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A NEW LC/MS/MS METHOD FOR DETERMINATION OF LISINOPRIL IN HUMAN PLASMA

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ABSTRACT. A simple and sensitive liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) method for the quantification of lisinopril in human plasma was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a mobile phase of 11:89 (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water at 45 ºC with a flow rate of 1 mL/min. A 1% ammonia solution in acetonitrile was added post column. The detection of lisinopril was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer with electrospray positive ionisation. The human plasma samples (0.25 mL) were deproteinized with 12% perchloric acid in water and aliquots of 15 µL from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity ($r > 0.9946$), precision (CV < 11.3 %) and accuracy (bias < 7.0 %) over the range of 1.29-129 ng/mL plasma. The lower limit of quantification (LLOQ) was 1.29 ng/mL and the recovery was between 97.5-105.9 %. The method is not expensive, it needs a minimum time for plasma sample preparation and has a run-time of 5.0 min for instrument analysis (retention time of lisinopril was 4.5 min). The developed and validated method is very simple, rapid and efficient, with wide applications in clinical level monitoring, pharmacokinetics and bioequivalence studies of lisinopril.

Keywords: *lisinopril, LC/MS/MS, therapeutic drug monitoring*

INTRODUCTION

Lisinopril, (S)-1-[N2-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline (Fig.1), is an orally active angiotensin converting enzyme inhibitor, primarily used in the treatment of hypertension, congestive heart failure and acute myocardial infarction, with benefits in diabetic nephropathy [1] and diabetic retinopathy [2]. It has a hydrophilic molecule. After oral administration the bioavailability of lisinopril is about 25%, but widely variable between individuals (6 to 60%). Lisinopril has a long half-life and is eliminated by kidneys, in the unchanged form, without being metabolized [3]. Elimination rate of lisinopril decreases with old age and kidney or heart failure. Therapeutic drug monitoring and dose

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adjusting can be necessary in elderly patients or in patients with significant renal impairment to avoid drug accumulation and the risks of dosage-related side effects.

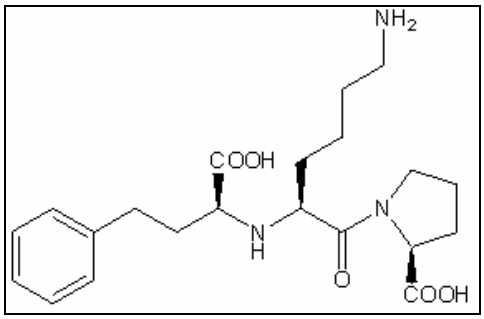

Figure 1. Chemical structure of lisinopril

Several methods involving radioimmunoassay [4], gas-chromatography (GC) coupled with mass spectrometry (MS) [5] and high-performance liquid-chromatography (HPLC) with ultraviolet (UV) [6], fluorescence [7] or mass spectrometric (MS) [8-10] detection have been reported to determine therapeutic levels of lisinopril in plasma. Radioimmunoassay is very sensitive but requires radiolabels and antilisinopril serum and it is not available to all researchers. Because its molecule is thermolabile and ionic, lisinopril must be derivatised into a derivative suitable for GC analysis. The sensitivity of HPLC-UV methods is inadequate for the measurement of lisinopril in plasma and the fluorescence detection also requires a derivatization step to chromophore derivatives. The GC and conventional HPLC methods involve solid phase extraction for the sample preparation. Both extraction and derivatization are time-consuming steps; they increase the cost of assay and can affect the recovery.

The LC/MS or LC/MS/MS method offers considerable advantages by its powerful performances: speed, selectivity, sensitivity and robustness [11, 12]. Sample preparation is more simple and rapid and often includes only precipitation of proteins (PP) [8, 13] or extraction before chromatographic analysis [9, 10, 14-16].

The aim of this work was to develop and validate a new simple and efficient LC/MS/MS assay for the quantification of lisinopril in human plasma for application in therapeutic drug monitoring and bioavailability studies.

