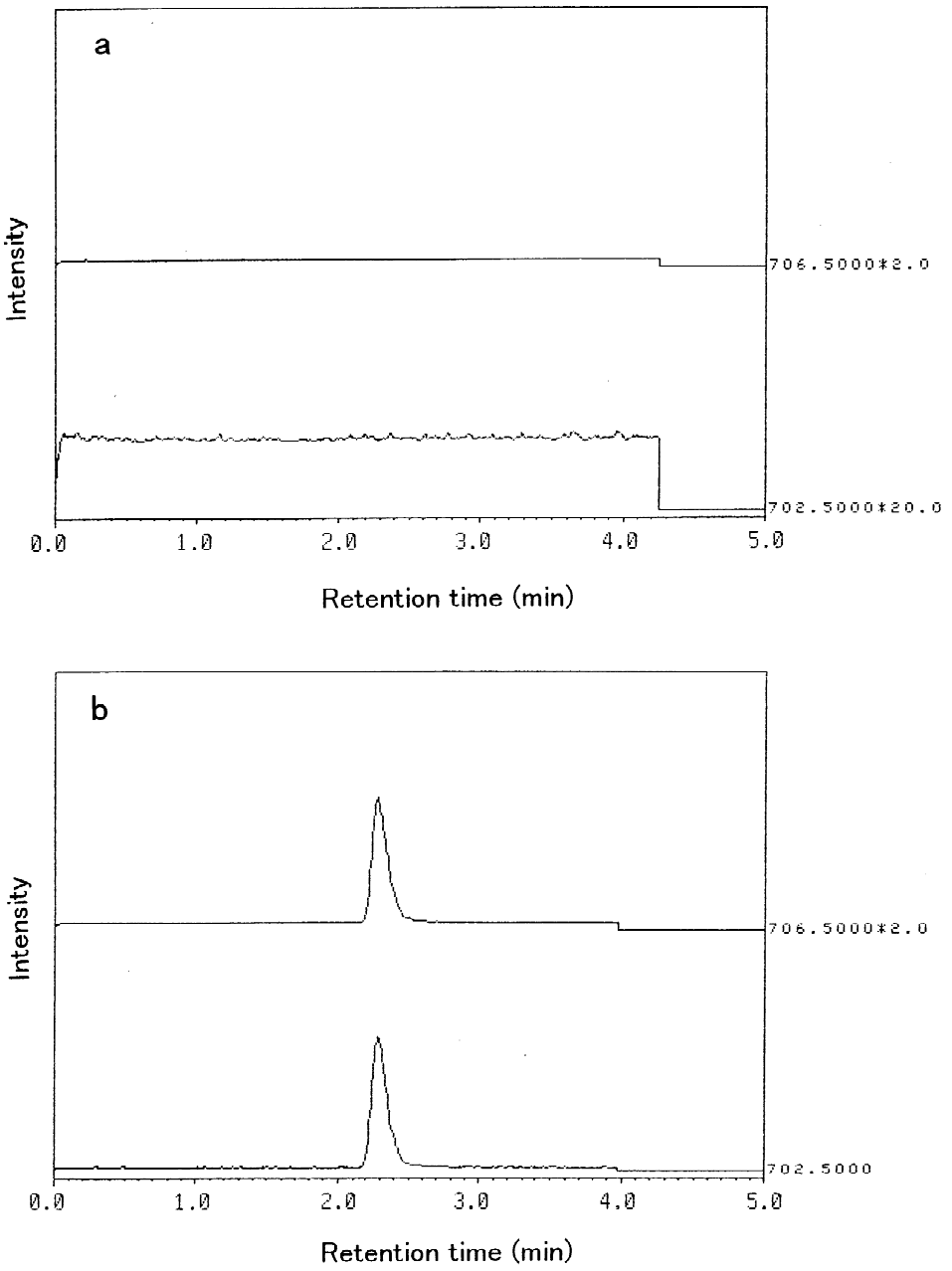

Fig. 3. Selected ion chromatograms of human blank plasma, (a), and a quality control sample spiked with 20 ng of TZT-1027 and 10 ng of I.S., (b). The m/z 702.5 corresponds to TZT-1027 and the m/z 706.5 corresponds to the I.S.

