Cancer Chemother Pharmacol (2006) 58: 561–569
DOI 10.1007/s00280-006-0211-z

ORIGINAL ARTICLE

Yilong Zhang · Yousheng Hua · Doris M. Benbrook
Joseph M. Covey · Guowei Dai · Zhongfa Liu
Kenneth K. Chan

High performance liquid chromatographic analysis and preclinical pharmacokinetics of the heteroarotinoid antitumor agent, SHetA2

Received: 25 May 2005 / Accepted: 7 February 2006 / Published online: 14 March 2006

Abstract *Background*: SHetA2 {[(4-nitrophenyl)amino][2,2,4,4-tetramethylthiochroman-6-yl)amino]methanethione], NSC 726189} is a sulfur-containing heteroarotinoid, which selectively inhibits cancer cell growth and induces apoptosis without activation of nuclear retinoic acid receptors (RARs). The objective was to develop and validate a HPLC/UV method for the determination of SHetA2, and study the pharmacokinetics of SHetA2 in the mouse. *Methods*: SHetA2 and the internal standard, methylated XK469 (MeXK469) were isolated from 0.2 ml of mouse plasma by solid phase extraction. The analytes were separated on a narrow-bore C18 column, with the mobile phase consisting of 60% acetonitrile in water at a flow rate of 0.2 ml/min. UV detection was set at 341 nm. Pharmacokinetic studies of SHetA2 were carried out in mice following i.v. bolus dose at 20 mg/kg and oral administrations at 20 and 60 mg/kg. *Results*: The standard curves were linear between 25 and 2,500 nM and the lower limit of quantification (LLOQ) was 25 nM. The within-run coefficients of variation (CVs) were 11.1% at 10, 9.4% at 100, and 5.2% at 2,500 nM and the respective between-run CVs were 10.9, 3.1, and 1.5% (all $n = 5$). The recovery was 85.8% for SHetA2 and 80.6% for MeXK469. Following i.v. bolus dose, plasma concentrations of ~10 μM were achieved at 5 min in mice and declined biexponentially with detectable levels at 60 h. The data were fitted with a two-compartment model, which gave a mean initial $t_{1/2}$ of 40 min and terminal $t_{1/2}$ of 11.4 h ($n = 6$). The total body clearance was ~1.81 l/h/kg. The volume of distribution at steady state (V_{dss}) was 20.8 l/kg. Plasma protein binding was found to be 99.3–99.5% at low micromolar concentrations. Plasma concentration data for the i.v. and p.o. doses were also fitted interactively to a two-compartment deconvolution model. From this result, oral bioavailability values of 15% at 20 mg/kg and 19% at 60 mg/kg were obtained. *Conclusions*: A highly sensitive HPLC/UV method for the quantification of SHetA2 in plasma has been developed to support pharmacokinetics of SHetA2 in the mouse. Pharmacokinetic behaviors of this drug appear to be favorable for future development.

Keywords Preclinical pharmacokinetics · Heteroarotinoid antitumor agent · SHetA2 · Hplc

Y. Zhang · Y. Hua · G. Dai · Z. Liu
College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA

D. M. Benbrook
Department of Obstetrics and Gynecology and Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA

J. M. Covey
The National Cancer Institute, Rockville, MD 20852, USA

K. K. Chan (✉)
Colleges of Pharmacy and Medicine and Public Health, The Ohio State University, Columbus, OH 43210, USA
E-mail: chan.56@osu.edu
Tel.: +1-614-2920627
Fax: +1-614-2927766

Introduction

SHetA2 {[(4-nitrophenyl)amino][2,2,4,4-tetramethylthioochroman-6-yl)amino] methane-thione], NSC 683864} is a novel drug that regulates growth, differentiation and apoptosis in cancer cells with minimal effects on normal cells in vitro [1, 2]. These differential effects are maintained in animal models of ovarian cancer, teratogenesis and skin irritancy [3, 4]. SHetA2 belongs to the class of drugs called heteroarotinoids (Hets), which were originally developed as retinoids targeted at the nuclear retinoic acid receptors (RARs) [5]. All Hets contain a heteroatom in the cyclic ring of the classical arotinoid structure of retinoids which reduces the toxicity 1,000-fold [6]. While conformationaly restricted two-atom linker Hets bind

and activate the nuclear RARs, the three-atom linker flexible Hets (Flex-Hets) do not activate the RARs or exhibit any of the classical retinoid toxicities or teratogenic effects [1, 2, 4]. SHetA2 was chosen as the lead compound from a series of Flex-Hets because it exhibited the most potent apoptosis activity in cancer cells, while retaining the differential effect on normal cells [1, 2, 7]. The mechanism of SHetA2 apoptosis occurs through the intrinsic mitochondrial pathway, while the mechanism of SHetA2 differentiation appears to occur through regulation of gene expression [7, D. M. Benbrook, personal communication].

The ultimate biological outcome of SHetA2 treatment in vitro is dependent on the dose and duration, as well as, the status of the cell. Concentrations of 3 μM or higher SHetA2 are required to induce apoptosis in cancer cell lines within 24 h [7, D. M. Benbrook, personal communication]. If the drug is replenished every 2–3 days for 2 weeks, however, apoptosis can be induced with 1 μM SHetA2 in organotypic cultures of cancer cell lines [1]. Differentiation, however, can also be observed in these same organtoypic cultures treated with 1 μM SHetA2 [1]. The organotypic cultures consist of three-dimensional clumps of cells that have grown from single cells suspended in collagen. Within the same SHetA2-treated organotypic culture, some of the clumps exhibit glandular differentiation, while other clumps consist of cells undergoing apoptosis suggesting that the status of the cell can determine the ultimate fate upon treatment with 1 μM. At higher concentrations, however, apoptosis dominates over differentiation regardless of the cells status.

