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Chiral high-performance liquid chromatographic analysis of the enantiomers of XK469, a new antitumor agent, in plasma and urine

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Abstract

XK469 (NSC697887), (±)-2-[4-(7-Chloro-2-quinoxaliny)oxy]-phenoxy propionic acid, an analog of the herbicide Assure®, which possesses antitumor activity, especially against murine solid tumors and human xenografts, has recently been found to be the first topoisomerase IIβ poison. Both R(+) and S(−) isomers are cytotoxic, although the R-isomer is more potent. A chiral high-performance liquid chromatography (HPLC) assay that utilizes Chirobiotic T column for the measurement of enantiomers of XK469 in plasma has been developed with a limit of quantitation (LOQ) of 0.2 μg/ml using a 0.2 ml plasma sample. Chloroqinoxaline sulfonamide (CQS) was used as the internal standard and the assay has been validated in rat plasma. The within-run coefficient of variations (CVs) were 5.9, 5.0, and 3.1% for the S-isomer and 8.1, 4.2, 6.4% for R(+)-XK469 at 0.2, 1, and 2 μg/ml, respectively. The between-run CVs were 10.5, 5.3, and 1.9% for S(−)- and 10.9, 6.3, and 3.6% for R(+)-XK469. Using this chiral assay, a plasma concentration time data of R(+)-,S(−)-XK469 in a Fischer 344 rat receiving i.v. dosing of S(−)XK469 at 10 mg/kg was monitored. S(−)XK469 was found to be significantly converted to the R-enantiomer in circulation even when the S-enantiomer was administered. The predominant inversion from S(−)- to R(+)-XK469 was also observed in the mouse and dog plasma. In the rat, the plasma concentration–time profiles for both isomers follow two compartmental pharmacokinetics with the $t_{1/2\beta}$ for the R-enantiomer slightly longer and the clearance of the S-enantiomer higher than the R-enantiomer.

Keywords: Chiral HPLC; XK469; (±)-2-[4-(7-Chloro-2-quinoxaliny) oxy]phenoxy propionic acid

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1. Introduction

XK469 (NSC 697887), (±)-2-[4-(7-Chloro-2-quinoxaliny) oxy]phenoxy propionic acid (Fig. 1A), is one of the synthetic analogs of the herbicide Assure® that was originally synthesized by DuPont. XK469 has been found to possess antitumor activity by a disk-diffusion-soft-colony-formation-assay [1]. In vivo studies showed that XK469 exhibited broad activity against murine solid tumors such as colon 38, pancreatic 03, and mammary 16/C. In addition, XK469 was found to be highly cytotoxic against multidrug-resisitant tumors [1,2]. The mechanism of the antitumor action of XK469 has recently been found to be the first selective topoisomerase IIβ poison [3,4], which stabilizes the DNA-protein intermediate in the topoisomerase reaction, thus inhibiting DNA synthesis. About a 90% inhibition of DNA synthesis was detected after 24-h exposure of human colon carcinma cells to XK469 [5]. More recently, the topoisomerase IIβ poisoning effect was found to be strongly related to its cytotoxicity [4]. As a result of these activities, XK469 was selected for development by the National Cancer Institute.

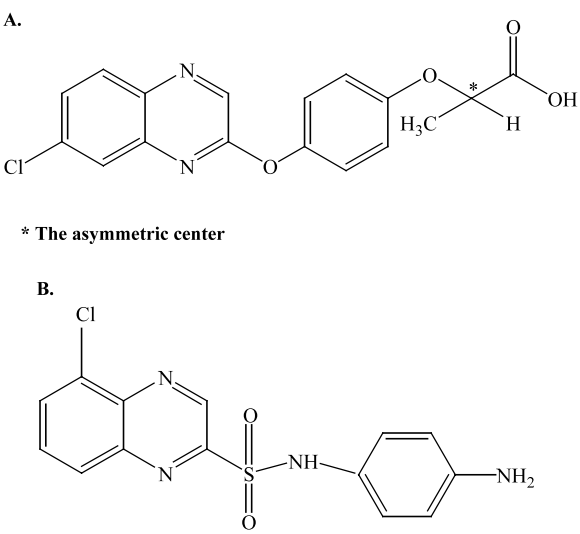

Fig. 1. Chemical structure of (A) XK469; (B) chloroquinoxaline sulfonamide.