RESULTS AND DISCUSSION

Sample preparation

In LC/MS/MS assays sensitivity depends on MS detection mode, but the method involved in sample preparation may influence the chromatographic background level and can generate matrix suppression effect. Several

researchers prefer to include in plasma sample preparation an extraction step to eliminate the impurities and to increase sensitivity (Table 1). Solid-phase extraction (SPE) is preferred as isolation method [9, 10, 14, 16] because of the amphoteric nature of lisinopril that would make the isolation difficult by simple liquid-liquid extraction (LLE). Huang et al. obtained constant extraction recoveries of lisinopril with a mixture of isopropanol and ethyl acetate (1:2, v/v) and pseudoephedrine hydrochloride as internal standard [15]. However, the extraction step increases the time of analysis and the costs and can affect the recovery. In scientific literature there were reported only two methods that used PP without extraction for determination of lisinopril in human plasma. Zhou et al. analyzed lisinopril by LC/MS after twice PP with methanol. They obtained a good sensitivity (LOQ of 2.5 ng/mL) and absolute recoveries between 94.4-98.2% [8]. Qin et al. used perchloric acid for PP. They obtained a better sensitivity (LOQ of 2 ng/mL), but the method requires the use of internal standard (absolute extraction recoveries of lisinopril were 55.1-58.6% for QC levels and absolute recovery of IS was 66.8%) [13]. Huang et al. reported the better LOQ. They quantified lisinopril in the linear range of 0.78-100 ng/mL plasma, but after isolation by LLE. The method also requires the use of internal standard (absolute extraction recoveries of lisinopril were 40.68-42.09% for QC levels) [15].

In our method we analysed volumes of only 0.25 mL plasma by PP with 12% perchloric acid (0.05 mL) and direct injection into the chromatographic system from supernatant after centrifugation. We obtained a very good sensitivity (LLOQ of 1.29 ng/mL) and better absolute recoveries (between 97.5 and 105.9%). Our method is more simple and rapid and offers a shorter time of analysis and a lower cost as compared with the other longer methods which used an extraction step prior to the chromatographic assay (Table 1). No matrix interference or ion suppression was observed from the plasma samples.

As the therapeutic doses effective in lowering blood pressure range from 10 to 80 mg of lisinopril per day and the therapeutic plasma levels of lisinopril are < 100 ng/mL, the LLOQ of 1.29 ng/mL established in our method can be accepted in bioequivalence studies and in routine purposes for therapeutic level monitoring of lisinopril in human plasma.

LC-MS assay

The chromatographic conditions, especially the composition of mobile phase, were optimized in several trials to achieve maximum sensitivity and symmetrical chromatographic peaks, a short retention time of lisinopril and consequently a shorter run time of analysis.

Several mobile phases containing varying percentages of organic acid were tested (Table 2). The best results were obtained with the mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA) in water (11:89, v/v) under isocratic conditions. By its amphoteric nature, lisinopril is ionized in aqueous solution whatever the pH is, thus giving a poor chromatographic

peak shape (tailing) on reversed-phase chromatography. The use of TFA as additive to aqueous phase has a large impact on the retention of lisinopril on C18 chromatographic column and also on its peak shape.

Table 1. Analytical characteristics of reported LC/MS and LC/MS/MS methods for the determination of lisinopril in human plasma or serum