SHetA2 also induced glandular differentiation in ovarian cancer xenografts in vivo when administered at 10 mg/kg/day [3]. Apoptosis was also increased in the xenografts from the SHetA2 treated animals, but the increase in percentage of cells undergoing apoptosis in tumors from the treated versus untreated control animals was not statistically significant [3]. No evidence of SHetA2-induced bone, skin or liver toxicity could be detected in this animal model [3]. Additional xenograft models performed in the National Cancer Institute's Rapid Access to Intervention Development (RAID) program did not detect tumor growth inhibition activity at 10 mg/kg/day, but tumor growth inhibition activity was observed at 60/mg/kg/day in one animal model [M. Hollingshead, personal communication]. Skin irritancy was not observed in an animal model when 0.01, 0.1, 1 and 10 mM SHetA2 were applied topically for 4 days [3]. Teratogenic effects were not observed at single dose administration of 2.5, 10 and 25 mg/kg SHetA2 in pregnant mice [4].

To appropriately design future clinical trials that will achieve plasma concentrations of SHetA2 sufficient to induce apoptosis in cancer and pre-malignant cells, an accurate detection method and preclinical pharmacokinetic study of SHetA2 is needed. In the present study, we developed and validated a HPLC/UV method for the determination of SHetA2, and investigated the stability and preclinical pharmacokinetics of SHetA2 in the mouse.

Materials and methods

Chemicals and reagents

SHetA2 and the internal standard (IS), methylated XK469 (MeXK469, Fig. 1) were provided by Drug Synthesis and Chemistry Branch, Division of Cancer treatment, the National Cancer Institute (Bethesda, MD, USA). C18 reversed-phase resin (octadecyl C18, 40 μm) was purchased from J. T. Baker (Philipsburg, NJ, USA). HPLC grade methanol and acetonitrile (ACN) were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade water was obtained from an E-pure water purification system (Barnstead, Dubuque, IA 52081). Mouse and rat plasma were purchased from Harlan Bioproducts (Indianapolis, IN). Dog plasma was a gift from Dr. James Dalton (The Ohio State University). Human plasma was obtained from the American Red Cross (Columbus, OH). Trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Instrumentation

A Shimadzu (Columbia, MD, USA) HPLC system, consisting of a SIL-10AD system controller, LC-10AD pumps, a SIL-10Advp autosampler, and a SPD-M10A PDA detector were used. The PDA detector was set to give the spectrum of 190–800 nm in every 0.64 s. All operations were controlled by EZstart 7.2 software (Columbia, MD, USA) in a Windows NT system.

For on-line HPLC-UV-MS analysis, a Finnigan LCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) coupled to the Shimadzu HPLC system described above was used. The mass spectrometer was operating with a background helium pressure of 1.75×10^{-3} Torr, a typical electrospray needle voltage of 4.5 kV, a sheath gas flow of 90 (arbitrary unit), and an auxiliary nitrogen gas flow of 45 (arbitrary unit), and a heated capillary

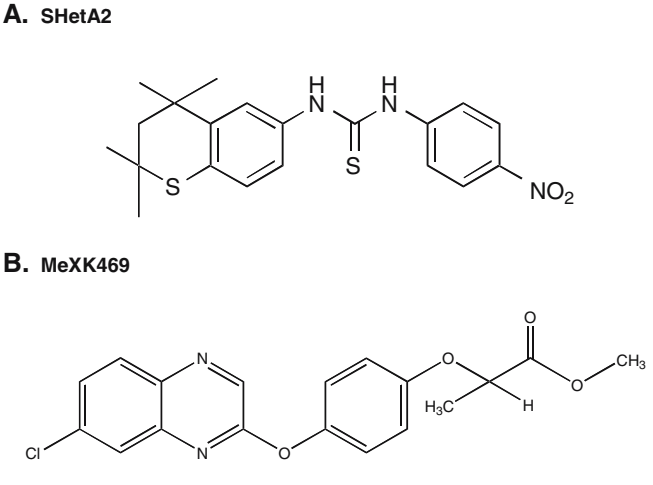

Fig. 1 Structures of SHetA2 (**a**) and MeXK469 (**b**)

temperature of 200°C. Triple play mode [8–10] was chosen for analyzing the reconstituted solutions obtained from sample extracts. Full mass scan was in the range of 100–750 Da, and zoom scan was performed on the most intense peak from the full scan. The mass spectrometer was tuned to its optimum sensitivity by infusion of SHetA2. All operations were controlled by Finnigan Xcaliber software in a Windows NT 4.0 system.

Chromatographic conditions for sample analysis

For HPLC-UV, a 5 μm, 2.1×250 mm^2 VYDAC C18 column (Grace, Columbia, MD) was used for drug analysis. The mobile phase consisted of 60% of ACN in water. The total run time for each analysis was 25 min at a flow rate of 0.2 ml/min. Ultraviolet detection was set at 341 nm. Chromatography was performed at ambient temperature. Peak areas for all analytes were obtained using electronic integration.

For on-line HPLC-UV-MS, the components were separated on the same VYDAC C18 column with a gradient system consisting of 10% ACN in 0.01%TFA as mobile phase A and 95% ACN in 0.01% TFA as mobile phase B. The elution was initiated from 100% A to 65% B in 5 min, to 100% B in 20 min, then maintained at 100% B for 5 min, returned to 100% A in 0.5 min, and re-equilibrated at 100% A for 14.5 min. All gradient elution were linear.

Sample preparation

Aliquots of mouse plasma (0.2 ml) were spiked with various amounts of SHetA2 standards and a constant amount of the IS and then were loaded onto pre-conditioned solid phase extraction (SPE) columns. After washing with 1 ml of 20% (v/v) ACN in water, the analytes were eluted with 2 ml of 100% ACN. The eluant was collected and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 100 μl of mobile phase (60% of ACN in water) and the solution was centrifuged at 15,900*g* for 10 min. A 50 μl aliquot was injected into the HPLC for analysis. Since the aqueous solubility of SHetA2 is low and believed to be in micromolar, a control experiment was performed, where the drug solution was made in 30% ACN in PBS or plasma to 10 μM and no precipitation was observed or after centrifugation.