XK469 possesses a chiral center, giving rise to the R(+)- and S(−)- enantiomers. The R(+)-enantiomer has been found to be approximately twice as effective as the S(−)-enantiomer in inducing the dose-dependent protein-DNA crosslinks [3]. A non-chiral high-performance liquid chromatography (HPLC) method for the analysis of XK469 has recently been reported and its preclinical pharmacokinetics of XK469 described [6], and the pharmacokinetics of the enantiomers has also been described in an abstract form using a normal-phase separation [7]. Two other chiral analytical methods, primarily designed for pharmaceutical applications have been documented [8,9], yet none is suitable for use in pharmacokinetic studies. We now describe the development of a simple chiral HPLC assay for the analysis of enantiomers of XK469 in plasma and urine and document the inversion of S(−)-XK469 to the R(+)-enantiomer in vivo.

2. Experimental

2.1. Chemical and reagents

S(−)-XK469 (NSC 698216), R(+)-XK469 (NSC 698215) and racemic XK469 (NSC 697887) were provided by the Drug Synthesis and Chemistry Branch, the National Cancer Institute (Rockville, MD). The internal standard chloroquinoxaline sulfonamide (CQS, Fig. 1B) was provided by Dr William Tong from Memorial Sloan-Kettering.

HPLC water was obtained from an E-pure water purification system (Barnstead, Dubuque, IA52081). Ammonium nitrate was purchased from Sigma (St Louis, MO). Methanol, acetonitrile (both HPLC grade), acetic acid, and ethyl acetate (reagent grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ketamine HCl injection (USP) was purchased from The Ohio State University Pharmacy (product of Ben Venue Labs, Inc, Bedford, OH, USA). Drug-free heparinized rat plasma was purchased from Harlan Bioproducts for Science, Inc (Indianapolis, IN, USA). All chemicals and reagents were used as received.

2.2. Instrumentation

A Schimadzu (Columbia, MD, USA) HPLC system, consisting of a SCL-10A*vp* system controller, two LC-10AT*vp* pumps, a SIL-10AD*vp* autoinjector, and a C-R5A Chromatopac recorder, was equipped with a Spectroflow 757 UV detector (ABI Analytical Kratos Division).

2.3. Chromatographic conditions

For the determination of XK469 concentration in rat plasma, an isocratic chromatographic condition was used. The mobile phase was composed of 30% methanol in 20 mM ammonium nitrate buffer, pH 4.0, and was delivered at a flow rate of 1.0 ml/min. Separation was achieved on a Chirobiotic™ T column, 5 μm particle size, 250 × 4.6 mm i.d. (ASTEC, Whippany, NJ), which was preceded by a Chirobiotic™ T guard cartridge, 20 × 4.0 mm. Ultraviolet detection was set at 330 nm. Chromatography was performed at ambient temperature.

For the analysis of XK469 in rat urine, because of the interference peaks, a gradient elution was employed with mobile phase A, consisting of 20 mM ammonium nitrate, pH 3.5, methanol (73:27, v/v) and mobile phase B consisting of 20 mM ammonium nitrate, pH 4.0, methanol (65:35, v/v). Acetic acid was used to adjust the pH of the mobile phase. The elution was initiated by 100% of A for 7 min, followed by a linear increase of B to 100% for 8 min. Then the % of B was linearly decreased to 0 in 10 min, at which time it was maintained for 7.5 min. The run time for each analysis was 32.5 min.

2.4. Sample preparations

2.4.1. Plasma

To a set of 13 × 100 mm glass tubes was each added an appropriate amount of the internal standard in 50 μl of methanol, followed by an addition of 0.2 ml of plasma sample or plasma spiked with various amounts of XK469 standards and 0.2 ml of acetic acid (2 N). One ml of acetonitrile was then used to precipitate the plasma proteins. Following centrifugation, the supernatant was transferred to a clean tube and the content was evaporated to dryness under a stream of nitrogen. The residue was reconstituted by an addition of 100 μl of the mobile phase, and a 50 μl aliquot was injected into the HPLC.