Reference	Matrix	Detection mode[a]	Mobile phase constituents[b]	Pre-treatment/ extraction[c]	LOQ[d] (ng/mL)	Run time (min)	Absolute recovery (%)
Zhou [8]	Plasma	ESI-MS, SIM	formic acid (pH 2.9)-MeOH-ACN (58:25:17, v/v)	PP with methanol	2.5	NA[e]	94.4-98.2
Tashtoush [9]	Plasma	ESI-MS, SIM		SPE	10	NA	91.69
Tsakalof [10]	Serum (1mL)	ESI-MS, SIM	50 mM ammonium formate buffer (pH 3)-ACN-MeOH (72:7:21,v/v)	SPE	6	6	88
Qin [13]	Plasma (0.5mL)	ESI-MS/MS, SRM (m/z 406→246)	10 mM ammonium acetate buffer (pH 5)-MeOH (70:30,v/v)	PP with perchloric acid	2	5	NA
Padua [14]	Plasma (0.5mL)	ESI-MS/MS, MRM (m/z 406.3>84.3)	ACN:water (60:40, v/v) with 20 mM acetic acid and 4.3 mM TEA	SPE	2	6.5	79.2-82.5
Huang [15]	Plasma (0.4mL)	ESI-MS/MS, SRM (m/z 406.1→246)	MeOH:0.1% formic acid (50:50, v/v)	LLE	0.78	5	NA
Kousoulos [16]	Plasma (0.5mL)	ESI-MS/MS, SRM (m/z 406.1→83.6)	75% MeOH, 25% 10mM formic acid and 5mM ammonium acetate in water (v/v)	SPE	2	2	NA

[a]SIM, selected ion monitoring; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; [b]ACN, acetonitrile; MeOH, methanol;TEA, triethylamine; [c]PP, protein precipitation; SPE, solid-phase extraction; LLE, liquid–liquid extraction; [d]LOQ, limit of quantification; [e]NA, not available.

Table 2. Influence of organic acid percent in aqueous mobile phase on retention time (RT) of lisinopril and its factor of capacity, k

Aqueous mobil phase	RT (min)	k
FA1 0.1%	0.75	2.8
TFA2 0.02%	1.5	6.5
TFA 0.04%	2.5	11.5
TFA 0.08%	4.8	23.0
TFA 0.1%	6.9	33.5
TFA 0.12%	7.2	35.0

[1]FA – formic acid, [2]TFA – trifluoroacetic acid

In 0.1% aqueous TFA (pH 1.9), lisinopril is transformed in a neutral adduct: the ionization of carboxylic groups is suppressed by the pH and the trifluoroacetate anion forms an ion-pair with ionized primary and secondary amino groups, neutralizing their charge. This way the liphophilicity of lisinopril is increased (Table 2) and the chromatographic peak becomes symmetric. However, there is also a drawback of using TFA in mass spectrometry: the strong ion-pair formed by TFA with analyte is no more ionized, so it cannot be "seen" by the mass spectrometer. The poor sensitivity due to TFA can be avoided by post-column adding a 1% ammonia solution in acetonitrile to neutralize the acid and to allow the ionization of lisinopril in electrospray ion source.

In the case of lisinopril, electrospray ionization (ESI) mode offers significantly higher signals compared to atmospheric pressure chemical ionization (APCI). The signal intensities of lisinopril obtained in positive ion mode were much higher than those in negative ion mode, so the former ionization mode was chosen. After the collision that induced the dissociation of lisinopril in ion trap mass spectrometer, the $[M+H]^+$ ion (m/z 406) produced some abundant ions (m/z 245, 246, 291, 309) at the optimum collision energy of 0.9 V (Fig. 2), thus, the detection of lisinopril was carried out in multiple reaction monitoring (MRM). The extracted ion chromatogram (EIC) of m/z (245, 246, 291, 309) from m/z 406 was monitored and analyzed. In the selected chromatographic conditions the retention time of lisinopril was 4.5 min and the analytical run-time was 5 min.

Assay validation

The method was validated in accordance with international regulations [17, 18]. Representative chromatograms of drug-free plasma and plasma spiked with lisinopril at LLOQ are shown in Fig. 3. No interfering peaks from the endogenous plasma components were observed in the retention time of lisinopril.

The calibration curves were linear over the concentration range of 1.29 – 129 ng/mL in human plasma, with a correlation coefficient greater than 0.9946. The LLOQ was 1.29 ng/mL. The values obtained for intra-day and inter-day precision and accuracy during the validation are shown in Tables 3 and 4, respectively.

