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Journal of Chromatography B, 700 (1997) 131–138

JOURNAL OF CHROMATOGRAPHY B

# Validated assay for the quantification of anastrozole in human plasma by capillary gas chromatography–$^{63}$Ni electron capture detection

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Received 30 December 1996; received in revised form 19 February 1997; accepted 20 February 1997

**Abstract**

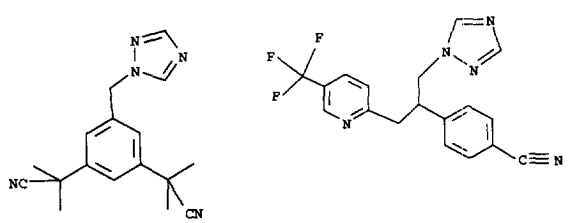
An assay was developed for the quantification of anastrozole [2,2′-[5-(1H-1,2,4-triazol-1-ymethyl)-1,3-phenylene]bis(2-methylpropiononitrile)] in human plasma using liquid–liquid extraction. Anastrozole and an internal standard were chromatographed and detected by gas chromatography with electron capture detection, using a combination temperature–pressure program. The range of the assay is 3 to 100 ng/ml. Anastrozole was quantified by comparing its peak area to that of an internal standard. A cross-validation of this assay was also successfully performed between several laboratories. © 1997 Elsevier Science B.V.

*Keywords:* Anastrozole

## 1. Introduction

Anastrozole (Fig. 1) is the active ingredient in Arimidex (trademark, the property of Zeneca Limited), which was recently approved by the FDA for the treatment of late-stage breast cancer in postmenopausal women. It is a potent aromatase (estrogen synthetase) inhibitor [1], which prevents androgen conversion to estrogens. The 1 mg/day dose of Arimidex is highly selective [2,3] and well tolerated by patients [4,5]. It has a half-life long enough to provide for once-daily dosing and this dose produces the maximum detectable estradiol suppression.

Due to the structure of anastrozole, liquid chromatography with UV, fluorescence, or electrochemical detection would not have been an appropriate method of analysis, since these detection methods did not have the sensitivity to yield a limit of quantitation low enough to allow full pharmacokinetic profiles of



(I) (II)

Fig. 1. Structure of anastrozole [2,2′-[5-(1H-1,2,4-triazol-1-ymethyl)-1,3-phenylene]bis(2-methyl propiononitrile)] (I) and the internal standard (II).

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*PII* S0378-4347(97)00129-1

anastrozole in the volunteers/patients from clinical studies. Therefore, gas chromatography with electron capture detection (GC–ECD) was tried and found to have adequate sensitivity and selectivity during method development. An internal standard (structure, Fig. 1), was used during this assay to correct for plasma extraction efficiency and variations in the ECD response.

## 2. Experimental

### 2.1. Instrumentation and conditions

HP 5890 Series II gas chromatographs with 7673B autosamplers and $^{63}Ni$ electron capture detectors from Hewlett-Packard (Wilmington, DE, USA), were utilized for sample analysis. They were equipped with split/splitless capillary injectors, electronic pressure controllers and direct injection liners. The analytical columns were Durabond 17 cross-linked fused-silica capillary, 50% phenyl–methyl silicone, 15 m×0.32 mm I.D., 0.25 μm film thickness from J&W Scientific (Folsom, CA, USA), and 5 m×0.32 mm deactivated guard columns from Restek (Bellefonte, PA, USA) were also used. These systems were coupled to VG Multichrom data acquisition software, version 2.0, for data collection. The injector and detector temperatures were 350°C, and the make-up flow was 50 ml/min argon–methane (95:5). The split vent flow was 65 ml/min, the purge vent flow was 5 ml/min and the splitless time was 10.0 min. A temperature and pressure program was used, and is summarized in Table 1.

### 2.2. Reagents and standard solutions

Anastrozole and the internal standard were synthesized by Zeneca Pharmaceuticals (Macclesfield, UK). Stock solutions of anastrozole and the internal standard were prepared in methanol (HPLC grade, Fisher) and spiking solutions were prepared in 0.9% sodium chloride, irrigation (USP). Two 1 mg/ml stock solutions of anastrozole were prepared. One set of spiking solutions was used for quality control sample preparation, and the other set was used to spike plasma standards. The concentration of the internal standard stock solution was 1 mg/ml.