2.4.2. Urine

To 0.5 ml of rat urine sample was added a fixed amount of the internal standard, followed by an addition of 0.5 ml of acetic acid (2 N). The acidified urine sample was extracted by 4 ml of ethyl acetate. The ethyl acetate extract was evaporated to dryness by a stream of N_2, and the residue was dissolved in 100 μl of the mobile phase A.

2.5. Assay validation

Plasma samples for calibration curves were prepared by spiking 0.2 ml of drug-free rat plasma with various amounts of racemic XK469 and a constant amount of the internal standard. The linearity was evaluated in the concentration range of 0.2–2 μg/ml in 0.2 ml of rat plasma. The recovery was estimated by comparing the peak area ratio of the extracted analyte to the unextracted internal standard to that of the unextracted pairs at two concentrations of 0.5 and 2 μg/ml.

The within-day precision was determined in six replicates at concentrations of 0.2, 1.0, and 2.0 μg/ml on the same day. The between-day precision was evaluated in six replicates at the above concentrations on six different days. The mean values of concentrations and the coefficients of variation were calculated. The accuracy of the assay was evaluated by comparing the theoretical concentrations with the corresponding calculated concentrations.

2.6. Stability

Stability of XK469 in rat plasma was evaluated at −80, 4, and 37 °C at a concentration of 1 μg/ml. A tube containing 5 ml of rat plasma spiked with 1 μg/ml each of R(+)- and S(−)-XK469 was kept at −80 °C. At the time schedule of 0, 1, 2, 3, 4, 5, 6, 7 and 8 week, the plasma

was thawed at room temperature and duplicate samples of 0.1 ml were taken for drug analysis. Another 5 ml of rat plasma spiked with 1 μg/ml XK469 was kept at 4 °C refrigerator, and duplicate samples of 0.1 ml were removed at 0, 1, 3, 6, 10, 17, 21, and 30 day. The third rat plasma sample, 5 ml at 1 μg/ml, was incubated in a water bath shaker (Precision Scientific, Chicago, IL, USA) at 37 °C. Duplicate samples of 0.1 ml were taken at the time schedule of 0, 15, 30, 60, 120, 240, 360, 660, 1440 min for drug analysis.

2.7. Preliminary pharmacokinetic study of XK469 in the Fischer rat

A Fischer 344 rat weighing 262 g was used in this study. The right jugular vein of the Fischer 344 rat was cannulated under ketamine anesthesia (100 mg/kg) about 17 h prior to drug administration. The animal was kept in a metabolism cage beginning 1 h after the surgery and for the duration of the experiment. The rat was given S(−)-XK469 intravenously at 10 mg/kg. The compound was first dissolved in a small volume of sodium carbonate solution then diluted in normal saline, and the pH value of the dosing solution was approximately 8. The dosing solution, 0.5 ml, was given to the animal through the jugular vein cannula followed by flushing the cannula with 0.5 ml of normal saline. At the time schedule of 0 (predose),15, 30, 60, 120, 240, 480, 720, 1440, 1800, 2160, 2880, 3300 and 4320 min after dosing, approximately 0.25 ml each of blood was drawn from the same cannula and the lost fluid was replaced by flushing the cannula with an equal volume of normal saline. Plasma was separated immediately by centrifugation. The cumulative urine was collected for 72 h, and all of the samples were kept frozen at −78 °C until analysis. A CD_2F_1 mouse was given S(−)-XK469 i.v. at 10 mg/kg and at 0.5 h following dosing, the blood sample was removed by cardiac puncture. The plasma was separated by centrifugation and kept at −80 °C until analysis. A beagle dog was given S(−)XK469 at 100 mg/kg, and serial blood samples were removed from a femoral vein cannula for a subsequent pharmacokinetic study. The 1 h time sample was selected for drug analysis by the chiral HPLC method.

2.8. Data analysis

Plasma concentration–time profiles were analyzed by WinNonlin (Pharsight Corporation, Mountain View, CA, USA) software, version 3.0.