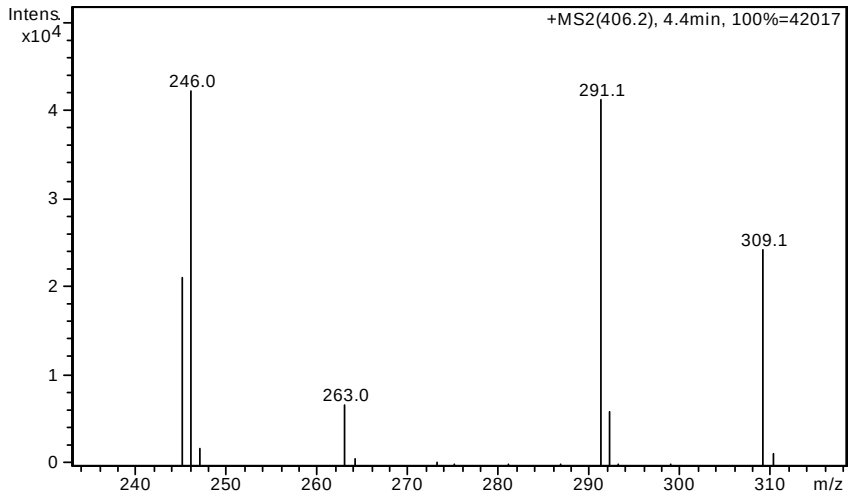

Figure 2. MS/MS spectra of lisinopril

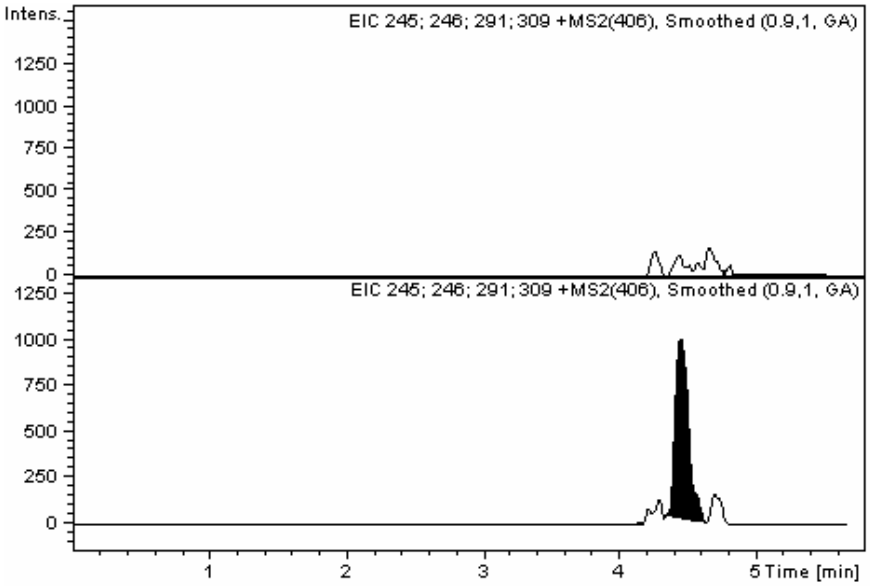

Figure 3. Representative chromatograms of (up) blank plasma and (down) plasma spiked with lisinopril at lower limit of quantification (1.29 ng/ml) (retention time – 4.5 min)

Table 3. The intra-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of lisinopril in human plasma (n = 5)

$c_{nominal}$ ng/mL	Mean c_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
1.29	1.26±0.10	8.2	-2.8	98.9±8.7
3.87	3.72±0.32	8.5	-3.9	105.9±9.2
19.36	19.83±1.31	6.6	2.4	99.1±6.6
51.62	53.16±1.71	3.0	3.0	99.0±3.2

