RESEARCH ARTICLE

Quantization of Metaxalone in Human Plasma using High - Performance Liquid Chromatography- Tandem Mass Spectroscopy

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ABSTRACT

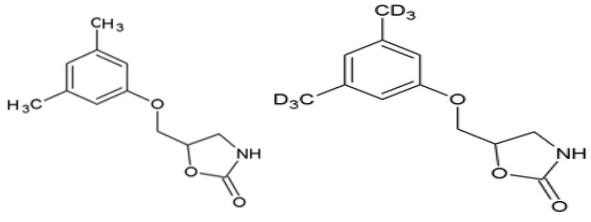
Present study reports the development and validation of Metaxalone in human plasma by LC-MS/MS using electron spray ionization technique. Metaxalone-d6 was used as internal standard. The chromatographic separation of analyte and internal standard was achieved by using ACE 5µ C18, 100 X 4.6 mm column as stationary phase and 10 mM Ammonium Acetate: Methanol with Acetonitrile (60:40) as mobile phase at a flow-rate of 1 ml/min. MS detection was performed at transitions of m/z 222.100/161.200 and 228.200/167.200 in multiple reaction monitoring for Metaxalone and Metaxalone-d6 at positive mode. Metaxalone was extracted from the plasma by liquid-liquid extraction using 0.2M NaOH as treatment buffer and Ethyl acetate as extraction solvent. The present method was found to be linear over the concentration range of 25.006 - 12059.526 ng/ml (r^2-0.9983). The limit of quantification of Metaxalone in plasma was found to be 25.006ng/ml. The retention times of Metaxalone and Metaxalone-d6 were found to be 2.01 min and 1.98 min, respectively. The analyte was found to be stable under various stability tests such as freeze-thaw, bench top, wet extract, dry extract, auto sampler and interim studies. This simple, rapid and specific validated method was successfully applied for the faster analysis of Metaxalone in human plasma in bioavailability and bioequivalence studies.

Keywords: LC-MS/MS, human plasma, Metaxalone, Metaxalone-d6, liquid-liquid extraction, validation.

INTRODUCTION

Metaxalone, 5-(3, 5-dimethylphenoxymethyl)-1, 3-oxazolidin-2-one is a muscle relaxant used to relax muscles and relieve pain caused by strains, sprains, and other musculoskeletal conditions with relatively low side effects. The mechanism of action of metaxalone in humans has not been established, but may be due to general central nervous system depression. Metaxalone has no direct action on contractile mechanism of striated muscle, the motor end plate, or the nerve fibre.

Various analytical methods, such as spectrophotometry[1-3], HPLC with Diode array detection[4], HPLC with UV detection[5], RP-UPLC[6]. LC-MS/MS methods have been previously reported for the estimation of Metaxalone. A method for the quantification of Metaxalone in rat plasma using Phenytoin as an internal standard[7]. The retention time of Metaxalone and Phenytoin were found to be 1.60 and 1.83 min respectively. The calibration curve was linear (r2 > or = 0.99) ranging from 0.98 to 998 ng/ml. Another LC-MS/MS method in which Metaxalone-d3 was used as internal standard and drug was extracted from the plasma by using solid phase extraction [SPE] technique[8]. Another method where Galantamine was used as internal standard and the drug was extracted by using liquid-liquid extraction[9]. The present study describes development and validation of a simple, specific, rapid and sensitive liquid chromatography – tandem mass spectrometry [LC-MS/MS] method for the determination of Metaxalone in human plasma with a limit of quantification [LOQ] of 25.006 ng/ml during a 3 min run time using Metaxalone-d6 as internal standard. The structures of Metaxalone and Metaxalone-d6 were displayed in Figure 1.



Metaxalone Metaxalone-d6

Fig.1: Structures of Metaxalone and Metaxalone-d6

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MATERIALS AND METHODS

Reagents and chemicals:

Metaxalone [98.00 % purity], Metaxalone-d6 [98.60 % purity] were obtained from Toronto Research Chemicals Inc. HPLC grade methanol, Acetonitrile, Ammonium acetate, Ethyl acetate were purchased from Merk, Mumbai. High purity water was prepared through a Milli-Q water purification system [Synergy UV Millipore, USA].