3. Results

3.1. HPLC assay and validation

Assay validation was performed in rat plasma. Representative HPLC chromatograms of an extract of drug-free rat plasma and an extract from 0.2 ml rat plasma standard spiked with 0.1 μg of each of the XK469 enantiomers and 0.15 μg of the internal standard are shown in Fig. 2. As shown, S(−)-XK469, R(+)-XK469 and the internal standard were baseline separated. There were no endogenous interference peak eluted at the same retention times of S(−)-XK469 (12.6 min), R(+)-XK469 (16.8 min) or of the internal standard (24.3 min). The limit of quantitation (LOQ) was set at 0.2 μg/ml in 0.2 ml of rat plasma based on 3 × signal to noise ratio.

Linearity was observed between the concentrations of 0.2–2 μg/ml monitored in 0.2 ml rat plasma, with the linear regression coefficients (r^2) of >0.99 achieved routinely. The within- and between-day accuracy and precision values of the assay are summarized in Tables 1 and 2. Briefly, the within-day coefficients of variation were 5.9, 5.0 and 3.1% for the S-isomer and 8.1, 4.2, 6.4% for R(+)-XK469 at 0.2, 1.0 and 2.0 μg/ml, respectively ($n=6$). The between-day coefficients of variation were 10.5, 5.3, and 1.9% for S(−)-XK469, and 10.9, 6.3 and 3.6% for R(+)-XK469, respectively ($n=6$).

3.2. Recovery and stability in rat plasma

The mean recovery values ($n=3$) were found to be 92.9 ± 2.6 and 95.8 ± 1.4% for S-isomer, and 93.4 ± 1.4 and 97.6 ± 0.6% for R-isomer at 0.5 and 2.0 μg/ml (all $n=3$), respectively.

It was found that both enantiomers of XK469 were stable in rat plasma at −80 °C, with no loss observed following eight freeze per thaw cycles for

Table 1
Within-day precision and accuracy in rat plasma ($n = 6$)[a]

Nominal concentration (μg/ml)	Observed concentration of S(−)-XK469 (μg/ml)[b]	CV (%)	Accuracy (%)	Observed concentration of R(+)-XK469 (μg/ml)[b]	CV (%)	Accuracy (%)
0.2	0.20 ± 0.01	5.9	100.7	0.20 ± 0.02	8.1	100.5
1.0	1.12 ± 0.06	5.0	111.6	1.09 ± 0.05	4.2	109.4
2.0	2.13 ± 0.07	3.1	106.5	2.05 ± 0.13	6.4	102.5

[a] 0.2 ml of rat plasma was used.
[b] Expressed as mean ± S.D.

Table 2
Between-day precision and accuracy in rat plasma ($n = 6$)[a]

Nominal concentration (μg/ml)	Observed concentration of S(−)-XK469 (μg/ml) [b]	CV (%)	Accuracy (%)	Observed concentration of R(+)-XK469 (μg/ml) [b]	CV (%)	Accuracy (%)
0.2	0.20 ± 0.02	10.5	101.3	0.22 ± 0.02	10.9	108.6
1.0	1.03 ± 0.05	5.3	102.8	1.00 ± 0.06	6.3	100.1
2.0	2.02 ± 0.04	1.9	100.9	2.04 ± 0.07	3.6	102.0

[a] 0.2 ml of rat plasma was used.
[b] Expressed as mean ± S.D.