### 2.3. Preparation of standards and quality control samples

Control human plasma (0.5 ml), obtained from drug-free volunteers, was spiked with anastrozole

Table 1
Gas chromatograph oven temperature and pressure program

| Parameter | Oven temperature program (A) | Pressure program (B) |
|---|---|---|
| Initial temperature (A) or pressure (B) | 180° C | 40 p.s.i.[a] (15 ml/min) |
| Initial time | 1 min | 0.30 min |
| Program rate | 50°C/min | 99.0 p.s.i./min |
| Temperature (A) or pressure (B) 2 | 240°C | 13.1 p.s.i. (3.0 ml/min) |
| Time 2 | 5.8 min | 0.43 min |
| Program rate 2 | 50°C/min | 1.92 p.s.i./min |
| Temperature (A) or pressure (B) 3 | 280°C | 15.4 p.s.i. (3.0 ml/min) |
| Program rate 3 | N/A | 28.3 p.s.i./min |
| Final pressure | N/A | 38.3 p.s.i. (10 ml/min) |
| Final time | 1.2 min | 5.8 min |
| Equilibration time | 0.5 min | 5.5 min |

[a] 1 p.s.i. = 6894.76 Pa.

and internal standard solutions to achieve a standard curve of anastrozole in concentrations of 3.0 through 100 ng/ml. Analytical standards, prepared at the same concentrations as the extracted plasma standards to determine relative recovery of anastrozole, were dried down under nitrogen, and reconstituted in 100 μl ethyl acetate (EtAc) (HPLC grade, Fisher).

Four levels of quality control samples (QCs) were prepared to cover the concentration range of the assay. Control human plasma was spiked with anastrozole solutions to yield 7.50, 15.0, 40.0 and 75.0 ng/ml. QCs were analyzed with calibration standards to determine concentration, and if the values were within acceptable range (±15% of theoretical), the QCs were used during runs quantitating study samples.

### 2.4. Extraction procedure

To 0.5 ml of a plasma sample (spiked standards, QC, blanks and clinical samples), 25 μl of an 2.5 μg/ml internal standard spiking solution was added, and the samples were vortexed to mix thoroughly. Then, two drops of concentrated $NH_4OH$ (Ultrex, J.T. Baker) were added, the samples were vortexed and 5 ml methyl-*tert.*-butyl-ether (MTBE) (HPLC grade, Burdick and Jackson) were added. The samples were vortexed for 30 s and centrifuged for phase separation. The organic layer was removed, transferred to a clean tube and dried under nitrogen using a warm waterbath (below 45°C). The residues were reconstituted in 100 μl ethyl acetate, and transferred to autosampler vials.

### 2.5. Recovery, calibration and quantitation

Analytical and calibration standards were analyzed in the same chromatographic run during validation, to calculate relative recovery. The raw data was collected using Multichrom software, and the standards were calibrated with a linear fit program using $1/x$ weighting. The following calculations were used: area ratio $= mx + b$ ($m$ = slope, $b$ = intercept and $x$ = concentration of anastrozole in ng/ml) where the area ratio = area (anastrozole)/area (internal standard). To solve for $x$: $x = [\text{area ratio} - b]/m$.

### 2.6. Specificity and plasma interferences

Plasma was collected from 20 healthy, drug-free volunteers, as well as from individuals after ingesting caffeine, aspirin, acetaminophen, ibuprofen, aldactone, spironolactone, propranolol, NPH insulin, vitamins and folic acid. The effect of hemolysis was tested by taking a sample of whole blood from a drug-free volunteer and splitting it in half. One half of this sample was frozen at −70°C, while the other half was centrifuged to separate the plasma from red blood cells. The frozen-then-thawed whole blood was mixed with the separated plasma in the following percentages: 90:10, 80:20, 70:30, 60:40 and 50:50 plasma–hemolyzed blood. These samples were extracted using the tested method, and analyzed for recovery and interferences. Instrument carry-over was also investigated by running blank plasma samples and ethyl acetate after the highest concentration standard.

