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A simple, selective validated method for simultaneous estimation of zuclomifene and enclomifene in human plasma by liquid chromatography - tandem mass spectrometry

Arvind G.Jangid[1,2]*, Rajesh H.Tale[1], Vikas V.Vaidya[1]

[1]School of Chemical Sciences, Swami Ramanand Teerth Marathwada University, Nanded - 431 606, (INDIA)

[2]Accutest Research Laboratories (I) Pvt.Ltd, S.G. Highway, Ahmedabad - 380 054, (INDIA)

E-mail: jangid_arvind@rediffmail.com

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ABSTRACT

A simple and selective method for estimation of zuclomifene and enclomifene in human plasma was developed and validated using glipizide as internal standard. The analytes were chromatographed on ACQUITY UPLC® BEH C18 1.7µ (2.1 mm x 100 mm) reverse phase column under isocratic conditions using 5 mM Ammonium Acetate in water (pH 4.00 ± 0.05): methanol (38:62 v/v) as the mobile phase and detected by tandem mass spectrometry in positive ion mode. The ion transition recorded in multiple reaction monitoring mode were m/z 406.2→ 100.0 for zuclomifene and enclomifene and *m/z* 446.1→321.0 for internal standard. Simple solid phase extraction method used for extraction of analytes and internal standard from plasma. Linearity in plasma was observed over the concentration range 0.102 – 15.225 ng/mL for zuclomifene and 0.149 – 22.275 ng/mL for enclomifene. The method was validated for sensitivity, matrix effect, accuracy and precision, linearity, recovery and stability studies. The lower limit of quantification of zuclomifene and enclomifene was 0.102 ng/mL and 0.149 ng/mL respectively. The mean recovery for zuclomifene and enclomifene was 88.2 % and 85.7 % respectively. The coefficient of variation of the assay was less than 11.7 % and 12.3 % and accuracy of 91.3% to 105.7% and 91.2% to 106.6% for zuclomifene and enclomifene respectively. The validated method can be applied to pharmacokinetic study of clomifene.

KEYWORDS

Clomifene;
Zuclomifene;
Enclomifene glipizide;
LC-MS-MS;
Human plasma.

INTRODUCTION

Clomifene citrate is a synthetic anti-oestrogen, structurally related to diethylstilbestrol, and is a first choice therapy to treat women absent or irregular ovulation due to hypothalamic-pituitary dysfunction associated with normal basal concentration of endogenous oestradiol[1,2]. Clomifene is a mixture of two geometric isomers (zuclomifene and enclomifene, Figure 1a and 1b respectively). The United States Pharmacopoeia (USP) specifies that official clomifene citrate preparations must contain between 30% and 50% of the Z isomer[3]. However, significant differences in the biological activities of the isomers have been demonstrated both in vitro and in vivo[4,5]. Clinical trials utilizing purified isomers have shown that zuclomifene is

much more potent in ovulation induction and may actually account for most of activity of the clomifene citrate formulation[6].

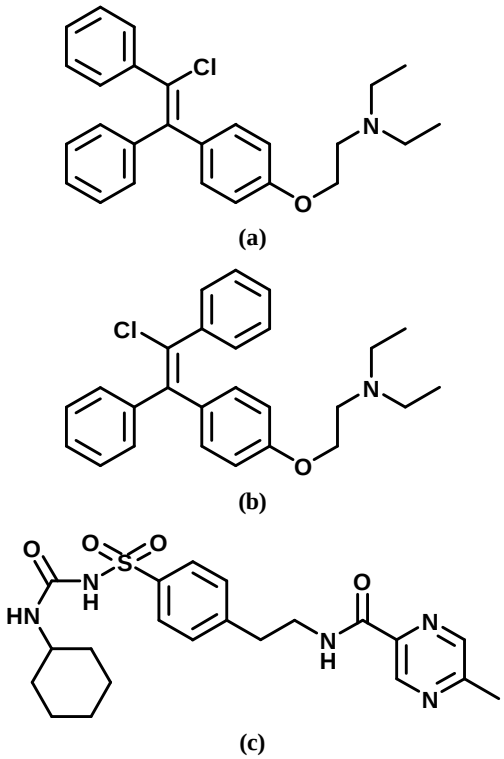

Figure 1 : Chemical structure for (a) Zuclomifene, (b) Enclomifene and (c) Glipizide