Assay validation

For calibration curves, 0.2 ml of drug-free mouse plasma was spiked with 20 μl of ACN containing appropriate concentrations of SHetA2 standard and 20 μl of 2.8 μM IS to result in final concentrations of SHetA2 of 0.025, 0.0625, 0.125, 0.25, 0.625, 1.25, and 2.5 μM. The samples were processed and assayed by HPLC described as above. Calibration curves were constructed using peak area ratios of the analyte to the IS plotted against analyte concentrations. The linearity was evaluated in the concentration range of 25–2,500 nM (0.025–2.5 μM) in 0.2 ml of mouse plasma.

The within-run precision of this assay was evaluated at 0.025, 0.25, 2.5 μM for SHetA2. Five replicates were determined at each concentration. The between-run precision was evaluated at the above concentrations on five different days. The mean values of concentrations and the coefficients of variation (CVs) were calculated. The accuracy of the assay was evaluated by comparing the theoretical concentrations with the corresponding calculated concentrations.

Stability

The stability of SHetA2 in mouse, rat, dog and human plasma were evaluated at 37, 22, and 4°C. Briefly, aliquots of plasma were spiked with ShetA2 to a final concentration of 2.5 μM and then incubated at 37°C in a water bath (Precision Scientific, Chicago, IL, USA). After 0, 2, 4, 6, 8, 12, 18, and 24 h of incubation, duplicate aliquots of 0.2 ml each were removed and processed for SHetA2 analysis. Similar studies were performed in a 4°C refrigerator and in a 22°C water bath.

The stability of SHetA2 was also evaluated at 4 and 37°C in 0.067 M sodium dihydrogen phosphate (NaH_2PO_4) buffer (PH 7.4). Due to the low aqueous solubility of SHetA2, 30% ACN was added to the phosphate buffer. During incubation at the appropriate temperatures, duplicate aliquots of 0.2 ml each were removed at 0, 2, 4, 6, 8, 12, 18, and 24 h and the samples were processed for SHetA2 analysis as described above.

Characterization of decomposed products

The decomposition of SHetA2 in mouse plasma was investigated at 37°C for 24 h at 250 μM. Following incubation in a water bath (Precision Scientific, Chicago, IL, USA), aliquots of 0.2 ml were removed and treated with 0.8 ml ice-cold ACN. After centrifugation at 15,900*g* for 10 min, the supernatant was collected and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1.0 ml of the HPLC mobile phase and the solution was centrifuged at 15,900*g* for 10 min. A 50 μl aliquot was injected into the HPLC-UV-MS for analysis.

Protein binding

Plasma protein binding of SHetA2 was determined using the ultracentrifugation method. Briefly, an appropriate amount of SHetA2 was added to mouse plasma to achieve drug concentrations of 10, 50, and 125 μM. After vortexing for 30 s and standing at room temperature for

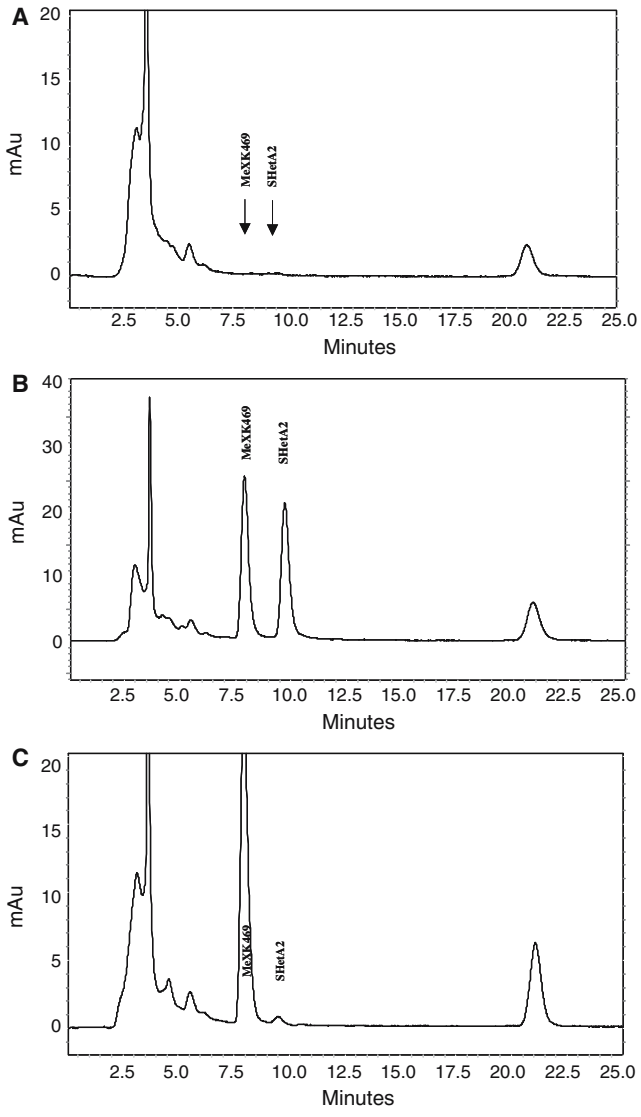

Fig. 2 A representative HPLC chromatogram of **a** mouse blank plasma, **b** extract from mouse plasma pre-spiked with the internal standard (IS) (2.8 μM) and SHetA2 (1.25 μM), and **c** extract from mouse plasma pre-spiked with the IS (2.8 μM) and SHetA2 (0.025 μM) (LLOQ)

10 min, a 0.2 ml aliquot of plasma was removed from each sample for analysis of the total drug concentration. The remaining plasma samples were placed in 3.5 ml Polyallomer Bell-top centrifuge tubes (Beckman, Fullerton, CA) and sealed. The samples were centrifuged at 540,000*g* for 22 h at 4°C. The tube caps were then carefully opened and the plasma was found to separate into three distinct layers, top white layer (lipoproteins), mid clear layer (protein-free plasma), and bottom solution layer (proteins). The mid layer was collected via a syringe for analysis of free drug concentration. Protein binding was calculated by the following equation:

$$\%\text{Protein bound} = \frac{\text{Total SHetA2} - \text{Free SHetA2}}{\text{Total SHetA2}} \times 100$$