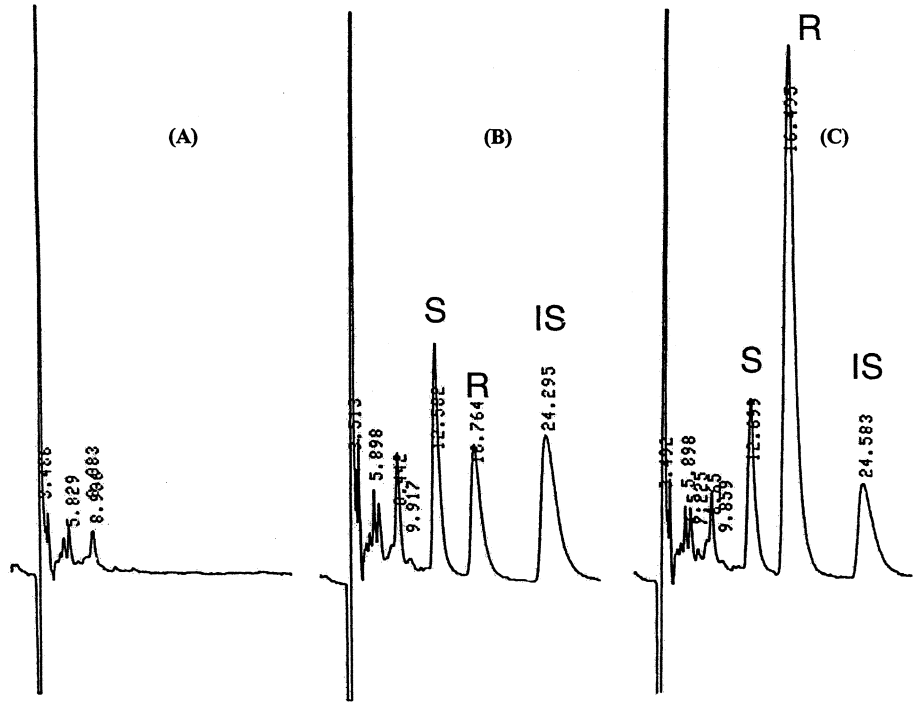

Fig. 2. A representative HPLC chromatogram of (A) drug-free rat plasma extract; (B) extract from rat plasma spiked with 0.5 μg/ml each of R(+)- and S(−)-XK469; and (C) extract from a plasma sample collected from a rat 5 min after dosing with i.v. S(−)-XK469 at 10 mg/kg.

8 weeks. There was no significant degradation of XK469 observed at 4 °C for 30 days, nor at 37 °C for 24 h.

3.3. Preliminary pharmacokinetics of XK469 in a Fischer 344 rat and detection of chiral inversion of S(+)XK469

A representative plasma concentration-time profile of XK469 in a rat given S(−)-XK469 at 10 mg/kg is shown in Fig. 3. As shown, a significant amount of R(+)-XK469 was detected in circulation up to 72 h after drug administration. The concentration of the S-enantiomer declined rapidly, and was below the detection limit after only 5–8 h.

The concentrations declined biexponentially with time, and the data were fitted to a two-compartmental pharmacokinetic model. The $t_{1/2\alpha}$ of S(−)-XK469 was about 5 min and $t_{1/2\beta}$ was 4.2 h, following an i.v. bolus dose of S(−)-XK469. The apparent $t_{1/2\alpha}$ of generated R(+)-XK469 was about 35 min and $t_{1/2\beta}$ was 24.7 h. The levels of the generated R(+)-XK469 in plasma were significantly higher than those of the parent S-isomer (Figs. 2 and 3) and the terminal $t_{1/2}$ was also significantly longer. In mouse plasma, at 0.5 h following i.v. dosing of S(−)-XK469 at 10 mg/kg, R(+)XK469 was also detected at higher level than the S-enantiomer (Fig. 4A). Similarly, in the beagle dog plasma 1 h after dosing with S(−)XK469 at 100 mg/kg, R(+)XK469 was also detected at significantly higher level than the S-isomer (Fig. 4B). These data indicated that S(−)XK469 undergoes rapid inversion to its R(+)isomer. No inversion was detected when R(+)XK469 or S(−)XK469 was incubated in rat plasma.

No detectable S(−)-XK469 was observed in the pooled 72 h rat urine, based on the detection limit of 0.2 μg/ml in 0.5 ml. About 7.0% of the administered S(−)XK469 dose was excreted as the R-enantiomer in 72 h urine.