Methods for the measurement of the isomers have been reported on HPLC using post-column photochemical derivatisation followed fluorescence detection[7-10]. Although these methods reported detection limits of less than 1 ng/mL, some required the use of up to 5 mL of plasma volume per sample. A LC-MS method for simultaneous determination of clomiphene isomers in plasma of patients has been reported with a LOD (limit of determination) of 35 pg/mL and 7 pg/mL for zuclomifene and enclomifene respectively[11]. This method involved 1 mL plasma volume and liquid-liquid extraction method in sample preparation. A 100µL sample injected on column. A LC/MS/MS method has reported for stereoselective quantitation of clomifene in human plasma[12]. The method involved solid phase extraction in samples preparation using 500 µL of plasma sample. A LLOQ of 0.174 ng/mL and 0.218 ng/mL reported for zuclomifene and clomifene respectively.

In the present study a robust, simple and selective method have been developed and validated to estimate zuclomifene and enclomifene in human plasma, with a total run time of 10 min. A very good chromatographic separation achieved between these two isomers using small particle size column. A 300µL of plasma volume used in sample preparation. Solid phase extraction technique has been applied which ensured a lot more sample clean up resulting in better selectivity and consistent recovery. Glipizide (Figure1c) was used as an internal standard.

EXPERIMENTAL

Materials and chemicals

The working standard of clomifene citrate as mixture of two isomers (zuclomifene/ Enclomifene: 40.6:59.4) procured from Synthon (Netherlands) and Glipizide was obtained from USV (Mumbai, India). HPLC grade methanol and acetonitrile were purchased from J.T. Baker INC (Phillipsburg, NJ, USA). Formic acid of AR grade was procured from Merck Ltd (Mumbai, India). Phenomenex Strata-X (30mg, 1mL) SPE cartridges were procured from Spincotech Pvt. Ltd., (India). Milli- Q water purification system from Millipore (Bangalore, India) used for preparation of Type-I reagent grade water. Blank human plasma with Na Heparin as anticoagulant was used for preparation and calibration and quality control samples.

Liquid chromatography and mass spectrometric conditions

The liquid chromatography system (Waters, Massachusetts, USA) coupled with mass spectrometer consisted of acquity UPLC binary solvent manager, autosampler and temperature controlled compartment for column. The small particle size analytical column, ACQUITY UPLC® BEH C18 1.7µ (2.1 mm x 100 mm) from waters (Massachusetts, USA) was used for separation of analyte and internal standard. A buffer of 5 mM ammonium acetate in water (pH 4.00 ± 0.05, adjusted with acetic acid) and methanol in ratio of 38:62 (*v/v*) was pumped at flow rate of 0.230 mL/min a mobile phase. Auto sampler temperature was set at 5 °C and the injection volume was 7µL. The column oven temperature was maintained at 40 °C and the total LC run time was 10 min.

The Waters Quattro Premier XE (Waters, Massa-

chusetts, USA) LC-MS-MS apparatus was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion *m/z* 406.2 to the product ion *m/z* 100.0 for zuclomifene and enclomifene and the transition of the protonated molecular ion *m/z* 446.1 to the product ion *m/z* 321.0 for the internal standard, Glipizide. The instrument response was optimized for clomifene isomers and glipizide by infusing a constant flow of a solution of the drug dissolved in mobile phase.