results were obtained from dog, rat, monkey and mouse plasma (Table 5). TZT-1027 was stable in plasma when stored below −15°C for at least 3 weeks, and through at least three freeze–thaw cycles. Since no degradation of TZT-1027 in plasma was seen during storage in a freezer, we extended the

Table 1
Recovery of TZT-1027 in plasma

Added (ng)	Recovery[a] (%)	RSD (%)
Human plasma (1 ml)		
0.5	73.5±11.0	15.0
1	78.6±9.4	12.0
20	83.8±1.1	1.3
Dog plasma (1 ml)		
0.5	79.1±7.8	9.9
1	83.9±1.8	2.1
20	87.5±2.5	2.9
Rat plasma (1 ml)		
0.5	83.5±8.8	10.5
1	81.1±5.6	6.9
20	99.1±3.7	3.7
Monkey plasma (0.5 ml)		
0.5	79.0±2.8	3.5
1	88.4±3.0	3.4
20	92.6±0.8	0.9
Mouse plasma (0.1 ml)		
0.5	86.7±2.2	2.5
1	85.1±1.1	1.3
20	94.3±2.5	2.7

[a] Data are expressed as mean±SD (n=3).

period of preservation. Using human plasma, the mean values when stored below −15°C for 6 months were within 8.2% of the initial values. Thus, TZT-1027 in plasma appears to be stable when stored frozen.

3.7. Application to clinical sample analysis

The present method is successfully applied to the determination of plasma concentrations of TZT-1027

Table 2
Calibration curve obtained from the determination of TZT-1027 on different days

Species	n	Slope (Mean±SD)	Intercept (Mean±SD)
Human	6	0.103±0.005	0.023±0.018
Dog	4	0.114±0.009	0.012±0.021
Rat	4	0.112±0.011	0.019±0.016
Monkey	4	0.107±0.006	0.014±0.019
Mouse	4	0.116±0.013	0.017±0.015

Table 3
Intra-day accuracy and precision for the determination of TZT-1027 in plasma

Added (ng)	Found[a] (ng)	Bias (%)	RSD (%)
Human plasma (1 ml)			
0.25	0.209±0.028	−16.4	13.4
0.5	0.428±0.019	−14.4	4.4
5	4.80±0.19	−4.1	4.0
20	19.7±0.1	−1.5	0.6
100	97.7±1.5	−2.3	1.6
Dog plasma (1 ml)			
0.25	0.272±0.017	8.8	6.3
0.5	0.508±0.031	1.6	6.1
5	5.03±0.13	0.7	2.5
20	20.1±0.3	0.3	1.4
100	98.8±1.3	−1.2	1.3
Rat plasma (1 ml)			
0.25	0.267±0.017	6.8	6.4
0.5	0.526±0.031	5.2	5.9
5	5.27±0.13	5.5	2.4
20	21.0±0.3	4.8	1.4
100	99.2±1.9	−0.8	1.9
Monkey plasma (0.5 ml)			
0.25	0.249±0.016	−0.4	6.4
0.5	0.488±0.010	−2.4	2.0
5	4.86±0.08	−2.8	1.7
20	19.3±0.1	−3.5	0.3
100	96.5±1.5	−3.5	1.6
Mouse plasma (0.1 ml)			
0.25	0.259±0.018	3.6	6.9
0.5	0.494±0.036	−1.2	7.3
5	5.08±0.11	1.6	2.2
20	19.9±0.5	−0.5	2.3
100	96.5±1.6	−3.5	1.7

[a] Data are expressed as mean±SD (n=5).

Table 4
Inter-day accuracy and precision for the determination of TZT-1027 in human plasma

Added (ng)	Found[a] (ng)	Bias (%)	RSD (%)
0.25	0.240±0.033	−4.0	13.8
0.5	0.485±0.055	−3.0	11.3
5	4.92±0.23	−1.7	4.7
20	19.8±0.1	−1.0	0.7
100	97.9±1.4	−2.1	1.4

[a] Data are expressed as mean±SD (n=3).

