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Development of an LC–MS/MS assay to determine plasma pharmacokinetics of the radioprotectant octadecyl thiophosphate (OTP) in monkeys

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ABSTRACT

Octadecenyl thiophosphate (OTP), a synthetic analogue of the lysophospholipid growth factor lysophosphatidic acid (LPA), significantly reduces mortality following a lethal dose of $LD_{80/30}$ radiation exposure in a mouse model of whole-body irradiation. To facilitate dose scaling between species, we developed a novel liquid chromatography/tandem mass spectrometry (LC–MS/MS) for the preclinical pharmacokinetic characterization of OTP in monkeys. Sample extraction was carried out using a butanol based liquid–liquid extraction method. A partially deuterated OTP analogue was used as internal standard (IS). OTP and IS were separated by reversed-phase liquid chromatography on a C-8 column using 10 mM ammonium acetate and acetonitrile. A triple quadrupole mass spectrometer operating in the negative electrospray ionization mode with multiple reaction monitoring was used to detect OTP and IS transitions of m/z 363.1 → 95.0 and 403.1 → 95.0. The method was applied to determine pharmacokinetic parameters in monkeys receiving a single oral OTP dose (3 mg/kg). OTP is readily absorbed with a relatively long half-life which supports further preclinical testing of OTP as a radioprotectant in monkeys.

1. Introduction

Radiation exposure during the course of cancer radiotherapy or a nuclear accident may induce severe damage to normal tissues depending upon radiation dose, exposure rate and quality. The most radiosensitive tissue is the bone marrow, where irreversible myelosuppression may occur following a dose of as little as 1 Gy whole-body radiation exposure. Gastrointestinal mucositis develops following higher doses of radiation exposure (>8 Gy) [1,2]. There are currently no therapeutically satisfactory radioprotectants or radiomitigators that alleviate radiation injury when applied after exposure. OTP or octadecenyl thiophosphate, a synthetic analogue of the lysophospholipid growth factor lysophosphatidic acid (LPA), has been identified as the prototype of a unique class of potent radioprotectants that act through activation of LPA receptors, a family of eight G protein coupled receptors (GPCRs) [3]. Studies have shown that OTP is a partial agonist for LPA1 and LPA3 compared to LPA and a potent and full agonist for LPA2 and LPA5 with an EC_{50} in the nanomolar range [4]. Preclinical tests using a mouse model of whole-body irradiation revealed that OTP at doses in the low mg/kg range significantly ameliorated radiation-induced gastrointestinal injury, as well as prevented death following a lethal dose of $LD_{80/30}$ radiation exposure. Experiments conducted with knockout mice indicate that the molecular target of OTP is the LPA2 receptor [4].

LPA is rapidly degraded by phospholipase- and lipase-mediated deacylation or phosphatase-mediated dephosphorylation rendering it inactive. We have shown previously that the thiophosphate analogue OTP is resistant to the phosphatase activity of lipid phosphate phosphohydrolase type 1 (LPP1) obtained from mouse embryonic fibroblasts [4]. Resistance to LPP1 degradation should lead to enhanced stability of OTP *in vivo*. Development of a novel analytical method for the detection of thiophospholipids in biological matrices was required to test the *in vivo* stability of OTP. OTP lacks a chromophore, thus, we chose electrospray liquid chromatography–tandem mass spectrometry (LC–MS/MS) as it is widely applied by pharmaceutical scientists in the quantification of drugs and their metabolites in various biological matrices [5–8]. Herein, we report a sensitive, specific, and reproducible triple quadrupole mass spectrometric assay with deuterium labeled

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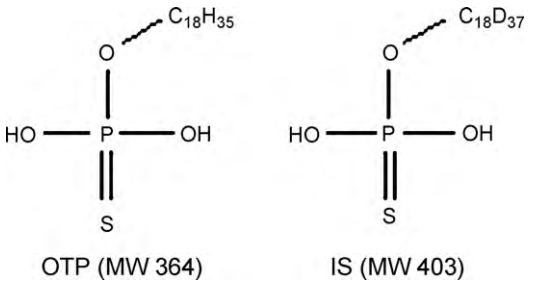

Fig. 1. Chemical structures of octadecyl thiophosphate (OTP) and the internal standard deuterated OTP.

internal standard (IS) applied to characterize the pre-clinical pharmacokinetics of OTP in monkeys.

