Development and Validation of a High-Performance Liquid Chromatography Method for the Quantification of Talazoparib in Rat Plasma: Application to Plasma Protein Binding Studies

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Abstract

A sensitive and selective RP-HPLC method has been developed and validated for the quantification of a highly-potent poly ADP ribose polymerase (PARP) inhibitor talazoparib (TZP) in rat plasma. Chromatographic separation was performed with isocratic elution method. Absorbance for TZP was measured with UV detector (SPD-20A UV-VIS) at λ_{max} of 227 nm. Protein precipitation method was used to extract the drug form plasma samples using methanol: acetonitrile (65:35) as a precipitating solvent. Method was shown sensitive and reproducible over 100-2000 ng/mL linearity range with LLQC of 100 ng/mL. TZP recovery was found to be >85%. Following analytical method development and validation, it was successfully employed to determine the plasma protein binding of TZP. It was found that TZP has high protein binding in rat plasma (95.76 ± 0.38 %) as determined by dialysis method.

Key word: Chromatography, Protein Binding, PARP inhibitor

Introduction

Poly(ADP-ribose)polymerase (PARP) enzymes are critical for recognition and repair of DNA breaks (Murai, Huang, Das, Renaud, Zhang, Doroshow, Ji, Takeda and Pommier 2012). PARP inhibitors (PARPi) were shown particularly effective in HR deficient cancers, but are also expected to have wide applications beyond *BRCA* mutations such as in sporadic tumors with 'BRCAness'(Lord and Ashworth 2008). More than 200 clinical trials on PARP inhibitors are either completed or currently active for various cancers ovarian and breast cancers, glioblastoma and others (https://clinicaltrials.gov). At present, many PARP inhibitors like olaparib, talazoparib (TZP), veliparib, niraparib and rucaparib are in clinical trials for different cancerous condition (Bryant, Schultz, Thomas, Parker, Flower, Lopez, Kyle, Meuth, Curtin and Helleday 2005, Lord and Ashworth 2008, O'Connor 2015, Rouleau, Patel, Hendzel, Kaufmann and Poirier 2010). Food and Drug Administration (FDA) has approved the PARP inhibitor, olaparib, for the treatment of ovarian cancer in 2014 and also classified olaparib as an advance remedy status for BRCA or ATM mutated castration-resistant prostate cancer (Helleday 2016, Liu and Matulonis 2016) .TZP, the most potent PARPi, was shown to be effective against a variety of cancer cells with EC_{50} in nanomolar concentrations (Wang, Chu, Feng, Shen, Aoyagi-Scharber and Post 2016). Clinical study with TZP monotherapy showed efficacy in a cohort of 39 patients with objective response rate of 65% in ovarian and peritoneal tumors, and 33% in breast cancer patients, is well tolerated (Engert, Kovac, Baumhoer, Nathrath and Fulda 2016, Livraghi and Garber 2015, Pulliam, Taverna, Lyons and Nephew 2015, Roche, Blum, Eiermann, Im, Martin, Mina, Rugo, Visco, Zhang and Lokker 2015, Smith, Reynolds, Kang, Kolb, Gorlick, Carol, Lock, Keir, Maris and Billups 2015). Like olaparib, TZP traps PARPs to DNA (Murai, Huang, Das, Renaud, Zhang, Doroshow, Ji, Takeda and Pommier 2012). The minimum toxic dose of TZP was 1 mg/day, with common adverse events of neutropenia, thrombocytopenia and anemia.

Currently three Phase III clinical trials on TZP are in progress (Murai, Huang, Das, Renaud, Zhang, Doroshow, Ji, Takeda and Pommier 2012, Smith, Reynolds, Kang, Kolb, Gorlick, Carol, Lock, Keir, Maris and Billups 2015).