Instrumentation:

API 3200 triple quadrupole instrument [Applied Biosystem SCIEX, Toronto, Canada] was used for mass spectroscopy. Shimadzu HPLC system [Shimadzu SIL HTC, USA] was used for chromatographic separation. Electron-spray ionization technique was used. Data was collected and processed using Analyst Software version 1.5.1 [Applied Biosystems MDS SCIEX, Toronto, Canada]. Ultra microbalance SE2 and Semi Microbalance CPA225D of Sartorius was used for weighing. A high speed desk centrifuge Sorvall Legend XTR of Thermo Scientific was used for centrifugation.

MS/MS conditions:

Detection of the ion was performed in MRM mode with positive polarity. Concentrations of 100 ng/ml and 100 ng/ml solutions of Metaxalone and Metaxalone-d6 were prepared in 80% methanol respectively for tuning the mass conditions. The precursor to product ion transitions for Metaxalone and Metaxalone-d6 were found to be m/z 222.100/161.200 and 228.200/167.200 respectively. The tuned conditions of Declustering Potential [DP] were 39V,40V; Collision energy [CE] were 16V,15V; Collision Cell Entrance Potential [CEP] were 19V,20V; Collision Exit Potential [CXP] 6V for analyte and internal standard respectively. Entrance Potential [EP] 10V, Heater temperature 500°C, Curtain gas 30 psi, Collision associated dissociation [CAD] 6 psi, Nebulizer gas [GS1] 40 psi, Heater gas [GS2] 45 psi and ion spray voltage [ISV]-5500V were optimized for both analyte and internal standard. Mass spectrums of parent and product ions of analyte and internal standard were represented in Figure 2 [A,B] and Figure 3 [A,B].