2. Materials and methods

2.1. Materials

OTP and partially deuterated OTP analogue (internal standard – IS) (Fig. 1) were synthesized by the Medicinal Chemistry Department, RxBio Inc. (Memphis, TN, USA) as described elsewhere [9]. All mobile phase solvents and water used were of HPLC grade and purchased from Fisher-Scientific (Pittsburgh, PA, USA). Monkey plasma was obtained from Bioreclamation Inc. (Liverpool, NY, USA).

2.2. Standard and quality control (QC) samples

Stock solutions of OTP and IS were prepared in methanol at a concentration of 1.0 mg/mL. The OTP stock solution was further diluted in methanol to make OTP working solutions for standards (20, 10, 5, 2.5, 1.0, .5, and .1 μg/mL) and QC samples (15, 3, and 2 μg/mL). The IS stock solution was further diluted in methanol to make an IS working solution (7.5 μg/mL). OTP working solutions were then spiked (10 times dilution) into monkey plasma to make calibration standards and QC samples. The resulting concentrations of standards and QC samples were 2000, 1000, 500, 250, 100, 50, 10 ng/mL and 1500, 300, 20 ng/mL, respectively. Calibration standards and QC samples were prepared freshly daily.

2.3. Sample preparation

OTP and IS were extracted using a water-saturated butanol-based liquid–liquid extraction method. Briefly, plasma samples (50 μL) were spiked with IS (10 μL) in methanol followed by gentle vortexing and the addition of water-saturated 1-butanol (400 μL). The contents were then vortexed (high speed for 10 min) and centrifuged (10,000 rpm for 5 min). An aliquot (400 μL) from the top layer was carefully removed and transferred to a fresh vial. The organic layer was evaporated to dryness using an Eppendorf vacufuge concentrator (40 °C; 20 min) and reconstituted in methanol (100 μL) for LC–MS/MS analysis.

2.4. LC–MS/MS

OTP and IS were separated by reversed-phase liquid chromatography using a C8 analytical column (Symmetry, 3.5 μm, 2.1 mm × 100 mm, Waters Corporation, Milford, MA) and an injection volume of 20 μL with a 5 min run time. The mobile phase consisted of acetonitrile and water containing 10 mM ammonium acetate and was delivered at a flow rate of 0.3 mL/min at room temperature. A 3-min gradient liquid chromatographic approach was used to elute OTP and IS. At the start of the gradient, acetonitrile (B) was 30%; it was ramped to 70% in 1 min, held constant at this value for 1 min and then ramped back to 30% over 1 min. The LC–MS/MS system comprised an Applied Biosystems (AB) Sciex (Foster City, CA) API 3000 tandem mass spectrometer, equipped with a Turboionspray™ interface, a Shimadzu (Columbia, MD) LC-10ADvp pump and a Leap (Carrboro, NC) HTS PAL autosampler. The electrospray ion source was run in a negative ionization mode for all the experiments. The typical ion source parameters were: capillary 3.5 kV, declustering potential (DP) 32 V, focusing potential (FP) 130 V, entrance potential (EP) −12 V, collision energy (CE) 29 eV, collision cell exit potential (CXP) 10 V, deflector −348 V, channel electron multiplier (CEM) 2200 V, source temperature 325 °C. Quantification was performed in the multiple reaction-monitoring (MRM) mode, monitoring the transition of the m/z 363.1 precursor ion to the m/z 95.0 product ion for OTP and m/z 403.1 precursor ion to the m/z 95.0 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed using the software program Analyst (Version 1.3).

2.5. Method validation

Inter-day/intra-day precision and accuracy ($n = 3$) were evaluated by spiking known amounts of OTP and IS in plasma at three different concentrations (1500, 300, 20 ng/mL). Replicate OTP QC samples were stored at −80 °C to determine freeze–thaw cycle and long-term storage stability. Freeze–thaw sets of QC samples ($n = 3$) were thawed at room temperature then returned to the freezer for a minimum of 24 h. This process was repeated three times. Long-term stability of QC samples was analyzed after 1 month. Post-preparative autosampler stability was determined by reinjection of samples 12 h following initial injection. Analytical recovery was determined by comparing the pre-extraction spikes to the post-extraction spikes for QC samples (1500, 300, 20 ng/mL). For the post-extraction spike, the QC analytes were spiked into blank monkey plasma extract and mixed immediately prior to evaporation under a stream of nitrogen. The matrix effect was calculated by comparing the responses of post-extracted spiked sample and non-extracted sample. Specificity, defined as non-interference in the regions of interest of the analyte as well as IS with the endogenous substances, was evaluated using three separate lots of monkey plasma.