Electrospray ionization (ESI) was performed in the positive ion mode. The source temperature and desolvation temperature were 100 and 420°C respectively. Capillary voltage of 3.5 KV and extractor voltage of 2 V was used. Argon was used as the collision gas. The desolvation and cone gas optimized were 1050 L h^{-1} and 100 L h^{-1} respectively. Compound dependant parameters set for clomifene isomers and IS were cone voltage: 45 and 25 V; collision energy: 28 and 14 eV respectively. Q1 and Q3 were maintained at unit resolution and the dwell time was kept at 250ms. The collision activated dissociation (CAD) gas pressure was set at 2.5 e^{-3} mbar. Argon gas was used as CAD gas.

The instrument was interfaced with a computer running mass lynx version 4.1 software.

Preparation of standards and quality control samples

A 0.4mg /mL stock solution for clomifene (con. of 162.400 µg/mL and 237.600 µg/mL for zuclomifene and enclomifene respectively) and Glipizide were prepared by dissolving their accurately weighted compounds in methanol. Two separate stock solutions of clomifene were prepared for bulk spiking of calibration curve and quality control samples for the method validation experiment as well as the subject sample analysis. The stock solution of clomifene thus prepared was serially diluted to prepare working solution in required concentration range with diluent (methanol: water, 60:40 *v/v*). The calibration standards and quality control (QC) samples were prepared by spiking (5 % of the total plasma volume) with working solutions. Calibration standards were prepared at concentration of 0.102, 0.203, 0.508, 1.523, 3.045, 6.090, 9.135, 12.180, and 15.225 ng /mL for zuclomifene and at concentration of 0.149, 0.297, 0.743, 2.228, 4.455, 8.910, 13.365, 17.820, and 22.275 ng /mL for enclomifene. Similarly, quality control standards (QC's) were prepared at five different concentrations namely, 0.104 (LLOQ), 0.284 (LQC), 2.233 (M_1QC), 6.740 (M_2QC) and 11.368 (HQC) ng /mL for zuclomifene and at concentrations 0.151 (LLOQ), 0.416 (LQC), 3.267 (M_1QC), 9.860 (M_2QC) and 16.632 (HQC) ng /mL for enclomifene. Sufficient calibration standards and quality control standards were prepared to validate the method. Aliquots of the standards and quality controls were stored at -70 °C until used for sample processing.

Extraction procedure

The plasma samples (300 µl) were transferred to 1.7-mL clear tubes (Tarsons, India) added 30µl of internal standard (working solution of 0.8 µg /mL of Glipizide), vortexed to mix.. A 300 µl of 5% orthophosphoric acid in water (*v/v*) added to the sample. The samples were vortexed to mix for 30 sec and centrifuged for 5 minutes at 14000 rpm. After centrifugation the samples were loaded on phenomenex strata-X 30mg/1mL cartridge pre-conditioned with 1 mL methanol followed by 1 mL water. The plasma matrix was drained out from the extraction cartridges by applying positive nitrogen pressure. The extraction cartridges washed with 2 mL of 5% (*v/v*) methanol in water. The analytes and the internal standard were eluted with 1.0 mL of methanol. The collected samples dried under nitrogen at 60°C, and reconstituted with 300 µl of methanol: Buffer (60: 40). A 7 µL sample was injected.

Method validation

Selectivity

Selectivity was performed using 10 different sources of blank plasma comprising of 6 normal, two haemolysed and two lipemic. They were processed as per the extraction method and their response was assessed at the retention time of analytes and the internal standard with six LLOQ samples for zuclomifene and enclomifene prepared in the screened blank plasma with minimum interference.

Carry over

Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs. The design of the experi-

ment comprised blank plasma, LLOQ, upper limit of quantitation (ULOQ) followed by blank plasma to check for any possible interference due to carryover.

Linearity and lower limit of quantification

The linearity of the method was determined by analysis of three calibration curve plots associated with nine-point calibration standards. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. The calculation was based on the peak area ratio of analyte versus the area of internal standard. The concentration of the analyte were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x^2$). The linear regression (weighted with $1/concentration^2$) was found to be the simplest regression, giving the best results (r^2= 0.9948 and = 0.9938 for zuclomifene and enclomifene respectively). The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. The deviation of standards nominal concentration should not be more than ±15.0% except for LLOQ where it should not be more than ±20.0%.