4. Discussion

The primary objective of this study was to develop a chiral HPLC assay for the investigation of chiral pharmacokinetics of XK469. A non-chiral HPLC method was previously developed in our laboratory [6], and was incapable of characterizing the pharmacokinetic behavior of the individual XK469 enantiomers. A normal phase chiral method has recently been reported in an abstract form, with no assay validation data [7]. Others have reported a chiral HPLC method and a CE method for measurement of XK469 enantiomers, however, none of the assays have been validated, nor applied to biological samples [8,9]. The assay presented in this paper is a reliable, sensitive method for the analysis of animal samples and may be easily adopted in the planned clinical study of XK469.

One advantage of this assay is its simple sample preparation procedure. Due to the low pK_a value (< 3.0) of XK469 free acid (data from the National Cancer Institute), we first acidified the biological sample before extraction with organic solvent. This single step extraction afforded a recovery of over 90%. It was found that the low pH value of the mobile phase was essential for the separation of both XK469 enantiomers and the internal standard. At high pH values the retention times of the enantiomers are substantially decreased. The balanced methanol content in the mobile phase is also important; high water content caused an overlap between the peaks of R(+)-XK469 and the internal standard, but excessive methanol caused the interference from the biological matrix to overlap with the peaks of interest. After a number of attempts, we were able to optimize the chromatographic conditions as described above and successfully applied the assay to the preclinical pharmacokinetic study.

XK469 has been found to undergo a significant inversion from the S-enantiomer to the R-enantiomer in the mouse, rat, and dog, similar to that

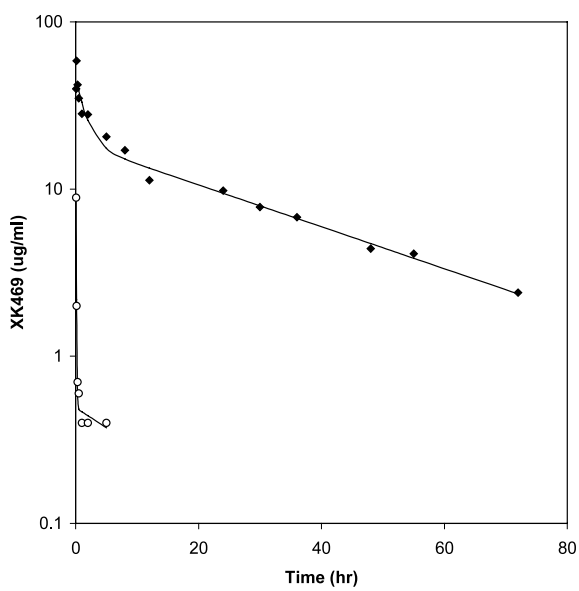

Fig. 3. A representative plasma concentration–time profile of S(−)-XK469 (○) and R(+)-XK469 (◆) in a rat given i.v. S(−)-XK469 at 10 mg/kg. The lines represent the computer fitted curve.

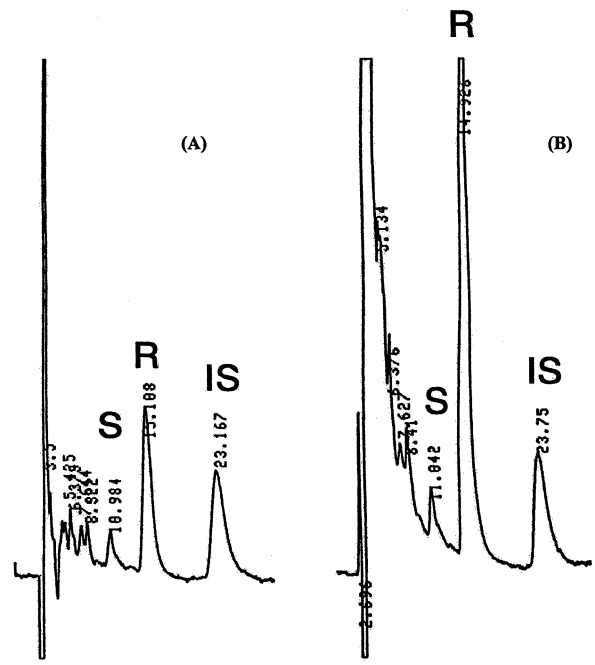

Fig. 4. A HPLC chromatogram of (A), extract from a plasma sample collected from a mouse 30 min after i.v. dosing of S(−)-XK469 at 10 mg/kg; and (B), extract from a plasma sample collected from a beagle dog 1 h after i.v. dosing of S(−)-XK469 at 100 mg/kg.