2.6. OTP Pharmacokinetics

The study was conducted in strict adherence with the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Resource Council (National Academy Press, Washington, DC, 1996). Anesthetized (ketamine HCl; 10 mg/kg) female rhesus macaques, housed at the California National Primate Research Center in accordance with American Association for Accreditation of Laboratory Animal Care standards, received a single sterile-filtered (0.22 μm) oral OTP dose (3 mg/kg; 2% propanediol and 1% ethanol v/v in PBS, pH 7.4). EDTA-anticoagulated blood was collected at pre-dose and 0.5, 1, 2, 4, 6, 8,24, 32, 48, 56, 72 and 80 h and plasma obtained by centrifugation (900 rpm at room temperature). Plasma was immediately frozen and stored at −80 °C until analysis.

OTP plasma concentration–time data were analyzed by non-compartmental methods. The area under the plasma concentration–time curves from time 0 to infinity ($AUC_{0-\infty}$) was calculated by the trapezoidal rule with extrapolation to time infinity. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$, where λ_z represents the terminal phase elimination constant determined from the slope of the regression line of the last three points of the concentration time profile.

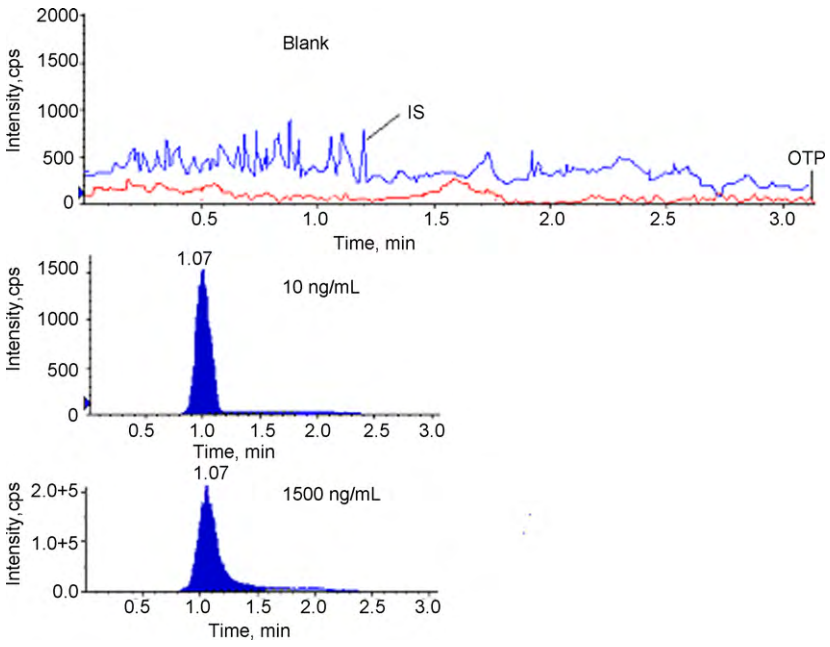

Fig. 2. MRM chromatograms: blank plasma sample showing no interferences of analyte and IS. OTP spiked at 10 ng/mL (LLOQ) and 1500 ng/mL (HQC) in monkey plasma.

3. Results

3.1. Mass spectrometry

The ionization and fragmentation of OTP were studied using electrospray ionization tandem mass spectrometry in positive and negative modes. The molecular ion [M−H] was observed at *m*/*z* 363.1 in negative mode. There were no significant molecular ion peaks observed in positive mode. The collision-induced dissociation of the [M−H] precursor ion at *m*/*z* 363.1 produced an intense product ion at *m*/*z* 94.9 at the optimum collision energy of 52 eV. Similarly the molecular ion (M−H) and the product ion of deuterated OTP (IS) were observed only in negative mode at *m*/*z* 402.5 and 95.1. MRM based on the *m*/*z* 363.2 → 95.1 and *m*/*z* 403.1 → 95.1, transitions were specific for OTP and IS. OTP and IS eluted at retention times of ∼0.97 and ∼1.3 min, respectively during the 3-min gradient chromatography.