### 2.7. Accuracy and precision

Recovery was calculated by comparing the peak areas of the extracted plasma standard curve to that of the analytical standard curve of identical theoretical concentrations. The analytical and extracted standards were analyzed during the same run on each day of validation. Inter-day and intra-day statistics, including mean and R.S.D., were generated for each standard concentration and QC level during each run.

### 2.8. Cross-validation

A blinded cross-validation study was conducted with laboratories in the US, UK and Japan. Spiked matrix samples were prepared and analyzed in each of the three laboratories, with the remainder of each sample sent to one other laboratory for analysis and comparison. The samples included concentrations in the range of 3.0 to 100 ng/ml, as well as blanks and samples higher than 100 ng/ml, which had to be diluted, and analyzed again. Each standard was prepared in duplicate and had to be quantitated and calculated to be ±15% of the theoretical concentration. If both standards did not meet this criteria, it would not be used in the calibration. If two standards at the limits of quantitation concentrations were

unacceptable, the assays limit of quantitation would change accordingly for that run, and all samples not bracketed by the new limits would have to be reanalyzed with acceptable standards at the limit of quantitation.

### 2.9. Stability of samples

Stability of anastrozole and the internal standard was tested in the extraction and injection solvents, as well as in thawed spiked plasma standards. Stability was also tested in frozen QC samples at all four concentrations, which had been in long-term storage (2 years at −10°C).

## 3. Results

### 3.1. Specificity and plasma interferences

Although small peaks were occasionally evident at the retention times of anastrozole and the internal standard in blank samples, their sizes were relatively small and did not interfere with the quantification of either compound. However, as the number of samples analyzed increased, GC maintenance was done more regularly, and this was shown to decrease carry-over. Three syringe washes of MeOH and EtAc after each sample injection, and regular maintenance of the GC injection liner and columns kept carry-over to a maximum of only 0.1%. It was also determined that hemolysis had no effect on the recovery of anastrozole or the internal standard.

### 3.2. Limits of detection, quantitation and linearity

The limit of detection for this assay is approximately 0.5 ng/ml, but the precision of the concentration determination was poor at this low concentration, due to background noise interference. The limit of quantitation was defined as three times background noise. A 3.0 ng/ml concentration was as low as this method could reproducibly quantitate anastrozole. The validated concentration range of this assay with undiluted samples is 3.0–100 ng/ml. However, the method could be extended, by dilution with control plasma, of up to 100-fold, which increases the range to 10 μg/ml. Over the range of 3.0–10 μg/ml, a linear fit with $1/x$ weighting was used with satisfactory results. The data are fitted to a line by the equation $y=mx+b$, where $y$ = the anastrozole area/the internal standard area, $b$ = a calculated constant, and $m$ = slope. An $R$-square value was calculated, which is the square of the correlation coefficient. Over the six days of validation, the mean $R$-square value is 0.997, with a R.S.D. of 0.19%; the mean slope value is 0.00740, with a R.S.D. of 6.0%; the standard error was 0.0144. Fig. 2 shows typical chromatograms for an extracted control plasma blank, the 3.0 and 100 ng/ml extracted standard, and the 100 ng/ml analytical standard.

### 3.3. Accuracy and precision

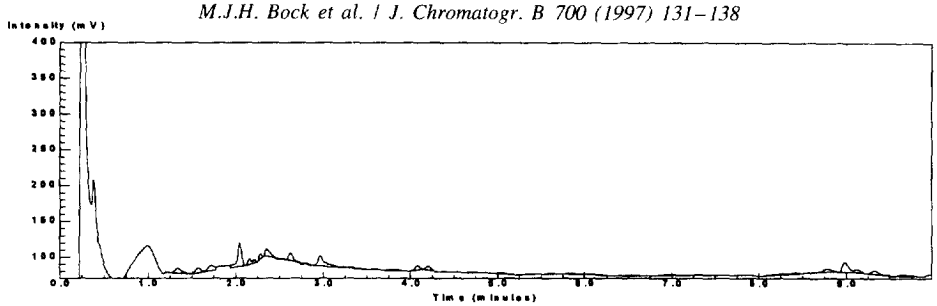
The average extraction recovery of anastrozole was 98.5% (91–109%) over the validated range of 3.0 to 100 ng/ml. Accuracy and precision statistics are summarized in Tables 2 and 3. The cross-validated samples were analyzed in two of the labs (US and UK or US and Japan), and a graph comparing these data can be found in Fig. 3. Both US/Japan and US/UK legs of the cross-validation show that the assay is rugged, performs comparably between the laboratories tested, and that it is valid to use this method to analyze study samples in any of the tested laboratories. During validation, the limits of quantitation did not change, and this occurred only rarely during study sample analysis.