Quantitation of a new compound in different analytical or bioanalytical matrix is always a crucial and necessity for drug discovery and development in a time and cost effective way (Shah 2007, Shah, Midha, Findlay, Hill, Hulse, McGilveray, McKay, Miller, Patnaik and Powell 2000). Chromatography is always a preferred option for quantitative analysis of the drug in a robust way. Our research laboratory has been developing novel nanoparticle formulation for targeted delivery of TZP to triple negative breast cancer. To the best of our knowledge, no bioanalytical method is published yet for TZP. We anticipate that development of a sensitive analytical method would accelerate the development of TZP and its formulations. Present study was aimed to develop and validate a sensitive and selective RP-HPLC method and apply this method to determine the plasma protein binding of TZP per FDA guidelines for industry on bioanalytical method validation.

Experimental

Material and methods

TZP and difluprednate (DFBA) were purchased from Selleckchem, Houston, TX, USA (Figure 1). Regenerated cellulose (RC) dialysis membrane with molecular weight cut-off 50 kD was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Methanol and acetonitrile (HPLC grade), ammonium acetate and glacial acetic acid were purchased from VWR International, Sugar Land, TX, USA. Phosphate-buffered saline (PBS, pH 7.4) was purchased from Mediatech Inc., Manassas, VA, USA. Buffer was filtered through a 0.45 µm cellulose membrane (Whatman International Ltd., Mailstone, England).

Stock solutions, calibration and quality control standards preparation

TZP and DFBA (used as internal standard, IS) were weighed accurately and stock solutions (1 mg/mL) of both drugs were made, 1 mg of TZP and DFBA dissolved in 1 mL of methanol and ACN, respectively. Mother stock solutions were used to make working stocks of the TZP and DFBA (100 μg/mL). Parallel dilution method was used to make analytical standards (AS) of TZP over the concentration range of 15.6, 31.25, 62.5, 125, 250, 500, 1000 and 2000 ng/mL in methanol for the estimation of TZP recovery in the rat plasma. Three replicates of quality control (QC) samples (100, 800 and 1600 ng/mL as low, medium and high quality control, respectively) were made separately. Calibration curve for TZP was made by adding 10 μL of working solution to 90 μL blank rat plasma over a concentration range of 100 to 2000 ng/mL.

Drug Extraction

Simple protein precipitation method was used for the extraction of TZP from plasma sample, by using methanol: ACN (65:35 ratios). To extract the control, QC plasma standard (90 μL rat plasma added with TZP and IS), and matrix blank samples (100 μL rat plasma without TZP and IS), 200 μL Methanol: ACN (65:35) was added as a precipitating solvent. Resulting mixture was vortexed for about 2 min and centrifuged at 8500 rpm for 10 min. 100 μL supernatant used for HPLC analysis.

HPLC setup and bioanalysis

RP-HPLC pump arrangement (LC-20A, Shimadzu) accompanied by degassing unit (DGU-20A, Shimadzu, Kyoto, Japan), auto-sampler (SIL-20A, with a 100-μL loop) was applied to inject the bioanalytical samples on a Luna C-18 column (5μm, 250mm x 3mm internal diameter, Phenomenex, USA) connected with a C-18 pre-column for the protection of main C-18 column. An isocratic elution method was used at a flow rate of 1 mL/min with 60:40 (v/v) ratios of methanol / ACN (65:35): DI water. Absorbance for TZP was measured with

UV detector (SPD-20A UV-VIS) at λ_{max} of 227 nm. Total run time was 10 min and RT for TP and DFBA were 7.65 and 6.5 minutes respectively (Figure 2). The calibration curve was found to be linear over the concentration range of 100 –2000 ng/mL (y = 55.021x - 1317.9, r^2= 0.9995).

Method validation

The RP-HPLC method validation was performed with regard to recovery from biosamples, accuracy, precision, intra and inters- day variability, sensitivity, specificity and reproducibility (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). The RP-HPLC method was validated as per US FDA guidelines for industry on bioanalytical method validation (Health and Services 2001, Matuszewski, Constanzer and Chavez-Eng 2003). Calibration standards linearity was determined between 100-2000 ng/mL concentration ranges for three days. Inter- and intra-day precision estimated using analysis of variance (one way) in terms of relative standard deviation (RSD) (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). The recoveries of TZP from the extracted samples were estimated by correlating the known concentrations of plasma samples. This whole process was recapitulated for concentration levels of 100, 800 and 1600 ng/mL, which represent the low, medium and high quality control (LQC, MQC, and HQC) standards respectively (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015).

