INTERNATIONAL JOURNAL
OF
PHARMACEUTICAL SCIENCES
AND
RESEARCH

NITISINONE IN HUMAN PLASMA: UPLC-MS/MS ASSAY VALIDATION AND STABILITY STUDIES

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ABSTRACT: A simple, precise, and rapid ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS method for determination of nitisinone in 20 µl human plasma was developed and validated. Nitisinone 6C13 was used as an internal standard (IS). Chromatographic analysis was performed on an Atlantis dC18 column (2.1 x 100 mm, 3 µm) using a mobile phase consisting of methanol and 10 mM ammonium-acetate (90:10, v;v) that was delivered at a flow rate of 0.25 ml/min. The eluents were monitored using electrospray ionization in the positive ion mode set at transition set of mass-to-charge (m/z): 330 → 217.92 and 336 → 217.91 for nitisinone and nitisinone-13C6 (internal standard, IS), respectively. The retention time of nitisinone and IS both were about 0.88 minutes, respectively. Relationship between nitisinone concentration and peak area ratio of nitisinone to the IS was linear ($R^2 \geq 0.9991$) in the range of 2–100 µg/ml and the intra- and inter-day coefficient of variations were 1.9% to 4.5% and 3.2% to 6.2%, respectively. Extraction recoveries for nitisinone and the IS were 93%, and 98%, respectively. Nitisinone stability was evaluated in processed samples (stored at room-temperature for 24 hours) and unprocessed sample (stored at room-temperature for 24 hours or at -20°C for 8 weeks) and after 3 freeze-thaw cycles was ≥ 91%..

INTRODUCTION: Nitisinone, 2-(2-nitro-4-trifluoromethyl benzoyl)-cyclohexane-1,3-dione (NTBC) is a FDA approved drug widely used in treatment of hereditary tyrosimia type-1 (HT-1). It can considerably lower the likelihood of HT-1 patients acquiring hepatocellular carcinoma and prevent the onset of liver disease [1-4].

According to nitisinone pharmacokinetic investigations, the mean maximal plasma concentration is 7.8 ± 1.4 µg/ml between 1.6 and 11.1 hours after the therapeutic dose of 1.0 mg/kg was administered as a capsule or liquid formulation [5, 6].

Determining the ideal dosage for an individual is crucial to prevent the hazardous effects of nitisinone overload. In general, NTBC blood concentrations between 40 and 60 mol/L are recommended [7]. Several assays, including coupled column chromatography [8], capillary electrophoresis method with photometric detection [9], high

performance liquid chromatography (HPLC) with ultraviolet [10] and tandem mass spectrometer (LC-MS/MS) detection are reported for the measurement of nitisinone levels in plasma samples. Most described assays uses more than twenty microliter of sample volume and/or run time for quantitative analysis ranged from 2 to 4 minutes [11-15]. Here, we present a simple precise and quick ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) assay for quantifying nitisinone using a 20-µl plasma sample. The protein precipitation assay uses 500 µl of acetonitrile. Each run's analysis was completed within one minute. The assay was validated in accordance with US-FDA regulations [16] and it has been utilized to evaluate the stability of nitisione under diverse clinical laboratory conditions.

MATERIAL AND METHODS:

Chemicals and Reagents: Nitisinone and Nitisinone-13C6 as internal standard (IS) were purchased from Toronto Research Chemicals Inc. Toronto ON, Canada. Ammonium acetate (AR-grade) and methanol (HPLC grade) were acquired from Fisher Scientific, NJ. USA. Water (HPLC grade) was generated by reverse osmosis followed by distillation and further purified by passing through a Milli-Q, Synergy System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia.

Equipment & Analytical Conditions: Ultra-performance-liquid-chromatograph (UPLC) integrated solvent and sample manager (Acquity) equipped with tandem mass spectrometer (MS/MS) Xevo-TQD and an interface Z-spray atmospheric pressure ionization (API) (Waters Corporation, Milford, MA, USA) were utilized for analysis. The analysis was performed using a reversed phase Atlantis C18 column (2.1 x 100 mm, 3 µm) protected on-line filter by guard column (0.2 µm x 2.1 mm). A mobile phase consists of 10 mM ammonium acetate and methanol (10:90, v:v) delivered in isocratic condition at a flow rate of 0.25 ml/minute. Electrospray-ionization-source was operated in the positive-ion-mode (capillary voltage 3.00 kV, cone voltage 38 volts). Nitrogen for nebulization/desolvation (1000 L/hr) and argon for collision (3.6×10^{-3} mbar) were used.

We found the optimum collision energy 20 eV and Dwell time 0.136 seconds for both nitisinone and the IS, respectively. The temperature of ion source and desolvation were maintained at 150°C and 500°C, respectively. Nitisinone and IS were detected in the positive ion mode, using electrospray ionization (ESI) at the following transitions of mass to charge (m/z) 330 → 217.92 and 336 → 217.91, respectively.

Standard & Control Samples: Stock solution of nitisinone and IS were prepared in methanol at concentration in 100 µg/ml. Seven nitisinone standard working solutions (2, 5, 10, 15, 30, 60, and 100 µg/ml) and three quality control solutions (2, 50 and 90 µg/ml) were prepared in blank human nitisinone plasma. All solutions (10 ml each) were vortexed for one minute in 15 ml polystyrene culture tube (16 x 125 mm), and stored at -20 ºC until used.