### 3.4. Stability of samples during extraction and analysis

Stability of anastrozole was tested over the concentration range of 3.0 to 100 ng/ml. The drug substance is stable for at least seven days in human plasma at room temperature, 3 h in the extraction solvent (MTBE), and 72 h in injection solvent (EtAc). Long-term stability in human plasma has also been determined for 2 years when stored at −10°C.

### 3.5. Proof of applicability

This assay was used to analyze samples from a clinical study after anastrozole was administered orally to volunteers at 1 mg/day. Quality control

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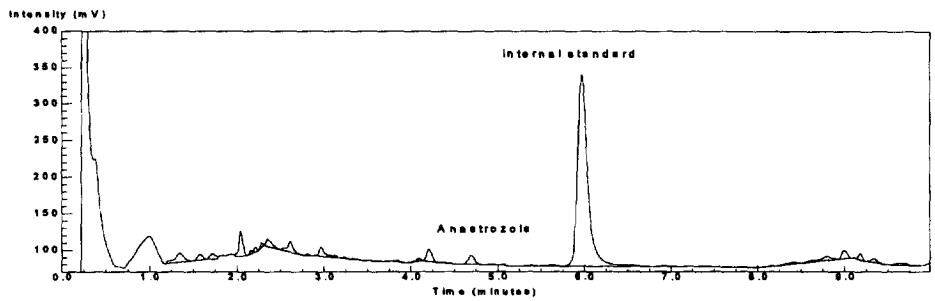


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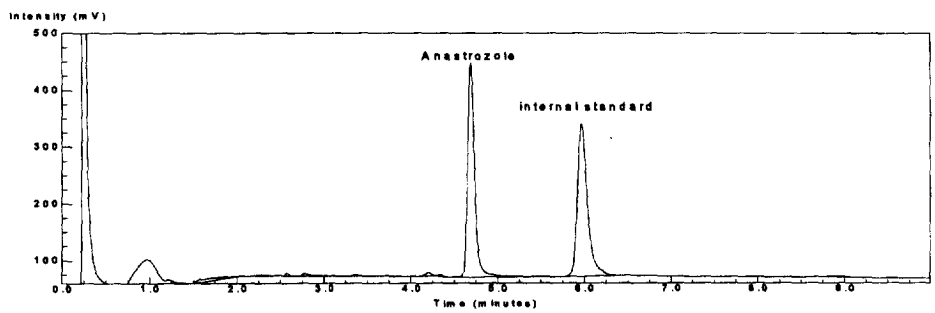


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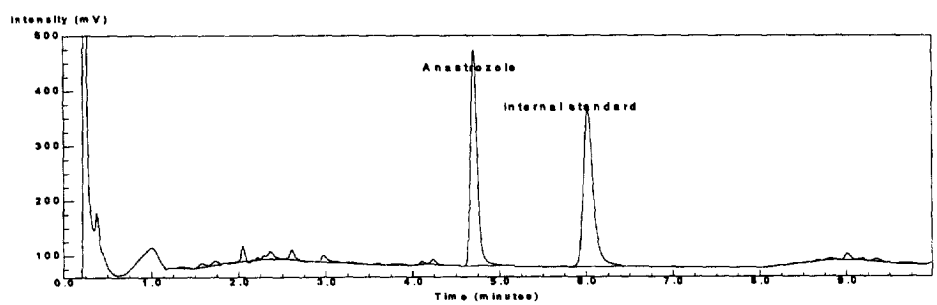


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Fig. 2. Typical chromatograms: (A) extracted blank human plasma; (B) 3.0 ng/ml extracted standard; (C) 100 ng/ml analytical standard; (D) 100 ng/ml extracted standard.