Accuracy and precision

The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the five quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analysed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of relative error (%RE) and coefficient of variation (% CV) respectively.

Matrix effect

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal blank plasma samples in replicate (*n*=4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found were well within the acceptable limit set i.e. the RSD of area ratio to be within ± 15% at each level tested.

Recovery

Absolute recoveries of the analyte were determined at the three different quality control levels viz. LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the un-extracted samples (prepared in the reconstitution solution at the same concentrations as the extracted samples) representing 100% recovery.

Dilution integrity

The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of ½ and ¼th concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

Stability

All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against comparison samples of identical concentration. Stock solutions of zuclomifene, enclomifene and IS were checked for short term stability at room temperature and long term stability at 2-8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Bench top stability, autosampler stability (process stability), freeze thaw stability, and long-term stability in plasma were performed at LQC and HQC level using six replicates at each level. Freeze-thaw stability was evaluated by successive cycles of freezing (at -70°C) and thawing (without warming) at room temperature. To meet the acceptance criteria, the difference between the stability and fresh samples should be within ±15%.

RESULTS AND DISCUSSION

The mean absolute recoveries of zuclomifene determined at 0.284, 2.233, 6.740 and 11.368 ng /mL were 92.7% (RSD 5.6 %), 86.6.7 % (RSD 6.2 %), 86.1 % (RSD 3.5 %) and 87.3 % (RSD 3.2 %), re-

spectively. The mean absolute recoveries of enclomifene determined at 0.416, 3.267, 9.860 and 16.632 ng /mL were 92.7% (RSD 5.6 %), 86.6.7 % (RSD 6.2 %), 86.1 % (RSD 2.7 %) and 87.3 % (RSD 3.0 %), respectively. The mean absolute recovery of glipizide was 102.3 % (RSD 5.2 %).

Minimal matrix effect for analytes and internal standard was observed from the six different plasma lots tested. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 1.6 % and 1.2 for zuclomifene and enclomifene respectively. For the internal standard the RSD of the area response over both LQC and HQC levels was less than 3.0 %. This indicated that the extracts were "clean" with no co-eluting compounds influencing the ionization of the analytes and the internal standard.

The high selectivity of MS-MS detection allowed the development of a very specific and robust method for the determination of zuclomifene and enclomifene in plasma. Representative chromatograms obtained from blank plasma and blank plasma spiked with LLOQ standard is presented Figure 2(a) and Figure 2(b) respectively. No significant interfering peak of endogenous compounds was observed at the retention time of analytes in blank human plasma containing Na Heparin as the anti-coagulant in ten different plasma lots which was compared versus six replicates of extracted samples at the LLOQ level.