Table 4. The inter-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of lisinopril in human plasma (n = 5)

$c_{nominal}$ ng/mL	Mean c_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
1.29	1.38±0.16	11.3	7.0	100.9±3.9
3.87	3.70±0.21	5.6	-4.4	101.3±11.4
19.36	20.49±0.77	3.8	5.8	102.1±4.3
51.62	53.77±1.42	2.6	4.2	97.5±7.6

All values for accuracy and precision were within recommended limits (<15%). The absolute recovery values were between 97.5 and 105.9%. Lisinopril showed a good post-preparative stability (PPS) in autosampler at room temperature following sample processing for at least 24 h (CV% of -6.4% at lower level and -3.8% at higher level, respectively) and a good stability (RTS) in plasma for 4 hours at room temperature (CV of –6.6% at lower level and –1.7% at higher level, respectively). Lisinopril is found to be stable at three freeze-thaw cycles (the concentration was changed with 3.5% at lower level and with 9.1% at higher level, respectively) and it was also stable in plasma stored at –20°C for at least 5 months (concentration changed with 0.2% at lower level and with 3.0% at higher level, respectively).

Method application

The validated method for determination of lisinopril in human plasma was successfully applied in a bioequivalence study of a generic formulation with lisinopril versus Zestril after oral administration of a dose of 20 mg lisinopril to 20 volunteers. The mean drug plasma concentration – time curve of lisinopril obtained is shown in Fig. 4.

CONCLUSION

Our developed LC-MS/MS assay is simple, rapid, accurate and not expensive. In comparison with other published LC-MS/MS [13-16] or LC-MS [8-10] assays for therapeutic level monitoring of lisinopril in human plasma our method performs better in terms of volume of analyzed plasma sample, analyte recovery, speed (both sample preparation and chromatographic run-time) and costs, which are essential attributes for methods used in routine analysis. The method was validated over the concentration range of 1.29-129 ng/mL which covers therapeutic plasma levels of lisinopril. This new fast method was successfully applied in bioavailability study of lisinopril, but it can also be used in therapeutic drug monitoring of lisinopril in human plasma.

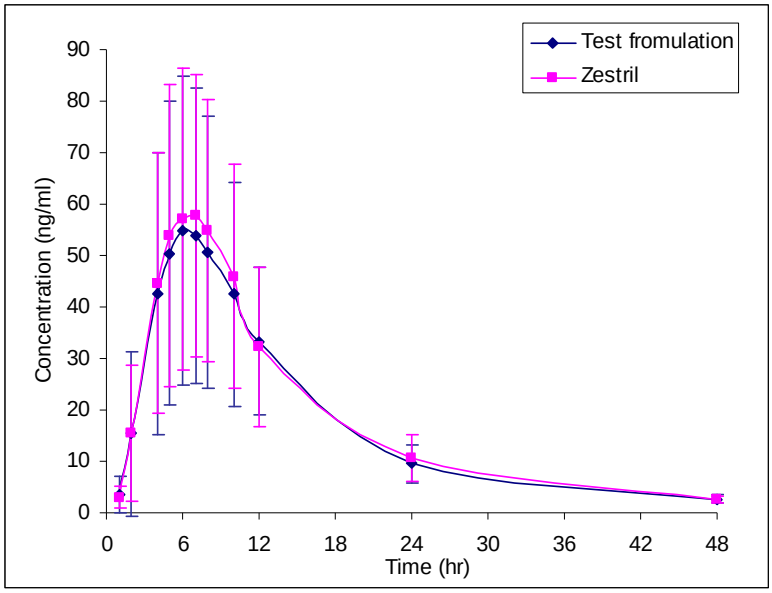

Figure 4. Mean plasma concentration – time curve for lisinopril after oral administration of a generic formulation containing 20 mg lisinopril and Zestril (20 mg lisinopril), respectively, obtained from healthy male volunteers (n=20, mean value and SD are plotted)