Table 5
Stability of TZT-1027 in plasma

Added (ng)	Treatment	Found[a] (ng)	Percentage of initial value (%)
Human plasma (1 ml)			
5	Day on preparation (Initial)	5.09±0.01	–
	Three freeze–thaw cycles	4.85±0.14	−4.7
	Stored below −15°C for 3 weeks	4.87±0.03	−4.5
80	Day on preparation (Initial)	85.5±3.1	–
	Three freeze–thaw cycles	83.2±2.7	−2.6
	Stored below −15°C for 3 weeks	84.0±1.5	−1.6
Dog plasma (1 ml)			
5	Day on preparation (Initial)	5.27±0.05	–
	Three freeze–thaw cycles	5.25±0.17	−0.3
	Stored below −15°C for 3 weeks	4.93±0.11	−6.4
80	Day on preparation (Initial)	81.3±1.6	–
	Three freeze–thaw cycles	82.8±2.7	1.8
	Stored below −15°C for 3 weeks	83.9±2.0	3.2
Rat plasma (1 ml)			
5	Day on preparation (Initial)	5.23±0.20	–
	Three freeze–thaw cycles	5.28±0.20	0.9
	Stored below −15°C for 3 weeks	5.08±0.15	−3.0
80	Day on preparation (Initial)	85.7±2.7	–
	Three freeze–thaw cycles	83.7±0.2	−2.3
	Stored below −15°C for 3 weeks	84.6±0.9	−1.2
Monkey plasma (0.5 ml)			
5	Day on preparation (Initial)	5.02±0.08	–
	Three freeze–thaw cycles	4.86±0.13	−3.2
	Stored below −15°C for 3 weeks	5.18±0.09	3.1
80	Day on preparation (Initial)	82.4±1.6	–
	Three freeze–thaw cycles	79.9±1.5	−3.0
	Stored below −15°C for 3 weeks	81.8±0.8	−0.8
Mouse plasma (0.1 ml)			
5	Day on preparation (Initial)	5.14±0.17	–
	Three freeze–thaw cycles	5.03±0.21	−2.2
	Stored below −15°C for 3 weeks	5.17±0.17	−0.5
80	Day on preparation (Initial)	86.0±1.1	–
	Three freeze–thaw cycles	84.2±1.4	−2.1
	Stored below −15°C for 3 weeks	78.9±0.8	−8.3

[a] Data are expressed as mean±SD (n=3).

in a clinical phase I study. The plot of plasma concentration versus sampling time obtained from a patient during and after single intravenous infusion (1 h) of TZT-1027 is shown in Fig. 4.

4. Conclusion

A sensitive LC–MS method for the determination of TZT-1027 in plasma was validated in a calibration range of 0.25–100 ng per 1 ml of human, dog and rat plasma, per 0.5 ml of monkey plasma, and per 0.1 ml of mouse plasma. The method consists of sample preparation by solid-phase extraction, followed by solvent extraction, chromatographic separation on a C_{18} column and then detection in SIM mode using APCI. The accuracy and precision show that the method is suitable for use in clinical trials and for pharmacokinetic and toxicokinetic studies of TZT-1027.

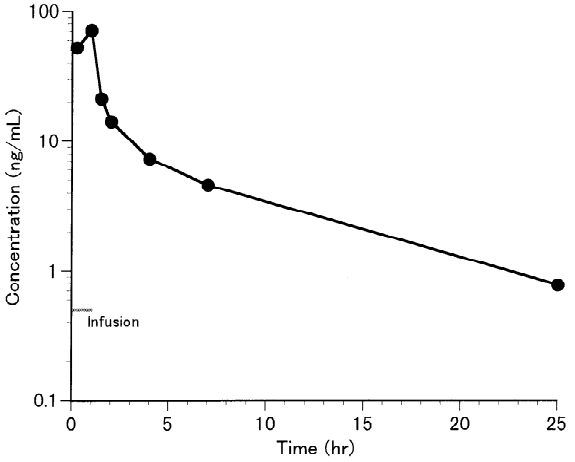

Fig. 4. Plasma concentrations of TZT-1027 in a patient during and after single intravenous infusion (1 h) of 1920 μg (1.35 mg/m^2).

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