3.2. Selectivity and sensitivity

Carryover, a potential problem arising especially with lipid-based drug analysis was tested by injecting blank samples immediately after a high QC sample (1500 ng/mL). A chromatographic peak with ∼2 to 5% of high QC peak area was observed in the blank sample at the same retention time. After a brief investigation, it was concluded that OTP sticking to metal surface during injection was causing the carryover problem. To eliminate this problem the injector needle height was adjusted to minimize the contact surface between analyte and needle, two kinds of wash solution was used between injection (wash solution 1: CCl3/IPA (50/50, v/v); wash solution 2: same as mobile phase), which significantly reduced carryover to less than 10% of the LOQ peak area.

Assay selectivity was assessed using blank samples spiked with either OTP or IS. Interfering peaks at the retention time of the analytes were not observed in blank extracted plasma from each monkey using the established method conditions. Additionally, no OTP and IS signals have been observed in any pre-dose monkey samples evaluated to date. The LOQ of OTP was 10 ng/mL for the plasma assay.

Fig. 2 displays representative chromatograms of blank plasma and plasma spiked OTP with or without IS. Blank plasma chromatograms show no interfering peaks at the observed OTP and IS retention times.

3.3. Linearity and lower limit of quantification

Standard curves were constructed by plotting ratio of peak areas of OTP and IS versus concentration and were linear in plasma. The correlation coefficient of OTP was found to be 0.997 for plasma samples or better.

The mean ± standard deviation ($n = 3$ replicates) of OTP slope and intercept of the regression curve were 0.0014 ± 0.0001 and −0.029 ± 0.037 for plasma. The assay was linear over the concentration range 10–2000 ng/mL. The limit-of-detection (LOD) ($S/N > 4$) and limit-of-quantitation (LOQ) ($S/N > 10$) were estimated to be 3 ng/mL and 10 ng/mL, respectively.

3.4. Precision and accuracy

The inter- and intra-day accuracy and precision values were calculated by taking high, mid and low quality control samples ($n = 3$) and are displayed in Table 1. The precision (% CV) of quality control plasma samples was less than 5.3% (intra-day), and 2.2% (inter-day), respectively. The accuracy was 103.8% (intra-day), and 103.9% (inter-day) or less for plasma, respectively. The values of precision and accuracy were acceptable in view of the international recommendation that the precision and accuracy should not exceed 15% except at the LOQ, which should lie within ±20% of the targeted concentration. The results indicated the method was reproducible.

3.5. Recovery from plasma

The calculated extraction recoveries for QC samples at three different concentration levels in monkey plasma samples ranged from 81.9 ± 12.1 to 87.4 ± 9.4%, proving that this method of extraction was suitable for plasma samples. The study indicated that an optimized butanol based liquid–liquid extraction method with plasma to butanol ratio 1:8, resulted in high extraction recoveries. Three different blank plasma samples from each monkey were tested for interfering peaks corresponding to OPT and IS MRM transitions. Method selectivity was demonstrated by the fact that no corre-

Table 1
Summary of OTP quality control sample validation in monkey plasma.

Nominal concentration (ng/mL)	Intra-day measured concentration (ng/mL)			Inter-day validation summary			
	Day 1	Day 2	Day 3	Mean	SD	% CV	% Accuracy
LQC 20	20.24	20.95	21.12	20.8	0.47	2.24	103.85
	19.82	19.02	20.26				
	20.91	19.22	20.92				
Mean	20.323	19.73	20.77				
SD	0.5498	1.06	0.45				
% CV	2.7051	5.379	2.167				
% Accuracy	101.62	98.65	103.8				
MQC 300	302.1	303.50	294.2	299.93	5.01	1.67	99.98
	294.5	294.10	305.2				
	291.2	312.50	294.4				
Mean	295.93	303.37	297.93				
SD	5.59	9.20	6.29				
% CV	1.89	3.03	2.11				
% Accuracy	98.64	101.12	99.31				
HQC 1500	1510.2	1490.2	1484.5	1494.97	13.5	0.90	99.66
	1507.2	1528.5	1521.2				
	1481.2	1492.8	1515.7				
Mean	1499.53	1503.83	1507.13				
SD	15.93	21.40	19.79				
% CV	1.06	1.42	1.31				
% Accuracy	99.97	100.26	100.48				

sponding peaks were observed (Fig. 2). The method is sensitive, selective, precise, and accurate for the determination of OTP in monkey plasma samples at very low concentrations (10 ng/mL) over a concentration range extending up to 2000 ng/mL.