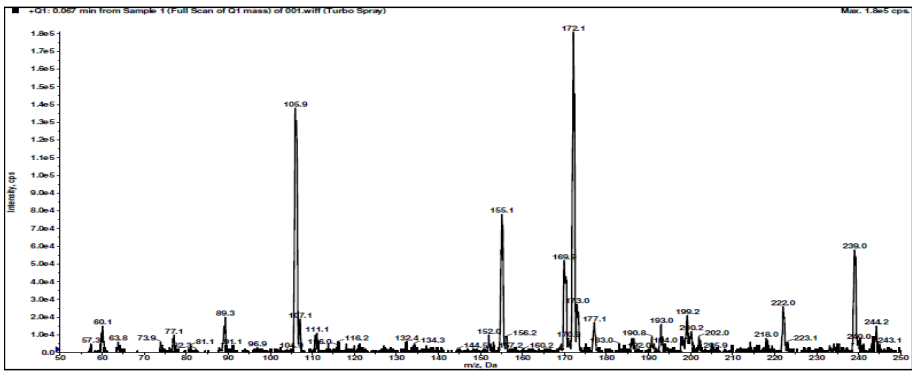

Fig. 2A: Parent ion scan of Metaxalone

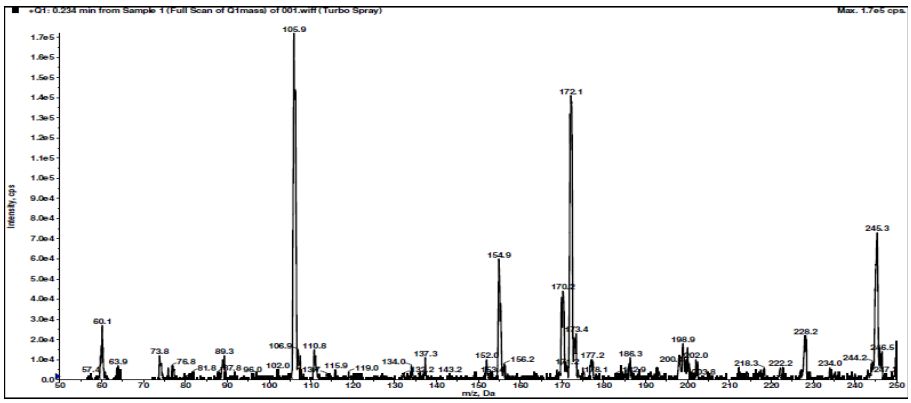

Fig. 3A: Parent ion scan of Metaxalone-d6

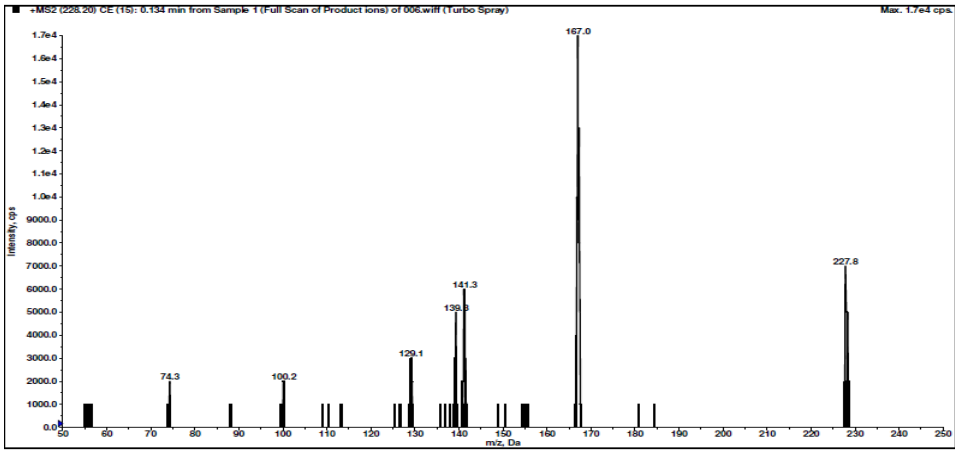

Fig. 3B: Product ion scan of Metaxalone-d6

Chromatographic conditions:

The liquid chromatographic separation was carried out using ACE 5µ C18, 100 X 4.6 mm analytical column and auto-injecton volume of 20 µl. Mobile phase was composed of 10mM Ammonium Acetate : Methanol with Acetonitrile (60:40) and was delivered at a flow rate of 1ml/min with run time of 3 min. Retention time of Metaxalone and Metaxalone-d6 were found to be 2.01 min and 1.98 min respectively [Figure 4].

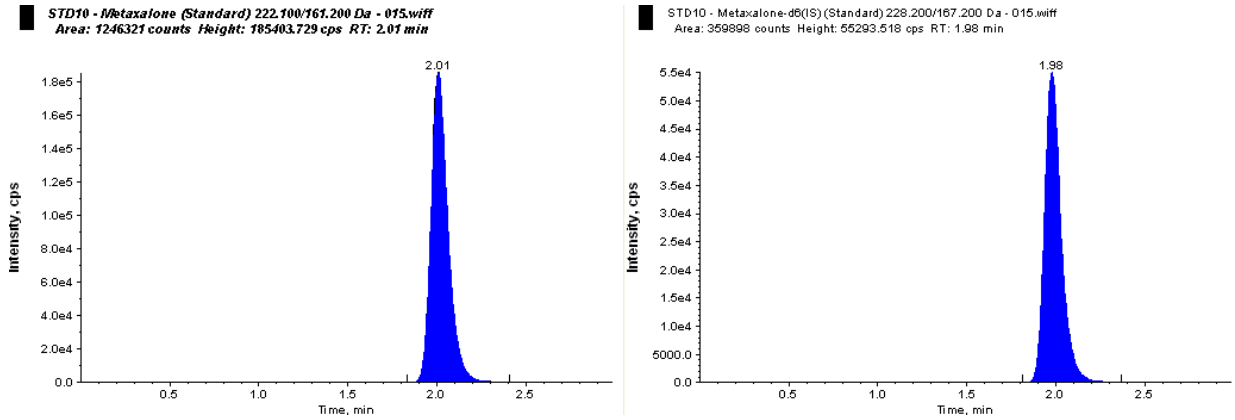

Fig.4: Retention time of Metaxalone and IS

Preparation of standards and quality control samples:

Standard stock solutions of Metaxalone and Metaxalone-d6 of concentration 2mg/ml, 0.2mg/ml were prepared in methanol. The internal standard solution was diluted to a concentration of 5µg/ml by using diluent 50% methanol. Calibration curve standard solutions were prepared by spiking stock solution into drug free human plasma to obtain 10 concentrations of 25.006, 50.012, 150.367, 601.469, 1202.938, 2405.875, 4811.751, 7235.716, 9647.621, and 12059.526 ng/ml by using diluent 50% Methanol. Three quality control [QC] samples, lower quality control [LQC] of 65.682 ng/ml, medium quality control [HQC] of 6012.040 ng/ml and higher quality control [HQC] of 9027.087 ng/ml were prepared in an analogous manner to the calibration standards. Matrix based samples were stored at -70±15^{0}C and stock solutions were stored at 2-8^0 C.