Pharmacokinetic study of SHetA2 in the mouse

CD2F1 mice (20.0–27.8 g, Harlan, Indianapolis, IN) were used in this study and six animals per time point were used. All animal procedures were performed according to a protocol in compliance with The Ohio State University Laboratory Animal Resources (ULAR) policies, which adhered to the guidelines and "Principles of Laboratory Animal Care" by National Institutes of Health. For intravenous administration, SHetA2 was first dissolved in PEG400 and ethanol mixture (v/v, 80/20) then diluted with normal saline. The final ratios of PEG400, ethanol, and saline in dosing solution were 57.1, 14.3, and 28.6%. Approximately 100 μl (adjusted by body weight) dosing solution was injected through the tail vein to result in an i.v. bolus dose of 20 mg/kg. The blood was removed by cardiac puncture at the time schedule of 0 (pre-dose), 0.08, 0.15, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 36, 48, and 60 h after dosing and was mixed with 3% (v/v) sodium heparin. For oral administration, SHetA2 was dissolved in sesame oil. Approximately 100 μl (adjusted by body weight) dosing solution was administered by gavage to result in a dose of 20 or 60 mg/kg. The blood was removed by cardiac puncture under CO_2 anesthesia at 0 (pre-dose), 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 36, and 48 h after dosing (up to 24 h for 20 mg/kg and 48 h for 60 mg/kg). The blood samples were centrifuged at 1,000*g* at room temperature for 5 min and the supernatant of each was collected and kept at −80°C until analysis. Plasma concentration-time data were analyzed by WinNonlin computer software (Pharsight, Mountain View, CA) using appropriate pharmacokinetic models.

Table 1 Within- and between-run precision and accuracy of SHetA2 in mouse plasma ($n = 5$)[a]

	Within-run			Between-run		
Nominal concentration (nM)	Observed concentration (nM)[b]	CV (%)	Accuracy (%)	Observed concentration (nM)	CV (%)	Accuracy
25	26.4 ± 3.0	11.1	106.1	22.7 ± 2.5	10.9	89.5
250	247.4 ± 23.2	9.4	99.2	251.6 ± 7.5	3.0	100.9
2,500	2459.6 ± 127.9	5.2	98.6	2465.1 ± 37.7	1.5	98.9

[a]0.2 ml of mouse plasma was used
[b]Expressed as mean ± SD

Table 2 Half-lives of SHetA2 in vitro at different media and temperatures

Medium/temperature	Half-lives (h)	
	37°C	22°C
Mouse plasma	12.7	42.4
Rat plasma	13.0	68.6
Dog plasma	12.7	63.3
Human plasma	12.7	45.6
PBS	54.7	–

Results

HPLC assay and validation

Assay validation was performed in mouse plasma. Representative HPLC chromatograms of an extract from drug-free mouse plasma and an extract from 0.2 ml mouse plasma spiked with SHetA2 at 1.25 μM and the IS at 2.8 μM are shown in Fig. 2. As indicated, there were no interfering peaks at the region of the analyte and the IS. SHetA2 and the IS were baseline separated, with the retention times of 9.7 and 7.9 min, respectively.

The relationship between peak area ratio and SHetA2 concentrations between 0.025 and 2.5 μM was linear, with a regression coefficient (r^2) > 0.99 achieved routinely. The lower limit of quantification (LLOQ) was 0.025 μM based on 3× signal-to-noise ratio using 0.2 ml of mouse plasma (Fig. 2).

The within-run precision and accuracy of SHetA2 was evaluated at three different concentrations with five replicates. Results are shown in Table 1. The within-run CVs were 11.1% at 0.025, 9.4% at 0.25, and 5.2% at 2.5 μM (all $n = 5$).

The between-run precision and accuracy of SHetA2 was determined at the same three concentrations on five different days. The results are shown in Table 2. The between-run CVs were 10.9% at 0.025, 3.1% at 0.25, and 1.5% at 2.5 μM (all $n = 5$)