Table 2
Inter-day accuracy and precision statistics generated during validation for extracted plasma samples and quality control samples

| Standard theoretical concentration (ng/ml) | Mean calculated concentration | R.S.D. | *n* | Relative recovery (%) |
|---|---|---|---|---|
| 3 | 3.06 | 4.3 | 12 | 98.6 |
| 5 | 4.65 | 6.5 | 12 | 96.8 |
| 7 | 6.87 | 6.0 | 12 | 102 |
| 10 | 9.78 | 2.2 | 12 | 103 |
| 30 | 31.5 | 2.5 | 12 | 109 |
| 50 | 50.5 | 7.2 | 12 | 96.0 |
| 70 | 71.4 | 2.4 | 12 | 95.0 |
| 86 | 84.7 | 5.0 | 12 | 94.1 |
| 100 | 96 | 5.4 | 11 | 91.2 |
| 7.5 QC | 7.08 | 7.8 | 11 | – |
| 15 QC | 14.7 | 8.0 | 12 | – |
| 40 QC | 38.1 | 8.1 | 11 | – |
| 75 QC | 78.1 | 5.9 | 12 | – |

Table 3
Intra-day accuracy and precision statistics generated during validation for extracted plasma samples and quality control samples ($n=2$ unless R.S.D. = –)

| Standard/QC (ng/ml) | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 |
|---|---|---|---|---|---|---|---|
| 3.0 | Mean | 3.11 | 3.05 | 3.10 | 2.80 | 3.18 | 3.09 |
| | R.S.D. | 0.410 | 0.0200 | 0.620 | 0.780 | 3.90 | 1.00 |
| 5.0 | Mean | 4.09 | 4.86 | 4.66 | 4.71 | 4.81 | 4.76 |
| | R.S.D. | 1.30 | 3.20 | 5.40 | 5.80 | 1.90 | 3.90 |
| 7.0 | Mean | 6.40 | 6.88 | 6.78 | 7.63 | 6.76 | 6.81 |
| | R.S.D. | 2.00 | 2.60 | 3.50 | 4.10 | 2.70 | 0.100 |
| 10.0 | Mean | 9.73 | 9.83 | 9.89 | 9.83 | 9.61 | 9.79 |
| | R.S.D. | 1.10 | 2.90 | 0.150 | 5.50 | 0.680 | 1.60 |
| 30.0 | Mean | 31.3 | 31.4 | 32.0 | 31.4 | 31.8 | 31.3 |
| | R.S.D. | 0.710 | 1.20 | 3.2 | 5.60 | 3.80 | 0.0810 |
| 50.0 | Mean | 46.2 | 50.8 | 52.5 | 51.8 | 49.7 | 52.4 |
| | R.S.D. | 20.3 | 0.170 | 0.370 | 3.10 | 1.40 | 1.30 |
| 70.0 | Mean | 71.2 | 70.9 | 72.3 | 70.7 | 71.8 | 71.6 |
| | R.S.D. | 0.220 | 0.13 | 3.1 | 5.80 | 2.80 | 2.20 |
| 86.0 | Mean | 87.4 | 85.3 | 80.7 | 83.3 | 84.5 | 86.9 |
| | R.S.D. | 2.40 | 0.590 | 12.8 | 5.70 | 1.40 | 0.450 |
| 100.0 | Mean | 94.0 | 98.0 | 99.1 | 98.7 | 98.8 | 88.2 |
| | R.S.D. | 2.20 | – | 0.970 | 2.40 | 0.110 | 9.90 |
| 7.5 QC | Mean | 7.29 | 6.34 | 7.48 | 6.72 | 7.00 | 7.45 |
| | R.S.D. | 3.00 | 12.7 | 7.70 | – | 0.410 | 4.10 |
| 15 QC | Mean | 13.5 | 13.3 | 16.2 | 14.8 | 15.3 | 15.1 |
| | R.S.D. | 5.30 | 6.30 | 4.00 | 4.00 | 5.50 | 1.40 |
| 40 QC | Mean | 39.1 | 34.6 | 38.4 | 37.3 | 38.3 | 40.6 |
| | R.S.D. | 11.8 | 16.1 | 4.20 | – | 0.480 | 2.20 |
| 75 QC | Mean | 76.5 | 71.6 | 83.2 | 76.9 | 78.6 | 81.8 |
| | R.S.D. | 1.50 | 10.7 | 0.560 | 0.610 | 1.00 | 0.770 |