Sample preparation:

50 µl of internal standard solution [5 µg/ml] was added into labeled ria vial tubes and spiked with 100 µl of plasma sample [respective concentration] into each tube and vortexed for 5s. 100 µl of 0.2M NaOH was added to the above ria vial tubes and vortexed for 30s. Then 1.3ml of Ethyl acetate was added and vortexed. Samples were centrifused for 5 mins at

temperature of 5^{0}C. 1ml supernatant layer was separated from the vials. Evaporate to dryness at temperature of 35^{0}C, initial pressure 5psi and gradually increased to 15psi. Reconstituted with 500 µl of reconstitution solution/mobile phase and analyzed.

RESULTS AND DISCUSSION

Method validation:

The study samples, QC samples and calibration standards were processed in accordance with the validated analytical method to ensure the acceptability of the analytical run. The analytical method was validated according to the guidance of US Department of Health and Human services Food and Drug Administration[10]. Each analytical run consists of blank samples [processed matrix sample without analyte and IS] and a zero blank sample [processed matrix with IS], calibration standards at a minimum of 8-10 concentration levels, at least six sets of 3 levels QC samples [low, medium, and high] and study samples.

Specificity:

The specificity of the method was determined by comparing the blank samples and a zero blank samples with that of plasma samples spiked with analyte to find out the interferences caused by endogenous substances. This method was found to be specific. Chromatograms of blank and zero blank were represented in Figure 5 and 6.