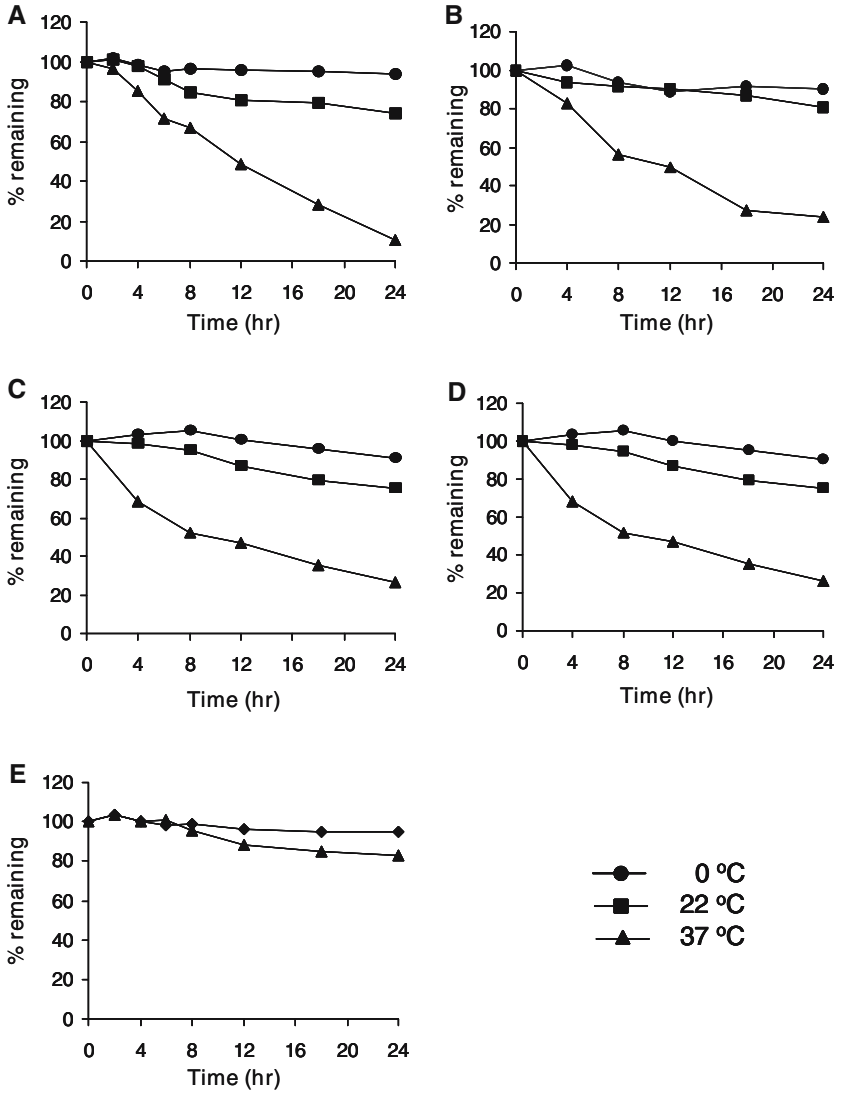

Fig. 3 Stability of SHetA2 in **a** mouse plasma, **b** human plasma, **c** rat plasma, **d** dog plasma, and **e** aqueous-ACN buffer

Stability of SHetA2 in mouse, rat, dog and human plasma

SHetA2 was found to be stable in mouse plasma at 4°C, with no significant loss after 24 h (Fig. 3a). However, at 22 and 37°C, SHetA2 concentrations were found to decline monoexponentially with half-lives of 42.4 and 12.7 h, respectively, as estimated by log linear regression (Fig. 3a). Similar results were obtained with rat, dog and human plasma (Fig. 3b–d). Comparison of SHetA2 plasma half-lives across the animal species at different temperatures is shown in Table 2. SHetA2 in aqueous buffer (0.067 M, pH 7.4, NaH_2PO_4 with 30% ACN) was found to be stable at 22°C with no significant decrease in concentration at 24 h (Fig. 3e and Table 2). However, the compound was found to undergo a first-order decay in this buffer at 37°C with a half-life of 54.7 h. The data suggest that SHetA2 degradation in buffer and in plasma increases with an increase in temperature. The relative contributions of spontaneous chemical decomposition and/or enzyme-mediated metabolism have not been determined, however.

Characterization of decomposed products

The decomposition of SHetA2 in mouse plasma was examined in more detail by HPLC-UV-MS. The HPLC-UV chromatogram (341 nm) of SHetA2 in mouse plasma at time 0 is shown in Fig. 4a. Following incubation at 37°C for 24 h, a new peak was observed with retention time of 11 min (Fig. 4b). The average mass spectrum of the peak between 11.1 and 11.3 min (to cover a time delay of 0.4 min between the UV detector and the mass spectrometer) gave a base peak at m/z 222.2 (Fig. 5a). This decomposition product was possibly produced by the cleavage of the thiourea connected to the fused ring moiety. The structure assignment is supported by the tandem mass spectrum of the ion at m/z 222 (Fig. 5b), which shows a fragment ion at m/z 166. Both ions were also found as fragment ions from the parent compound SHetA2 (Fig. 6). The proposed fragmentation pathways of SHetA2 are shown in Fig. 7. The species at m/z 222 appears to be produced from SHetA2 both as a metabolite/degradation product in plasma and as a fragment ion during MS analysis. The ion at m/z 166 is further produced from the ion at m/z 222 by a neutral loss of C_4H_8.

Protein binding

SHetA2 was found to be highly bound to mouse plasma proteins. The extent of binding was found to be 99.3 ± 0.1, 99.5 ± 0.1, and $99.4 \pm 0.1\%$ (mean ± difference, all $n = 2$) for SHetA2 at 10, 50, and 125 μM, respectively. There was no concentration dependence of binding over this concentration range in mouse plasma. A control experiment with 10 μM SHetA2 in 30% ACN in PBS was performed and no precipitation or significant change in concentration was found after centrifugation.

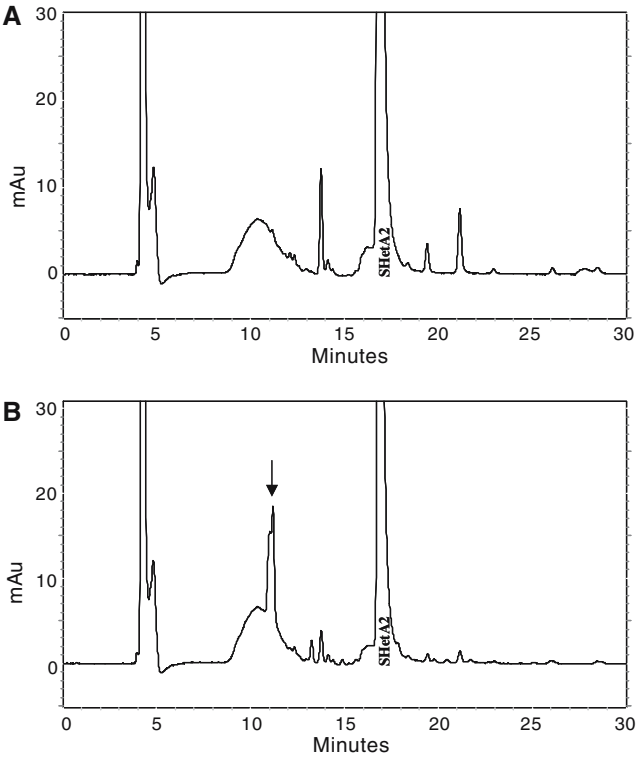

Fig. 4 LC-UV chromatograms of SHetA2 in mouse plasma **a** without incubation, and **b** following incubation at 37°C for 24 h (*arrow* shows the new peak after incubation)

Pharmacokinetics of SHetA2 in the mouse

Mean plasma concentration-time profiles of SHetA2 following i.v. and oral administrations to CD2F1 mice are shown in Fig. 8. Following a 20 mg/kg i.v. dose, plasma concentration reached ~10 μM at 5 min and then declined biexponentially with time. The compound was detectable in plasma for up to 60 h. The data were fitted to a two-compartment model using WinNonlin and the relevant pharmacokinetic parameters were computed (Table 3). The $t_{1/2\alpha}$ of SHetA2 was 40 min and $t_{1/2\beta}$ 11.4 h. The mean total body clearance was 1.81 l/h/kg and the volume of distribution at steady state (V_{dss}) was 20.8 l/kg.