Stability studies

Chemical stability of any compound in stock solution and biometrix is very critical parameter for its further development. Different stability studies like freeze-thaw stability, bench top stability, dry residue stability and long term stability studies were conducted to determine the stability of TZP in stock solution and in rat plasma. For determination of TZP stability, spiked control in rat plasma were made at 100, 800 and 1600 ng/mL concentrations, that

regard to LQC, MQC, HQC respectively, each with three sets of replicates (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015).

Protein binding determination

Plasma protein binding of TZP was determined by equilibrium dialysis bag method using Spectrum dialysis membranes of cellulose ester nature and molecular weight cut off (50 kD) with PBS buffer (pH7.4) as the dissolution medium. Study was carried out using 1 mg/ml of TZP dissolved in sprague dawley (SD) rat plasma placed in the dialysis bag (Barre, Chamouard, Houin and Tillement 1985, Giacomini, Abang and Blaschke 1982). Dialysis bag was dipped in a glass bottle containing 75 ml of the dissolution medium. The glass bottle was placed in an incubating orbital shaker (VWR, Houston TX, USA) maintained at 37±0.5 ^{o}C and 150 RPM. 500 µl samples were withdrawn at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 18 and 24 h and analyzed using HPLC.

Results and discussion

Extraction and recovery of the sample

At the beginning, different drug extraction procedures including protein precipitation with different organic solvents, liquid-liquid extraction (LLE), and solid-phase extraction (SPE) methods were tried and used to identify an optimal method (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). Finally, a single step protein precipitation method was considered, using methanol and ACN as precipitating solvent at 65: 35 ratio (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). TZP recoveries from the quality control samples were found to be consistent and more than 85%. Values are shown in Table 1. Recovery of TZP was found higher in 2 times dilution with precipitating solvent in comparison with 3 times dilution.

Precision and accuracy

A concentration range of 100 to 2000 ng/mL was set for the calibration range to quantitate TZP. $1/x^2$ weighting factor was used over this range of calibration curve and it was found that variance was comparable with different concentration values related to the r^2 (least-squares linear regression) values determined by uniform weighting and weighting factor 1/x (Kumar Hidau, Singh, Shahi, Mounika and Kumar Singh 2015, Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). r^2 values were always found to be more than 0.999. Intra and inter batch variation for TZP accuracy and precision evaluated from quality control samples at 100, 800 and 1600 ng/mL concentrations for five days (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). The method accuracy and precision were determined by % nominal values and % co-efficient of variance (% CV), respectively. Accuracy and precision values for 5 days are shown in Table 2. The back calculated concentration for LLOQ (lower limit of quantification) must be within ± 20% of nominal concentrations and all other quality standard (LQC, MQC and HQC) concentration values must be within ±15% of their nominal concentrations, according to present US Food and Drug Administration (FDA) guidelines(Food 2013, Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). The data values were found to be under the acceptance limits

Specificity of method

Method specificity was determined by comparison between the chromatograms obtained for the plasma spiked with TZP at LOQ to those acquired from plasma samples without drug. Specificity of this HPLC method was determined by preparing and analyzing individual as well as pooled rat plasma samples without drug. Chromatograms interference was also detected for all blank plasma samples visually. No interference was found in the area of TZP and DFBA retention time after analyzing plasma samples (Figure 2). These data reveal that the method was specific for the quantification of TZP in different QC samples.

Stability studies

Different stability studies were performed for the TZP. In freeze-thaw stability study, three freeze-thaw cycles between -80°C and melting ice temperatures were performed for QC samples. For bench-top stability studies, QC samples were placed at the room temperature up to 8 h. In dry residue stability studies, QC standards were extracted by a single step protein precipitation method and the dry residues were stored at -80°C for three days. The dry residues were reconstituted with methanol: ACN (65: 35) and analyzed by HPLC. Long-term stability was determined by storing the QC samples for 30 days at -80°C. (Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015). Different chemical stability data are presented in Table 3. All stability data points were found within the acceptance limit.