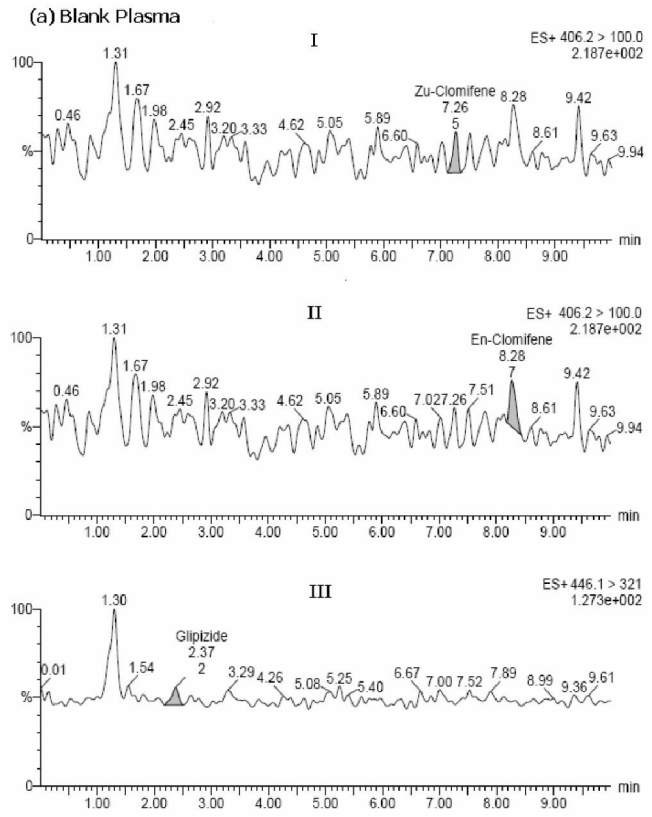

Figure 2a : Chromatogram of zuclomifene (I), enclomifene (II) and glipizide (internal standard, III) for blank plasma.

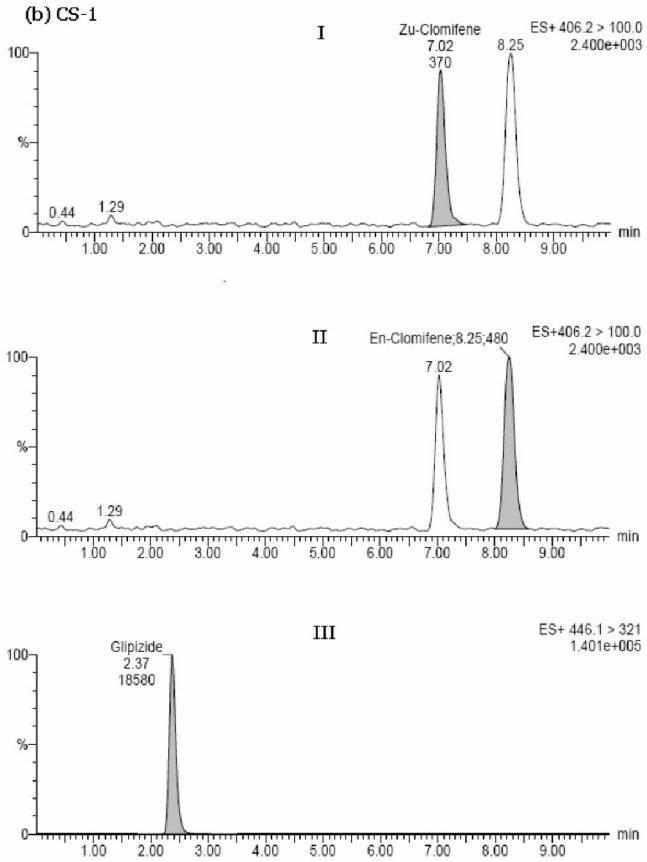

Figure 2b : Chromatogram of zuclomifene (I), enclomifene (II) and glipizide (internal standart, III) at LLOQ level.

The LLOQ, defined as that concentration of analyte which can still be determined with acceptable precision (%RSD < 20) and accuracy (bias within ± 20%) was found to be 0.102 and 0.149 ng /mL for zuclomifene and enclomifene respectively. Results of the intra-batch and inter-batch validation assays are presented in TABLES 1 and 2, respectively. The inter-batch and intra-batch precision were = 11.7 % and = 9.6 % whereas the inter-batch and intra-batch accuracy in terms of % bias were within the range of -1.4 to -8.7 and 0.0 to 5.8 for zuclomifene respectively. The inter-batch and intra-batch precision were = 12.3 % and = 4.4 % whereas the inter-batch and intra-batch accuracy in terms of % bias were within the range of -2.0 to -8.8 and 3.4 to 6.6 for enclomifene respectively.