SHetA2 was found to be unstable in mouse plasma at 37°C with a half-life of 12.7 h. Based on this data, clearance of SHetA2 is likely due to degradation/metabolism. Thus, the observed total clearance value of 1.81 l/h/kg is probably mainly attributed to this process. The large volume of distribution at steady state compared to mouse plasma volume (20.8 l/kg vs. 0.1 l/kg) indicates extensive tissue distribution and/or tissue binding.

Following p.o. administrations at 20 and 60 mg/kg, mean maximal plasma concentrations of 0.79 and 2.35 μM were observed at 2 and 3 h, respectively. The concentrations were detectable up to 24 h at 20 mg/kg and 48 h at 60 mg/kg. The oral bioavailability was estimated to be 15% at 20 mg/kg and 21% at 60 mg/kg, using the AUC and non-crossover method.

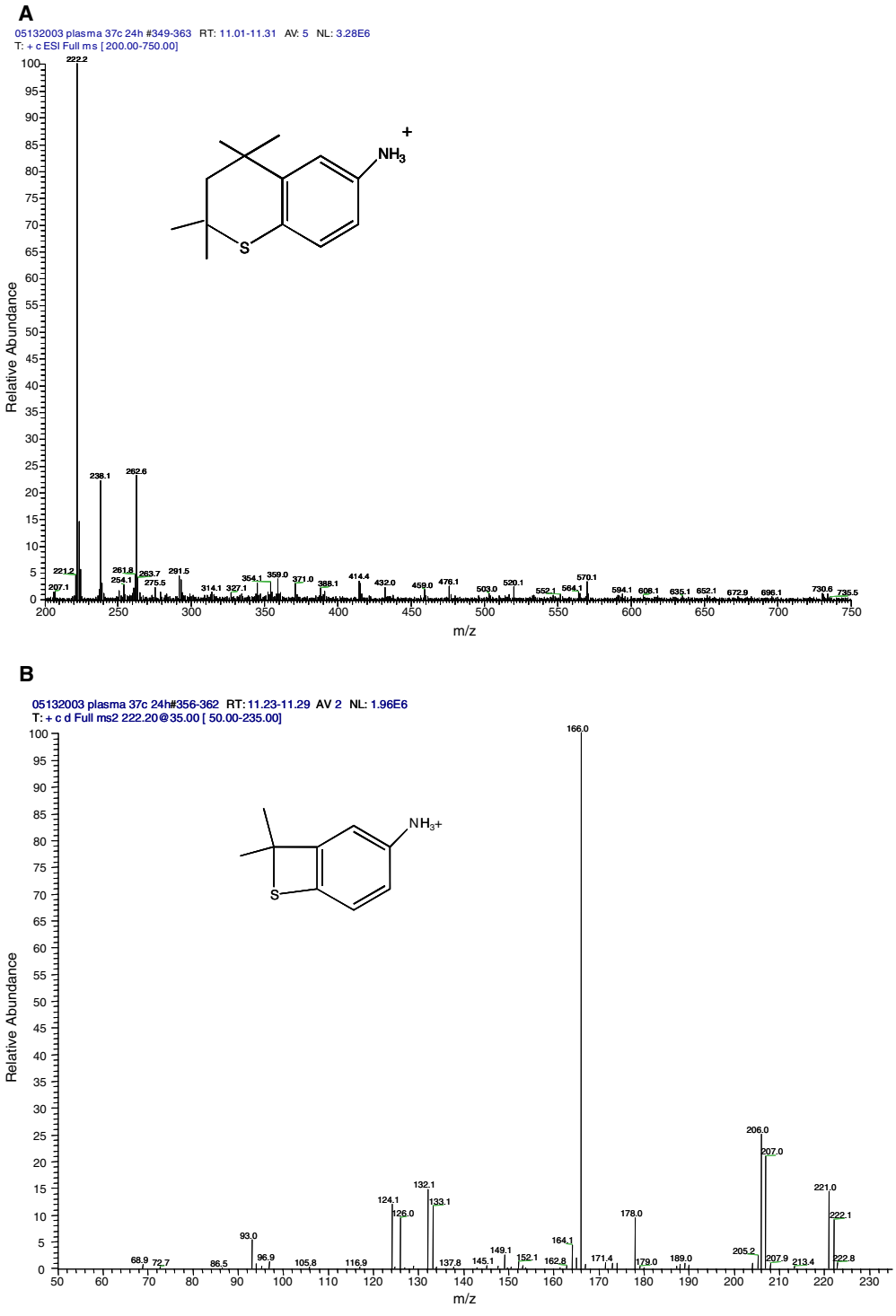

Fig. 5 Average mass spectrum of SHetA2 degradation product at the peak between 11.2 and 11.7 min in mouse plasma (**a**), and its tandem mass spectrum using the ion at m/z at 222 (**b**)

Plasma concentration-time data from both i.v. and all p.o. routes were also fitted simultaneously to a two-compartment oral model with deconvolution techniques [11], using WinNonlin and the relevant pharmacokinetic parameters are also shown in Table 3. The terminal half-life was 11.4 h, identical to that of i.v. dose. The oral bioavailabilities were estimated to be 14.5 and 19.0% for 20 and 60 mg/kg doses, respectively, which well agree with the individual fitted values. The absorption rate constant was 0.6 1 h^{-1}, and rather rapid. This model showed a negligible lag time at 20 mg/kg dose, but shows a small lag time of 0.15 h at 60 mg/kg.