Protein binding determination

The validated method was successfully applied to estimate plasma protein binding of TZP in SD rats at 1 mg/ml concentration (n=3). Association and dissociation of drug compound with plasma proteins is a dynamic process with execs of PBS buffer around the dialysis bag, acting as a sink for free drug removal. The percentage binding was then estimated from the % of drug remaining in the plasma after 24 h and the free amount of drug was evaluated from the dissolution medium. Protein binding for TZP was estimated 95.76 ± 0.38 % (Figure 3).

Conclusion

A sensitive, selective, and precise RP-HPLC bioanalytical method has been successfully developed and validated for quantification of TZP in rat plasma. Lower limit of quantification was found to be 100 ng/ml and linearity was found over a concentration range 100-2000 ng/ml. A time and cost effective protein precipitation method for sample extraction was developed with absolute recovery of more than 85%. Stability studies revealed that TZP has good stability throughout the storage period and sample processing under different conditions. The analytical method was successfully applied for the plasma protein binding

study. Results indicate that TPZ is highly protein bound compound. The validated HPLC method may be applied to various *in-vitro in-vivo,* preclinical, clinical, regulatory and exploratory studies like toxicokinetic, pharmacokinetics and drug-drug interaction studies (Gautam, Singh, Pratap and Singh 2010, Kumar Hidau, Singh, Shahi, Mounika and Kumar Singh 2015, Singh, Hidau, Misra, Kushwaha, Tiwari, Sharma and Singh 2015).

Conflict of interest

There is no conflict of interest to disclose.

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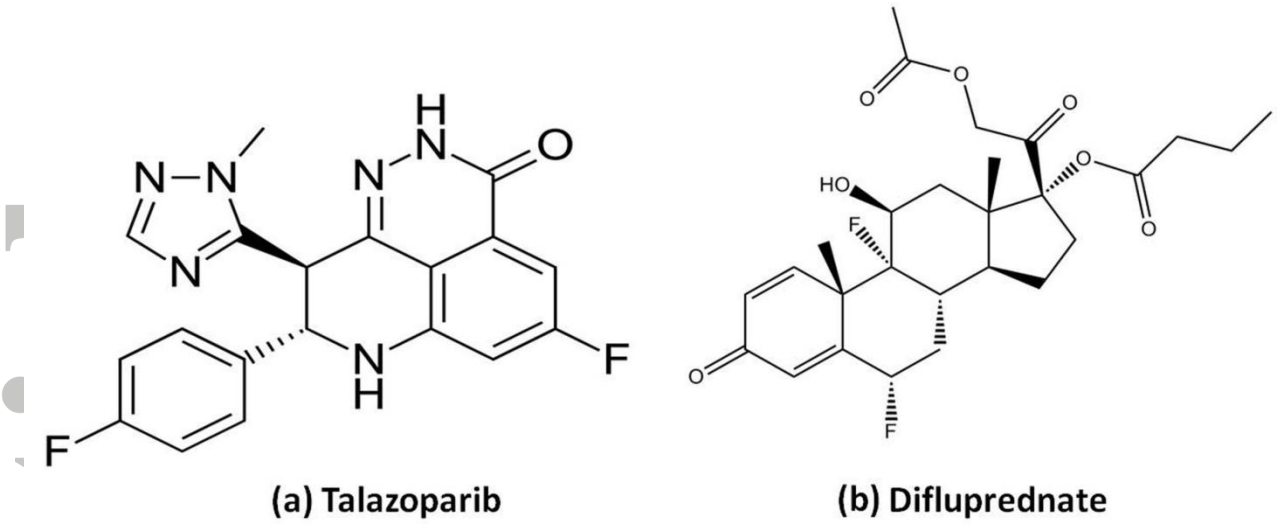

Figure 1: Chemical Structure of Talazoparib (a), IS Difluprednate (b).