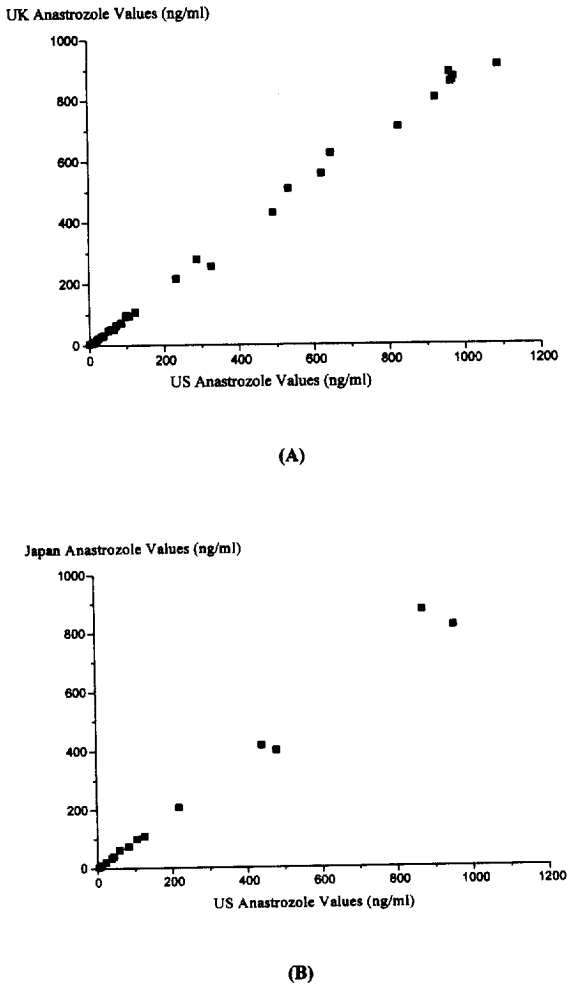


Fig. 3. Cross validation results between US/UK (A) and US/Japan (B) laboratories.

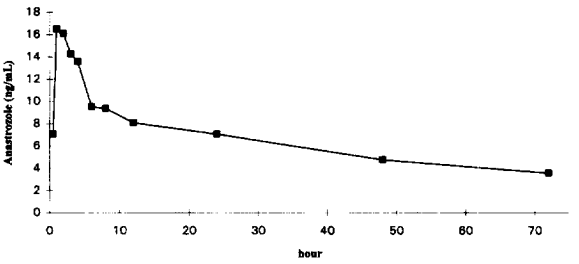


Fig. 4. Representative pharmacokinetic profile from one volunteer given 1 mg/day of anastrozole during a clinical study.

samples were analyzed during each run in duplicate to monitor the performance of the assay. Throughout the entire study, only 14 (4%) of the QC values were out of range (±15% of theoretical concentration), with at least one QC sample within acceptable range at each concentration level. The data generated from preparing clinical samples by this method was used to calculate pharmacokinetic parameters to support the clinical package submitted to the FDA. Table 4 summarizes the QC data from one clinical study, and Fig. 4 is a pharmacokinetic profile for one volunteer whose samples were analyzed using this method.

## 4. Conclusions

A method for the quantitation of anastrozole in human plasma has been developed and validated. The assay has also been successfully cross-validated in three independent laboratories, and was then used to analyze samples collected from clinical studies. These concentration values were then used to calculate pharmacokinetic data to support the NDA package for Arimidex, recently approved by the FDA.

Table 4
Quality control sample statistics of a clinical study of 40 analytical runs using the validated method

| Theoretical concentration (ng/ml) | Actual concentration (ng/ml) | R.S.D. (%) |
|---|---|---|
| 7.5 | 7.54 | 4.7 |
| 15 | 14.8 | 4.9 |
| 40 | 40.9 | 4.8 |
| 75 | 79.0 | 4.9 |

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