3.6. Stability

The results of three freeze–thaw cycles show that OTP was stable. The results of the long-term stability study suggested that OTP was stable upon storage −70 °C. Extracted and methanol-reconstituted OTP was stable and no significant changes were observed when left in the CTC-PAL autosampler for 12 h. Average sample analysis time was around 3 h and OTP was observed to be stable up to 2 freeze–thaw cycles.

3.7. Biological matrix

Matrix effects were consistent with no significant analyte ion suppression when the LC eluent was directed to waste from 0–0.6 to 2.0–3.0 min during the 3.0 min sample runtime. Matrix effects were consistently below 15%.

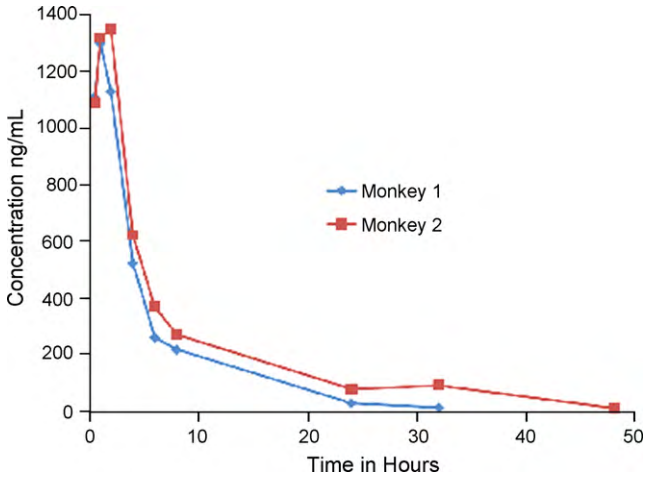

Fig. 3. OTP plasma concentration versus time profile. OTP (3 mg/kg) was orally administered as a single dose to monkeys followed by serial sampling of the plasma.

3.8. OTP Pharmacokinetics

Our method was applied to study the systemic exposure of OTP. Individual concentration vs. time profiles of OTP following oral administration of 3 mg/kg in monkeys are shown in Fig. 3. OTP plasma concentration declined exponentially after reaching a maximum plasma concentration at approximately 2 h. OTP exhibited a relatively long terminal half-life (~8 h) with a low volume of distribution (0.4 L/kg). The long plasma half-life of OTP was attributed to the fact that OTP was cleared very slowly from the plasma (~0.4 L/h/kg).

4. Discussion

LPA is rapidly inactivated via both phospholipase- and lipase-mediated deacylation and lipid phosphate phosphatase-mediated dephosphorylation; a fact which limits LPA's potential drug candidacy. A strategy for the design of stable LPA analogues was provided when it was hypothesized that thiophosphate substitution conferred resistance to LPP-mediated degradation as in the case of the ester-linked thiophosphate derivative (1-oleoyl-2-*O*-methyl-*rac*-glycerophosphothionate, OMPT) [10]. This hypothesis led us and others to design and synthesize novel metabolically stable thiophosphate derivatives (e.g., OTP) [9,11,12]. Recently, we provided the first direct evidence that thiophosphate derivatives were resistant to phosphatase activity by demonstrating that LPP1 derived from mouse embryonic fibroblasts was unable to dephosphorylate OTP [4]. Another important degradation pathway for LPA is via acylation of the sn-2 hydroxyl group. OTP lacks the sn-2 hydroxyl group of its congener LPA, which should further slow its systemic clearance and increase half-life *in vivo*. In order to test this hypothesis, we developed and validated a novel highly specific MRM-based LC–MS/MS assay to study the OTP's disposition in monkeys following subcutaneous administration.

In the current study, a reverse phase LC–MS/MS method using deuterated-OTP as IS was developed to quantify OTP in plasma samples. Several combinations of organic and aqueous mobile

phases and a variety of stationary phases (C8, C18 and silica) were attempted, but higher sensitivities with better peak shapes with minimal tailing and lower baselines were achieved using a C8 column and an ammonium acetate buffer (A) (5 mM, pH 7.0)/acetonitrile (B). Variations in mobile phase pH (pH 4, 5 and 9) did not enhance detection sensitivity, which is in agreement with observations made by Jemal et al. [13]. However, use of either a highly acidic or basic mobile phase was avoided because of possible degradation of OTP under these conditions.