Discussion

The pharmacokinetic results of this study demonstrate that SHetA2 has a long terminal half-life in the mouse

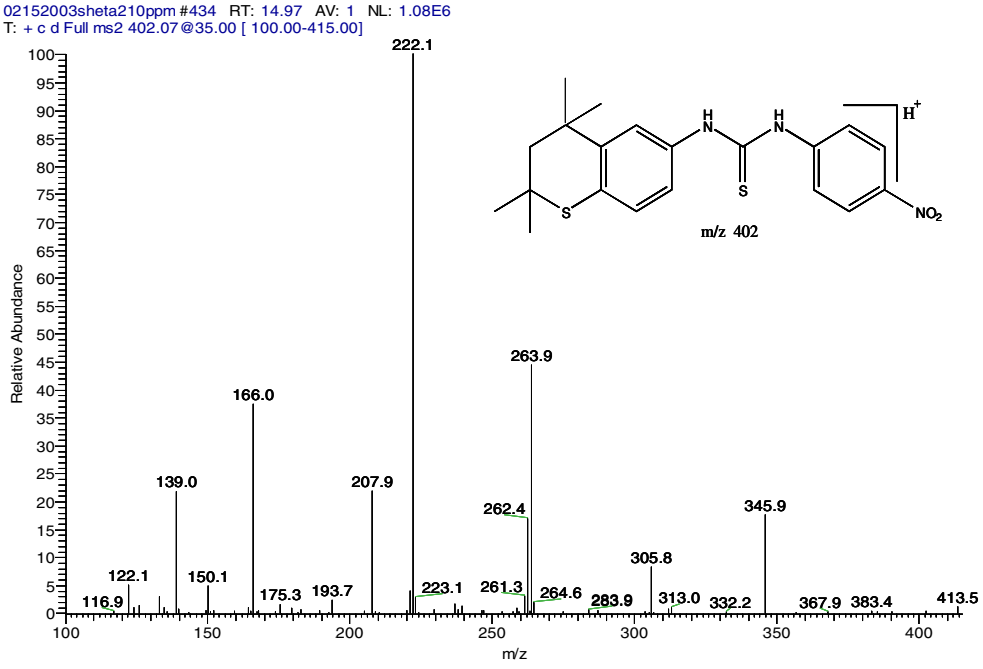

Fig. 6 Product ion mass spectrum of SHetA2 using $[MH]^+$

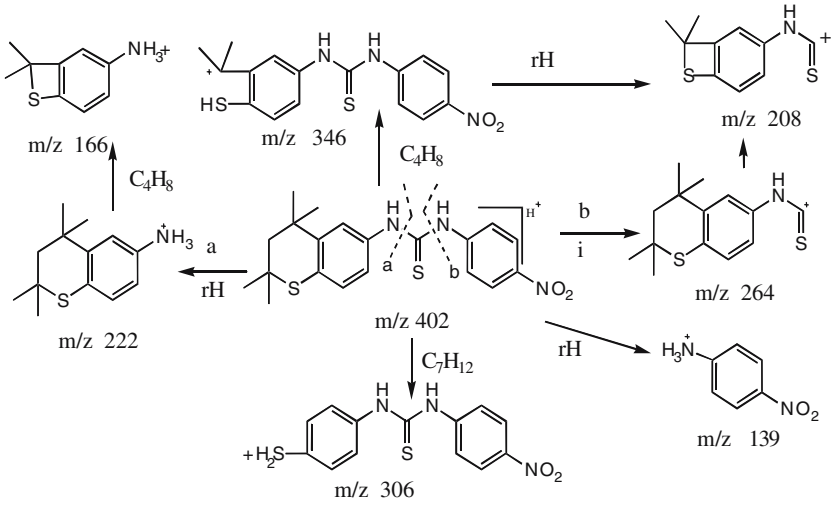

Fig. 7 Proposed fragmentation pathways of SHetA2

and is detectable in plasma for up to 60 h following intravenous administration. The in vivo and in vitro terminal half-lives of the drug are comparable and the stability half-lives in plasma were similar across four species. This indicates that dosing of SHetA2 at once per day may be sufficient in clinical trials. To maximize the activity of SHetA2 in cancer patients, it will be important to target plasma concentrations capable of inducing apoptosis in cancer cells, and thus irreversibly eliminating them from the body. The peak plasma concentrations achieved in mice after oral administration (0.79 and 2.35 μM) fell within the range of IC_{50} values of the growth inhibition curves for SHetA2 in the National Cancer Institute 60 cell line screen (0.37–4.6 μM) [3]. SHetA2 induction of apoptosis in cancer cells was observed in three different types of experimental settings: (1) monolayer cultures of cancer cell lines treated with 10 μM for 24 h [7], (2) three-dimensional organotypic cultures incubated with 1 μM SHetA2 that was replenished on Monday, Wednesday and Friday for 2 weeks [1], and (3) ovarian cancer xenograft tumors from animals treated with 10 mg/kg/day SHetA2 on 5 weekdays per week for 4 weeks [3]. These pharmacokinetic profiles therefore indicate that sufficient concentrations of SHetA2 shown to induce apoptosis in vitro, can be achieved and sustained in vivo, and that clinical trials can be designed to target exposure to apoptosis-inducing concentrations.

The volume of distribution for SHetA2 at steady state (20.8 l/kg) is much larger than the volume of mouse plasma (0.1 l/kg) and also much larger than the sum of the volumes of blood and most organ tissues indicating extensive tissue uptake and/or tissue binding.