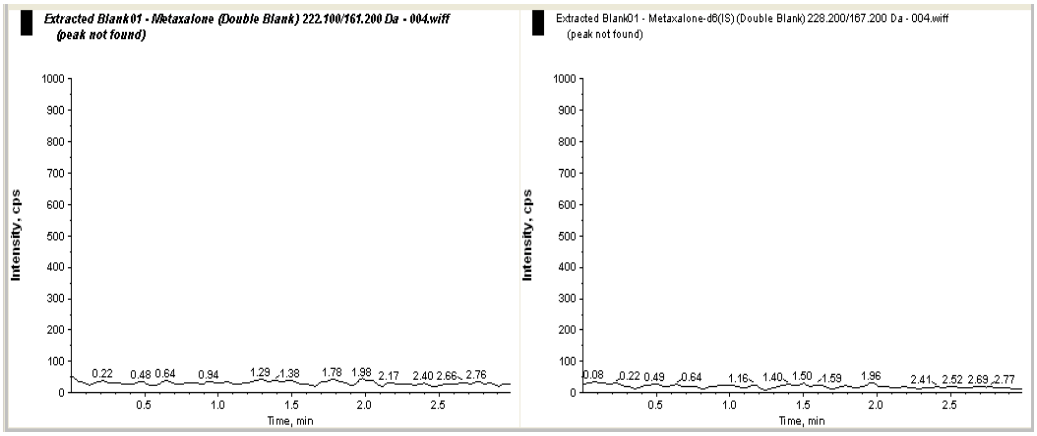

Fig.5: Blank of Analyte and IS

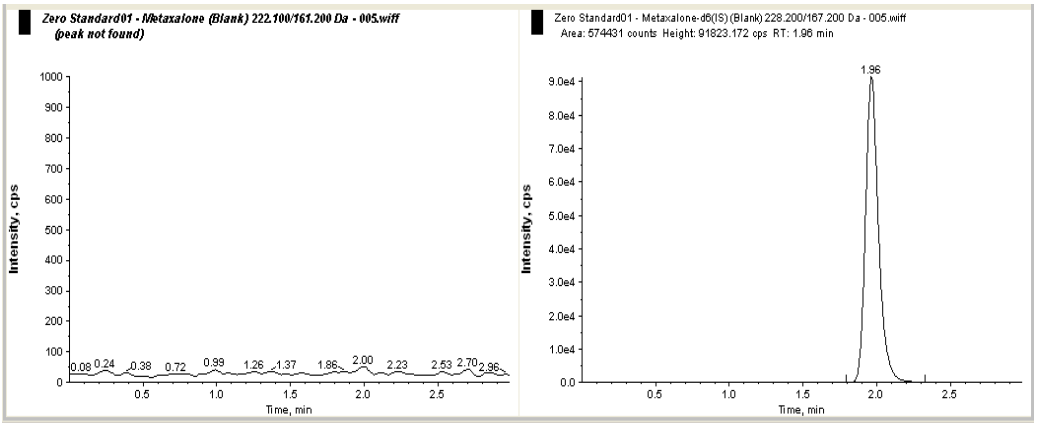

Fig.6: Zero blank of Analyte and IS

Linearity:

Calibration curve was linear over the concentration range of 25.006 - 12059.526 ng/ml for Metaxalone with correlation coefficient [r] 0.9997 [Figure 7]. The best linear fit and least square residuals for the calibration curve were achieved with a 1/x^2 weighing factor, giving a mean linear equation for the calibration curve in the form of y = mx + c, Where y was the peak area ratio of Metaxalone to Metaxalone-d6 and x was the concentration of Metaxalone. The lower limit of quantification was found to be 25.006 ng/ml.

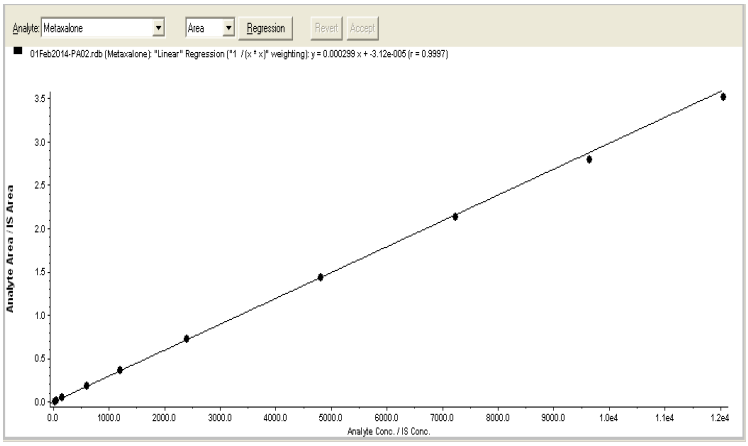

Fig.7 Calibration curve of Metaxalone

Precision and Accuracy:

The method precision and accuracy of Metaxalone in human plasma was evaluated by using three sets of QCs at three concentrations of HQC, MQC and LQC [Figure 8A, B, C]. Intra-day precision was done on set 1 and set 2, whereas inter-day precision was done on set 2 and set 3.The overall precision of the method expressed as relative standard deviation and accuracy of the method. Inter day batch accuracy ranged from 93.9% to 105.3%. Inter day precision ranged from 0.7% to 8.7%. Intraday batch accuracy ranged from 93.9% to 105.3%. Intraday batch precision ranged from 0.7% to 8.7%.The mean concentrations, standard deviation [SD], coefficient of variation [% CV] were evaluated and their results were tabulated in table 1. T-test was performed for interday and intraday precision concentrations and they were found to be statistically non-significant. Therefore, the method was found to be precise.

Table 1: Precision and Accuracy studies of Metaxalone samples [ng/ml]

Conc [ng/ml]	**LQC** [65.682]			**MQC** [6012.050]			**HQC** [9027.087]		
	Set 1	**Set 2**	**Set 3**	**Set 1**	**Set 2**	**Set 3**	**Set 1**	**Set 2**	**Set 3**
Mean	61.93	64.57	63.64	5644.96	5967.27	5904.96	9214.19	9482.06	9503.83
SD [±]	1.007	1.187	1.925	493.23	67.71	38.39	226.06	119.05	96.72
CV [%]	1.6	1.8	3.0	8.7	1.1	0.7	2.5	1.3	1.0
Accuracy[%]	94.3	98.3	96.9	93.9	99.3	98.2	102.1	105.0	105.3

Extraction recovery:

Recovery of Metaxalone was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples [LQC, MQC and HQC] to mean peak areas of six unprocessed reference solutions. Recovery of internal standard Metaxalone-d6 was evaluated by comparing the mean peak areas of low, medium and high quality control samples to mean peak areas of unprocessed reference solutions of the same concentration. The results were represented in table 2.