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TABLE 1 : Intra-batch precision and accuracy (*n*=6) of zuclomifene and enclomifene in human plasma

Analyte	Spiked conc. (ng/mL)	Mean calculated conc. (ng/mL)	% RSD	% Bias
Zuclomifene	0.104	0.104	9.6	0.0
	0.284	0.296	3.7	4.2
	2.233	2.362	3.9	5.8
	6.740	7.041	4.7	4.5
	11.368	11.963	2.2	5.2
Enclomifene	0.151	0.161	4.4	6.6
	0.416	0.435	1.4	4.6
	3.267	3.484	3.2	6.6
	9.860	10.214	4.5	3.6
	16.632	17.195	2.8	3.4

TABLE 2 : Inter-batch precision and accuracy (*n*=6) of zuclomifene and enclomifene in human plasma

Analyte	Spiked conc. (ng/mL)	Mean calculated conc. (ng/mL)	% RSD	% Bias
Zuclomifene	0.104	0.101	7.9	-2.9
	0.284	0.28	8.2	-1.4
	2.233	2.171	9.5	-2.8
	6.740	6.156	11.7	-8.7
	11.368	10.637	9.0	-6.4
Enclomifene	0.151	0.148	10.1	-2.0
	0.416	0.404	8.7	-2.9
	3.267	3.196	10.7	-2.2
	9.860	8.988	12.3	-8.8
	16.632	15.468	8.2	-7.0

Bench top and processed (autosampler) stability for zuclomifene and enclomifene were performed at LQC and HQC levels. The results revealed that both analytes were stable in plasma for at least 12 h at room temperature and 56 h in auto sampler at 5 °C. It was confirmed that repeated freeze and thawing (five cycles) of spiked plasma samples at LQC and HQC level did not affect the stability of zuclomifene and enclomifene. The long term stability results also indicated that zuclomifene and enclomifene were stable in human plasma for up to 112 days at a storage temperature of -70 °C. This period of long term stability was sufficient enough to cover the entire storage period from first day of storage of the plasma samples to the last day of analysis.

During method development different options were evaluated to optimize sample extraction, detection parameters and chromatography. Best signal for the analyte was achieved with the ESI positive ion mode. 5% Orthophosphoric acid used in sample pretreatment to achieve good recovery. Solid phase extraction method optimized for sample preparation, which enables sufficient sample clean up. A mobile phase containing buffer 0.1 % (*v/v)* formic acid, ammonium acetate and formate salt at different molarity and acetonitrile in varying combinations was tried during the initial development stages. The effect of pH of buffer also checked on sensitivity, peak shape and resolution for zuclomifene and enclomifene. But the best separation, sensitivity and peak shape for zuclomifene and enclomifene was achieved using a mobile phase of 5mM ammonium acetate in water (pH 4.00 ± 0.05, adjusted with acetic acid) in combination with methanol (28:62 *v/v*). Use of small particle size column ACQUITY UPLC® BEH C18 1.7µ (2.1 mm x 100 mm) column resulted in desired separation for zuclomifene and enclomifene at flow rate (0.230 ml/min). The retention times for zuclomifene and enclomifene were ~ 7.0 and 8.3 minutes respectively. Retention time for glipizide was ~ 2.4 minutes.

Glipizide used as internal as ionization and extraction characteristics of glipizide were found to be similar to that of zuclomifene and enclomifene and hence it was selected as the internal standard of choice.

The validated method can be employed for analysis of zuclomifene and enclomifene in plasma.

CONCLUSION

A robust, simple and selective method for the simultaneous estimation of zuclomifene and enclomifene in human plasma was developed and validated, using ultra high-performance liquid chromatographic separation with tandem mass spectrometric detection. The validated method was used in a pharmacokinetic study in which 12 healthy volunteers were enrolled; each subject dosed 50mg clomifene citrate as a single oral dose. With a LLOQ of 0.102 ng /mL and 0.149 ng/mL for zuclomifene and enclomifene respectively. The method allows simultaneous estimation zuclomifene and enclomifene, two isomers of clomifene and involves simple sample preparation. Robust LC-MS-MS performance was observed, with acceptable variation in

instrument response within batches. This method is an excellent analytical option for rapid quantification of clomifene isomers in human plasma.

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