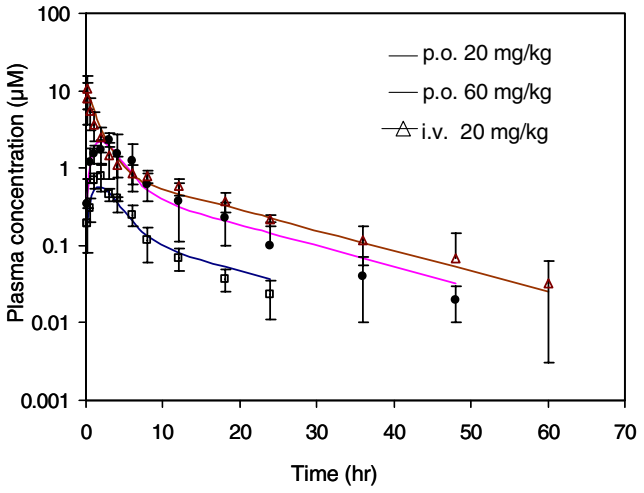

Fig. 8 Plasma concentration-time profiles of SHetA2 in the mouse following i.v. administration at 20 mg/kg and p.o. administrations at 20 or 60 mg/kg. The fitted curves represent simultaneously fitting of p.o. and i.v doses with each point representing mean ± SD ($n = 6$)

This is favorable for increasing exposure of cancer and premalignant cells to optimal concentrations of drug. Since the total body clearance of SHetA2 is larger (1.81 l/h/kg) than glomerular filtration rate in mice (0.84 l/h/kg), but smaller than liver blood flow, this suggests that clearance could be mainly attributed to degradation/metabolism. This contention is supported by the similar in vitro and in vivo half-lives of the drug. The major degradation pathway is likely to be cleavage of the thiourea bond adjacent to the fused ring system, possibly in part by non-enzymatic hydrolysis. Further work in the elimination kinetics and metabolism is necessary.

Table 3 Relevant pharmacokinetic parameters of SHetA2 in mouse plasma

PK parameter	i.v. Administration	p.o. Administration		Simultaneous fitting
	20 mg/kg	20 mg/kg	60 mg/kg	
$C_{5\ min}/C_{max}$ (μM)	10.9	0.79	2.35	-
T_{max} (h)	–	2	3	–
T_{lag}^{a} (h)	–	–	–	0.15
k_a (h^{-1})	–	0.65	0.53	0.61
$t_{1/2\alpha}$ (h)	–	1.07	1.31	1.13
α (h^{-1})	1.04	0.61	0.467	0.71
$t_{1/2\alpha}$ (h)	0.67	1.14	1.48	0.98
β (h^{-1})	0.061	0.067	0.074	0.06
$t_{1/2\beta}$ (h)	11.4	10.4	9.25	11.41
MRT (h)	11.5	7.16	9.67	–
Cl (l/h/kg)	1.81	–	–	–
V_{dss} (l/kg)	20.8	–	–	–
AUC∞ (μM h)	28.2	4.28	17.5	–
F_1^{b}		15.2		14.5
F_2^{c}	–	–	20.7	19.0

[a] t_{lag} lag time for 60 mg/kg group only since there was no lag time in p.o. 20 mg/kg group
[b] F_1 bioavailability of 20 mg/kg group
[c] F_2 bioavailability of 60 mg/kg group

The oral absorption of SHetA2 was rapid with a small lag time at the higher dose and bioavailability was moderate. The fact that reasonable fittings were generated by simultaneous fitting of all data from i.v. and p.o. doses indicated that pharmacokinetics of SHetA2 were linear and predictable in mice.

Conclusion

A highly sensitive HPLC/UV method for the quantification of SHetA2 in plasma has been developed. This method has been validated and applied for the preclinical pharmacokinetic study of SHetA2 in plasma. The pharmacokinetic profile in the mouse is reasonable for achieving and sustaining apoptotic concentrations of SHetA2 that could eliminate cancer and premalignant cells from the body.

References

1. Guruswamy S, Lightfoot S, Gold M, Hassan R, Berlin KD, Ivey RT, Benbrook DM (2001) Effects of retinoids on cancerous phenotype and apoptosis in organotypic culture of ovarian carcinoma. J Nat Cancer Inst 93:516–525
2. Liu S, Brown CW, Berlin KD, Dhar A, Guruswamy SB, Brown D, Gardner GJ, Birrer MJ, Benbrook DM (2004) Synthesis of flexible sulfur-containing heteroarotinoids that induce apoptosis and reactive oxygen species with discrimination between malignant and benign cells. J Med Chem 47:999–1007
3. Benbrook DM, Kamelle SA, Guruswamy SB, Lightfoot SA, Hannafon B, Rutledge TL, Gould NS, Dunn ST, Berlin KD (2005) Flexible heteroarotinoids (Flex-Hets) exhibit improved therapeutic ratios as anti-cancer agents over retinoic acid receptor antagonists. Inv New Drugs 23:417–428
4. Mic FA, Molotkov A, Benbrook DM, Duester G (2003) Retinoid activation of RAR but not RXR is sufficient for mouse embryonic development. Proc Natl Acad Sci 100:7135–7140
5. Benbrook DM (2002) Refining retinoids with heteroatoms. Minirev Med Chem 2:271–277
6. Benbrook DM, Madler MM, Spruce LW, Birckbichler PJ, Nelson EC, Subramanian S, Weerasekare GM, Gale JB, Patterson MK Jr, Wang B, Wang W, Lu S, Rowland TC, DiSilvestro P, Lindamood C III, Hill DL, Berlin KD (1997) Biologically active heteroarotinoids exhibit anticancer activity and decreased toxicity. J Med Chem 40:3567–3583
7. Chun K-H, Benbrook DM, Berlin KD, Hong WK, Lotan R (2003) Induction of apoptosis in head and neck squamous cell carcinoma (HNSCC) cell lines by heteroarotinoids through a mitochondrial dependent pathway. Cancer Res 63:3826–3832
8. Liu Z, Minkler PE, Lin D, Sayre LM (2004) Derivatization of amino acids with *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine for liquid chromatography/electrospray mass spectrometry. Rapid Commun Mass Spectrom 18:1059–1065
9. Liu Z, Floss HG, Cassady JM, Chan KK (2005) Metabolism studies of the anti-tumor agent maytansine and its analog ansamitocin P-3 using liquid chromatography/tandem mass spectrometry. J Mass Spectrom 40:389
10. Liu Z, Chan KK, Wang JJ (2005) Tandem mass spectrometric analysis of cyclophosphamide, ifosfamide and their metabolites. Rapid Comm Mass Spectrom 19(18):2581–2590
11. Madden FN, Godfrey KR, Chappell MJ, Hovorka R, Bates RA (1996) A comparison of six deconvolution techniques. J Pharmacokin Biopharm 24(3):283–299