Table 2: Extraction recovery data of analyte and internal standard

	Metaxalone			**Metaxalone-d6**		
	HQC	**MQC**	**LQC**	**HQC**	**MQC**	**LQC**
% Recovery	82.6	96.8	85.8	77.2	78.8	79.0
Overall standard deviation	7.40			1.00		
Overall %CV	8.4			1.3		

Re-injection Reproducibility:

The re-injection reproducibility was done by comparing the results of re-injection set of samples with that of the original set and results were represented in table 3.

Table 3: Results for reinjection reproducibility

Parameter	Observed concentration [ng/ml]	
	HQC	LQC
Average concentration	9330.6418	62.9647
Standard Deviation	168.59396	1.86245
CV[Precision %]	1.8	3.0
Nominal Concentration	9027.087	65.682
Accuracy [%]	103.4	95.9

Stability studies:

As a part of method validation, stabilities such as bench top stability, auto-sampler stability, freeze thaw stability, dry extract stability, wet extract stability [in refrigerator and on bench top] were validated. Six replicates were analyzed for each of low quality control [LQC] and high quality control [HQC] samples at each storage condition. The concentration of Metaxalone after each storage period was compared to the initial concentration as determined for the samples that were freshly prepared and processed immediately. The precision and accuracy for the stability samples must be within ≤15 and ±15%, respectively, of their nominal concentrations. The results were represented in table 4.

Table 4: Results for Stability studies

Stabilities	Time	HQC	LQC
Freeze-thaw	At 0 cycles	9430.8743	62.9308
	After 5 cycles	8870.9105	61.3738
	% Stability	94.0	97.0
Bench top	At 0 h	9590.2355	64.2960
	After 24 h	9484.6672	64.4192
	% stability	98.8	99.6
Wet extract at refrigerator	At 0 h	9590.2355	64.2960
	After 24 h	9554.8798	64.3198
	% stability	99.5	99.5
Wet extract at bench top	At 0 h	9430.8743	62.9308
	After 24 h	9114.6337	61.7992
	% stability	96.6	97.7
Dry extract	At 0 h	9430.8743	62.9308
	After 24 h	9030.0780	60.8283
	% stability	95.7	96.1
Auto sampler	At 0 h	9590.2355	64.2960
	After 48 h	9567.0347	65.0493
	% stability	99.7	100.6

Bench Top Stability [BTS]:

The stability of anlalyte in human plasma stored at room temperature [bench-top] was determined by processing bench top stability quality control samples after keeping them at room temperature approximately for 24h and quantifying them against the freshly spiked set of quality control samples.

Freeze-thaw Stability [FTS]:

The freeze-thaw stability was conducted by comparing the stability samples that had been frozen and thawed five times against freshly spiked quality control samples.

Wet Extract Stability:

Wet extract bench top and wet extract refrigerator stability of Metaxalone was determined by processing and reconstituting quality control samples, keeping them at room temperature and refrigerator [2-8^{o}C] approximately for 24h and quantifying them against freshly spiked set of quality control samples.

Dry Extract Stability:

The dry extract stability of Metaxalone was determined by processing quality control samples, keeping them in refrigerator for 24h and quantifying against freshly spiked set of quality control samples.

Auto injector Stability:

To assess the auto- injector stability of Metaxalone, quality control samples were stored into the auto-sampler for the stability period of 48h. These samples were then quantified against freshly spiked quality control samples.

CONCLUSION

A highly selective and rapid LC-MS/MS method employing liquid-liquid extraction for the determination of Metaxalone in human plasma has been developed and validated with a lower limit of quantification of 25.006 ng/ml. The validation data also demonstrates good precision, accuracy and high extraction efficiency. The validation data also demonstrates good precision, accuracy and high extraction efficiency. The validated method allows quantification of Metaxalone in the linear range of 25.006 - 12059.526 ng/ml. In conclusion, this paper describes a very simple and sensitive LC-MS/MS method for the quantization of Metaxalone suitable to monitor plasma concentrations during clinical pharmacokinetic and bioequivalence studies in humans.

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