EXPERIMENTAL SECTION

Reagents

Lisinopril was reference standard from AC Helcor SA, Romania. Acetonitrile and methanol of isocratic grade for liquid chromatography, formic acid, trifluoroacetic acid and 70% perchloric acid of analytical-reagent

grade were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled, deionised water pro injections was purchased from Infusion Solution Laboratory of The University of Medicine and Pharmacy Cluj-Napoca (Romania). The human blank plasma was supplied by the Bleeding Centre Cluj-Napoca (Romania) from healthy volunteers, men and women.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of two G1312A binary pumps, an in-line G1379A degasser, an G1329A autosampler, a G1316A column oven and an Agilent Ion Trap Detector 1100 VL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3.5 μm) column (Agilent Technologies) under isocratic conditions using a mobile phase of a 11:89 (v/v) mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid in water at 45 ºC with a flow rate of 1 mL/min. A 1% ammonia solution in acetonitrile was added post column with a flow rate of 0.1 mL/min by an another Agilent HPLC pump. The detection of lisinopril was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer with an electrospray ion (ESI) source, positive ionisation (capillary 4000 V, nebulizer 70 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 350ºC). The extracted ion current (EIC) chromatogram of m/z (245, 246, 291, 309) from m/z 406 was analyzed.

Standard solutions

A stock solution of lisinopril (0.963 mg/mL) was prepared by dissolving an appropriate quantity of lisinopril in methanol. Two working solutions (9.63 μg/mL and 129 ng/mL, respectively) were prepared by appropriate dilution in drug-free human plasma. These solutions were used to prepare plasma calibration standards with the concentrations of 1.29, 2.58, 5.16, 10.32, 20.64, 41.29, 82.58 and 129.04 ng/mL. Quality control (QC) samples of 3.87 ng/mL (lower), 19.36 ng/mL (medium) and 51.62 ng/mL (higher) were prepared by adding the appropriate volumes of working solutions to drug-free human plasma. The resultant plasma calibration standards and quality control standards were pipetted into 15 mL polypropylene tubes and stored -20ºC until analysis.

Sample preparation

Standards and plasma samples (0.25 mL) were deproteinized with 12% perchloric acid in water (0.05 mL). After vortex-mixture (10 s) and centrifugation (5 min at 5000 rpm), the supernatants (0.15 mL) were transferred in autosampler vials and 15 µL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing lisinopril with those obtained from different plasma blank samples (n=6).

The concentration of lisinopril was determined automatically by the instrument data system using peak areas and the external standard method. The calibration curve model was determined by the least squares analysis: $y = b + ax$, weighted (1/y) linear regression, where y - peak area of the analyte and x - concentration of the analyte (ng/mL).

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by the analysis of five different samples (n = 5) from each QC standards (at lower, medium and higher levels) on the same day. The inter-day precision and accuracy were determined by analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels).

The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

The relative recoveries (at LLOQ, lower, medium and higher levels) were measured by comparing the response of the spiked plasma with the response of standards in solvent with the same concentration of lisinopril as the plasma (n = 5).

The lisinopril stability in plasma at lower and higher levels (n=5) was investigated. For the post-preparative stability (PPS) study, the samples were prepared, analyzed immediately and after 12-24 h (kept in the autosampler of the HPLC system at 25ºC). The concentrations were calculated against the same calibration curve. For the long-term stability (LTS) study, the samples were stored below -20°C and analyzed during 5 months. The concentrations were calculated against calibration curve of the day and the mean values for the stored samples were determined and compared. For the freeze-thaw stability (FTS) study, the samples were subjected to three cycles of freeze-thaw operations in three consecutive days. After the third cycle the samples were analyzed and the concentrations were calculated against calibration curve of the day. The requirement for the stability studies of the drug is that the difference between the mean concentrations of the tested samples in various conditions and the nominal concentrations was in ±15% range.

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