Different extraction methods including protein precipitation (methanol, ethanol, and acetonitrile) and liquid–liquid extraction using chloroform and chloroform/methanol yielded very low recoveries of OTP (~20 to 30%). An optimized butanol based liquid–liquid extraction method with plasma to butanol ratio 1:8, resulted in high extraction recoveries. The method is sensitive, selective, precise, and accurate for the determination of OTP in monkey plasma samples at very low concentrations (10 ng/mL) over a concentration range extending up to 2000 ng/mL. The use of the stable labeled IS effectively corrected minor fluctuations during the extraction and sample analysis, including ion suppression and matrix effects in the ion source.

OTP pharmacokinetics in monkeys following oral administration was studied. The results show OTP was eliminated very slowly from the systemic circulation and as a result demonstrated a relatively long half-life of 7.66 h. In mice, dephosphorylated OTP was identified as the primary metabolite in plasma. This was somewhat surprising considering our *in vitro* findings that OTP was resistant to LPP-mediated dephosphorylation. It is possible that interspecies differences in LPP substrate specificity could account for this observation. However, this seems less likely considering the same dephosphorylated metabolite was formed at a much higher rate in mice following subcutaneous OTP administration (H. Kosanam, S. Ramagiri, Y. Kimura, V. Gududuru, W. Deng, G. Tigyi, D. Miller, and C.R. Yates, unpublished work). Another possibility is the existence of an unidentified (thio)-phosphatase whose activity varies dramatically across species.

In summary, preclinical pharmacokinetic studies of OTP in monkeys demonstrate that OTP is relatively long-lived as a result of inefficient elimination processes. The primary route of metabolism involves a thiophosphatase-like activity that apparently varies dramatically between species. Significant interspecies differences in thiophosphatase activity would have important implications in the pre-clinical and clinical development of OTP, particularly when developing interspecies scaling estimates of dose.

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References

[1] G.H. Anno, S.J. Baum, H.R. Withers, R.W. Young, Health Phys. 56 (1989) 821.
[2] C.N. Coleman, W.F. Blakely, J.R. Fike, T.J. MacVittie, N.F. Metting, J.B. Mitchell, J.E. Moulder, R.J. Preston, T.M. Seed, H.B. Stone, P.J. Tofilon, R.S. Wong, Radiat. Res. 159 (2003) 812.
[3] I. Ishii, N. Fukushima, X. Ye, J. Chun, Annu. Rev. Biochem. 73 (2004) 321.
[4] W. Deng, E. Shuyu, R. Tsukahara, W.J. Valentine, G. Durgam, V. Gududuru, L. Balazs, V. Manickam, M. Arsura, L. VanMiddlesworth, L.R. Johnson, A.L. Parrill, D.D. Miller, G. Tigyi, Gastroenterology 132 (2007) 1834.
[5] A. Espada, M. Molina-Martin, J. Dage, M.S. Kuo, Drug Discov. Today 13 (2008) 417.
[6] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[7] P. Song, B. Meibohm, C.R. Yates, Biotech. Suppl. (2005) 19.
[8] R.N. Xu, L. Fan, M.J. Rieser, T.A. El-Shourbagy, J. Pharm. Biomed. Anal. 44 (2007) 342.
[9] G.G. Durgam, T. Virag, M.D. Walker, R. Tsukahara, S. Yasuda, K. Liliom, L.A. van Meeteren, W.H. Moolenaar, N. Wilke, W. Siess, G. Tigyi, D.D. Miller, J. Med. Chem. 48 (2005) 4919.
[10] Y. Hasegawa, J.R. Erickson, G.J. Goddard, S. Yu, S. Liu, K.W. Cheng, A. Eder, K. Bandoh, J. Aoki, R. Jarosz, A.D. Schrier, K.R. Lynch, G.B. Mills, X. Fang, J. Biol. Chem. 278 (2003) 11962.
[11] G.D. Prestwich, Y. Xu, L. Qian, J. Gajewiak, G. Jiang, Biochem. Soc. Trans. 33 (2005) 1357.
[12] L. Qian, Y. Xu, Y. Hasegawa, J. Aoki, G.B. Mills, G.D. Prestwich, J. Med. Chem. 46 (2003) 5575.
[13] M. Jemal, Z. Ouyang, D.S. Teitz, Rapid Commun. Mass Spectrom. 12 (1998) 429.