previously reported [7]. This inversion may be unidirectional, as no detectable S(−)-enantiomer was observed in rat plasma following administration of R(+)XK469 [10]. It has been shown that 2-arylpropionates possessing an asymmetric center alpha to the carbonyl function undergo unidirectional chiral inversion from the R to S enantiomer [11–13]. Being a phenoxy propionic acid, this inversion of XK469 is probably through a similar mechanism common to 2-arylpropionates. The change in the stereochemical designation is a result of the Cahn–Ingold–Prelog priority for XK469, and not a difference in the absolute configuration, however.

Since XK469 possesses activity against a broad spectrum of solid tumors and its mechanism of action has recently been elucidated [3,4], the more active R(+)-XK469 was selected for preclinical development and subsequently clinical evaluation.

5. Conclusion

A simple chiral HPLC assay for the quantitation of XK469 enantiomers has been developed and validated with a LOQ of 0.2 μg/ml in 0.2 ml rat plasma. By using this method, preliminary pharmacokinetics in Fischer 344 rats following an i.v. administration of S(−)-XK469 was investigated. It was found that the S(−)-XK469 was largely converted to R(+)-XK469 in the rat, mouse, and dog. The plasma $t_{1/2}$ of the S-enantiomer was significantly shorter than that of the R-enantiomer, at least in the rat.

Acknowledgements

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References

[1] T.H. Corbett, P.M. LoRusso, L. Demchik, C. Simpson, S. Pugh, K. White, J. Kushner, L. Polin, J. Meyer, J. Czarnecki, L. Heiburn, J.P. Horwitz, J.L. Gross, C.H. Behrens, B.A. Harrison, R.J. McRipley, G. Trainor, Invest. New Drugs 16 (1998) 129–139.
[2] P.M. LoRusso, R. Parchment, L. Demchik, J. Knight, L. Polin, J. Dzubow, C.H. Behrens, B. Harrison, G. Trainor, T.H. Corbett, Invest. New Drugs 16 (1999) 287–296.
[3] H. Gao, K.-C. Huang, F.F. Yamasaki, K.K. Chan, L. Chohan, R.M. Snapaka, Proc. Natl. Acad. Sci. USA 96 (1999) 12168–12173.
[4] R.M. Snapka, H. Gao, D.R. Grabowski, D. Brill, K.K. Chan, L. Li, G.C. Li, R. Ganapathi, Biochem. Biophys. Res. Commun. 280 (2001) 1155–1160.
[5] B. Subramanin, A. Nakeff, F.V. Valeriote, Proc. Am. Assoc. Cancer. Res., 40 (1999), (abstr).
[6] K.K. Chan, C. Jiang, H. Zheng, M. Chiu, J.M. Covey, AACR-NCI-EORTC, Washington, DC, 1999, (abstr).
[7] R.A. Wiegand, T.D. Doyle, C.K. Grieshaber, P.M. LoRusso, J. Kassab, B.R.Jasti, R.E. Parchment. AACR-NCI-EORTC, Washington, DC, 1999, (abstr).
[8] J. He, A.P. Cheung, E. Struble, E. Wang, P. Liu, J. Pharm. Biomed. Anal. 22 (2000) 583–595.
[9] A.P. Cheung, E. Struble, N. Nguyen, P. Liu, J. Chromatogr. A 870 (2000) 179–188.
[10] H. Zheng, K.K. Chan, J.M. Covey, P. Tosca, N. Turner. Proc. Am. Assoc. Cancer Res., 42 (2001), (abstr).
[11] E.J.D. Lee, K. Williams, R. Day, G. Graham, D. Champion, Br. J. Clin. Pharm. 19 (1985) 669–674.
[12] J.M. Mayer, Acta Pharm. Nord. 2 (3) (1990) 197–216.
[13] R.D. Knihinicki, R.O. Day, K.M. Williams, Biochem. Pharmacol. 42 (1991) 1095–1911.