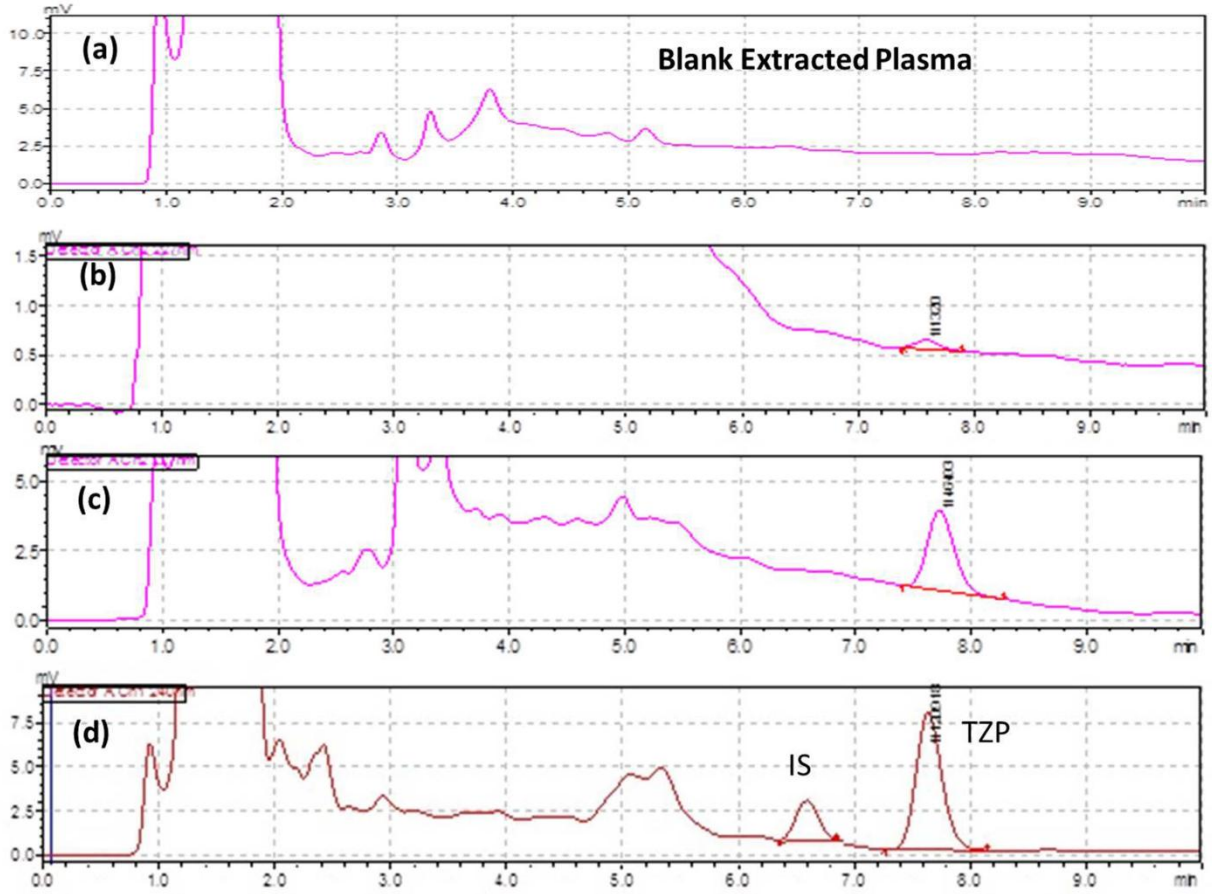

Figure 2: HPLC chromatograms: Blank extracted plasma (a), 50 ng/mL (b), Calibration standard Talazoparib at 125 ng/ml (c), spiked IS and Talazoparib at 1000 ng/ml (d).

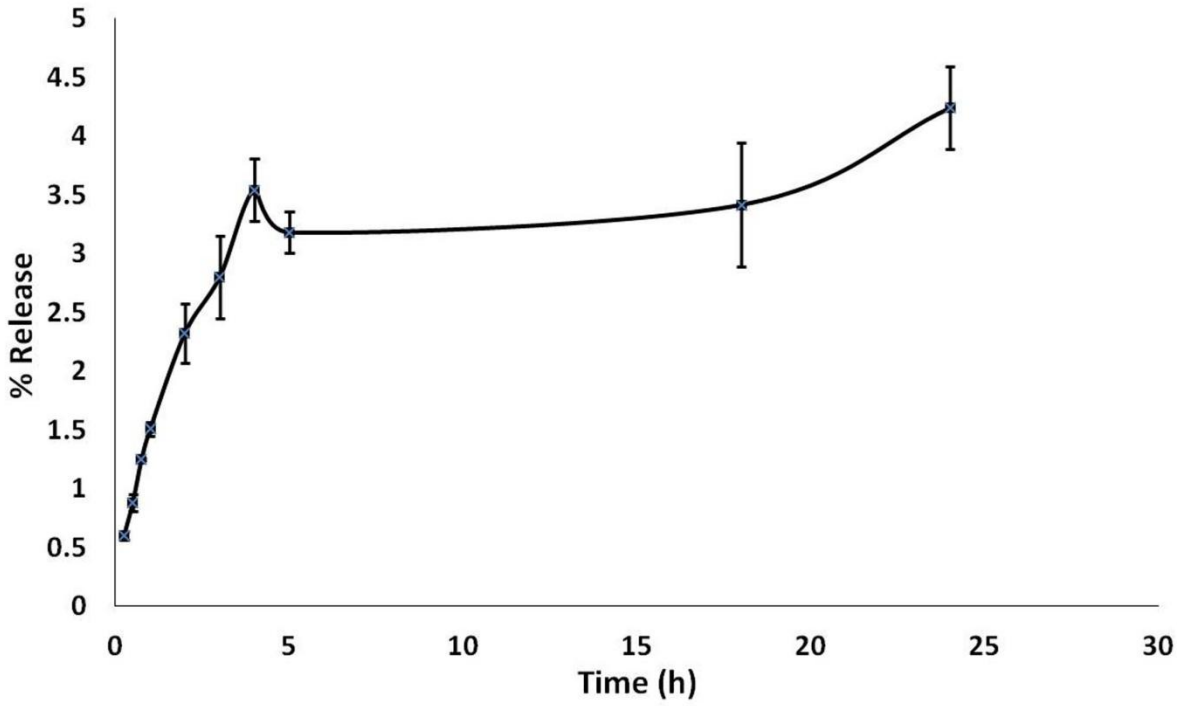

Figure 3: Release profile of percent drug remaining in the plasma after 24 h

Table 1: Recovery of spiked plasma samples at low, medium and high concentration respectively.

QC standard	Conc.(ng/mL)	Day1	Day2	Day3	Mean ± SD
LQC	100	98.56	85.35	91.02	91.65 ± 6.6
MQC	800	98.63	90.50	105.73	98.29 ± 7.6
HQC	1600	91.80	98.63368 88.44 91.80267	97.94	92.73 ± 4.8

Table 2: Intra- and inter-assay accuracy and precision for the TZP QC samples in the rat plasma

QC standard	Theoretical nominal Concentration (ng/mL)	Accuracy (%) (n=5)		Precision (%CV) (n=5)	
		Intra-day	Inter-day	Intra-day	Inter-day
LQC	100	-12.13	-11.18	6.7	-9.1
MQC	800	12.39	12.24	9.4	6.8
HQC	1600	13.85	14.44	8.0	5.9

Table 3: Stability of TZP in rat plasma after freeze-thaw cycles, bench top stability, dry residue stability and long term stability

Storage conditions	Nominal Concentration (ng/mL)	Concentration recovered (ng/mL)	% Recovery
After 3 Freeze-thaw stability	100	86.11 ± 3.25	85.57
	800	732.74 ± 48.3	91.59
	1600	1900 ± 109.2	114.34
Bench top stability for 8 h at ambient temperature	100	86.24 ± 9.12	85.69
	800	701.92 ± 22.16	87.74
	1600	1810.49 ± 44.05	113.15
Dry residue stability for 3 days	100	89.04 ± 7.44	89.04
	800	721.24 ± 37.67	90.15
	1600	1820.14 ± 48.97	113.75
Long term stability for 30 days	100	88.57 ± 8.12	88.57
	800	723.29 ± 20.85	90.40
	1600	1745.15 ± 61.53	109.19