Sample Preparation: Drug free plasma sample (blank), calibration curve or quality control (20 µl) each taken in Eppendorf (1.5 ml) micro-centrifuge tube. Then add 500 µl of acetonitrile containing IS (100 µg/ml) were mixed thoroughly by vortex one minute and centrifuged 10 minutes at room temperature. About 50-µl supernatant was transferred into an auto-sampler vial and 5 µl were injected to analyzed by UPLC-MS/MS with a run time of two minutes.

Recovery: Nitisinone and Nitisinone 13C6 (IS) recovery was examined by comparing peak-areas of extracted and un-extracted samples (five replicates, 6, 50 and 90 µg/ml) for nitisinone. Similarly, IS recovery determined at 100 µg/ml.

Matrix-effect: For the evaluation of matrix effect, we prepare nitisinone solution in methanol (standard) and plasma-extract-spiked solutions in methanol at three concentration (6.0, 50, and 90 µg/ml) and IS (100 µg/ml). Matrix effect was evaluated by comparing peak area response obtained by the analysis of samples spiked-after extraction and corresponding standard solutions.

Stability Studies: Nitisinone three QC samples (6, 50, and 90 µg/ml) were used stability studies: Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at

room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at –20°C for eight weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed, reconstituted, and stored at room temperature for 24 hours before analysis (auto-sampler stability).

Finally, fifteen aliquots of each QC sample were stored at –20°C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to –20°C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Assay-validation: The method validated according to standard procedures described in the US Food and Drug Administration (FDA)[12] The validation

parameter included specificity, recovery, linearity, accuracy, precision and stability

RESULTS & DISCUSSION:

LC-MS/MS Condition Optimization: The product and precursor ions of nitisinone and nitisinone 13C6 (IS) determine by infusing a standard mixture containing nitisinone and IS in the mass-spectrometer. We used a configured software program (Intellistart, Waters Corporation, Milford, MA, USA). **Fig. 1** depicts the MS/MS spectrum and chemical structures of these compounds. The best liquid-chromatographic conditions were obtained with a mobile phase of 2 mM ammonium-acetate and methanol (10:90, v: v) with a flow rate 0.25 ml/min. The mass-spectrometry acquisition was achieved with multiple-reaction-monitoring in positive-ion mode. The retention time of nitisinone and IS both were about 0.88 minutes, respectively.
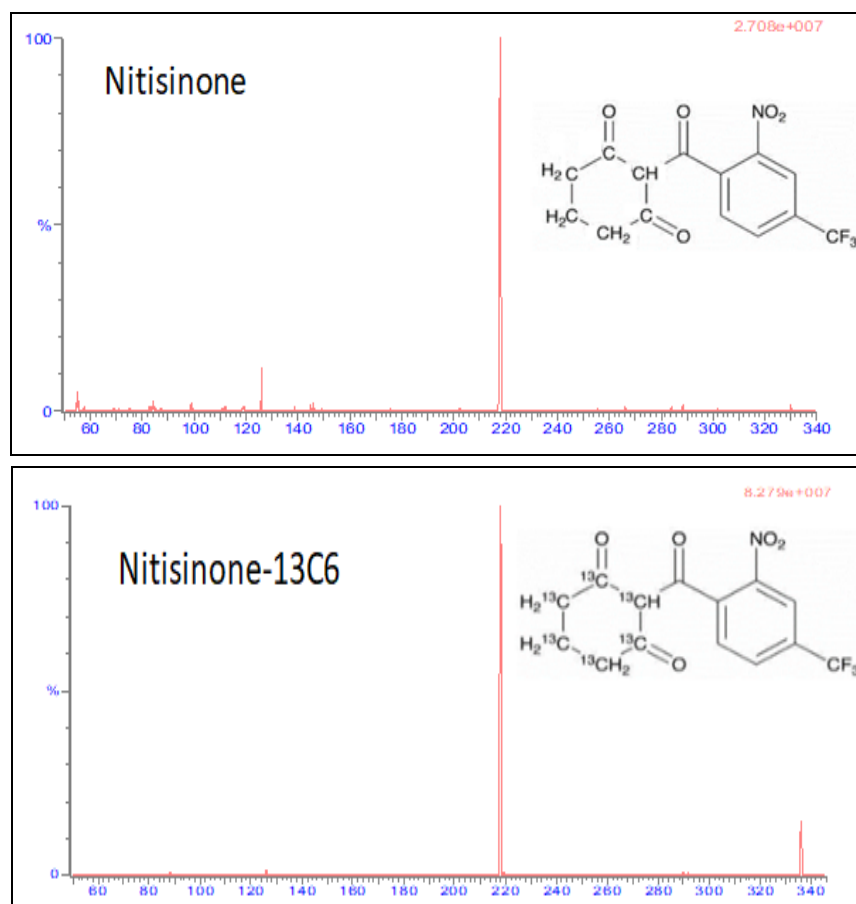
FIG. 1: MS/MS SPECTRUM AND CHEMICAL STRUCTURES OF NITISINONE, AND NITISIONE-13C6 (INTERNAL STANDARD)

Effect of Matrix: Measurement of plasma matrix effect on response of nitisinone and IS were carried out by comparing the peak response obtained from

extract-spiked plasma samples and corresponding standard solution prepared in methanol. No significant difference in response was observed.

Method Validation:

Specificity: The method's specificity was established in order to confirm the interference of endogenous components from blank plasma. Plasma samples from six distinct batches were examined. Nitisinone and IS were not co-eluted with any endogenous substance. **Fig. 2** depicts a representative chromatogram of drug free, IS and nitisinone spiked concentration (6, and 90 µg/ml) low and high of quality control, respectively.
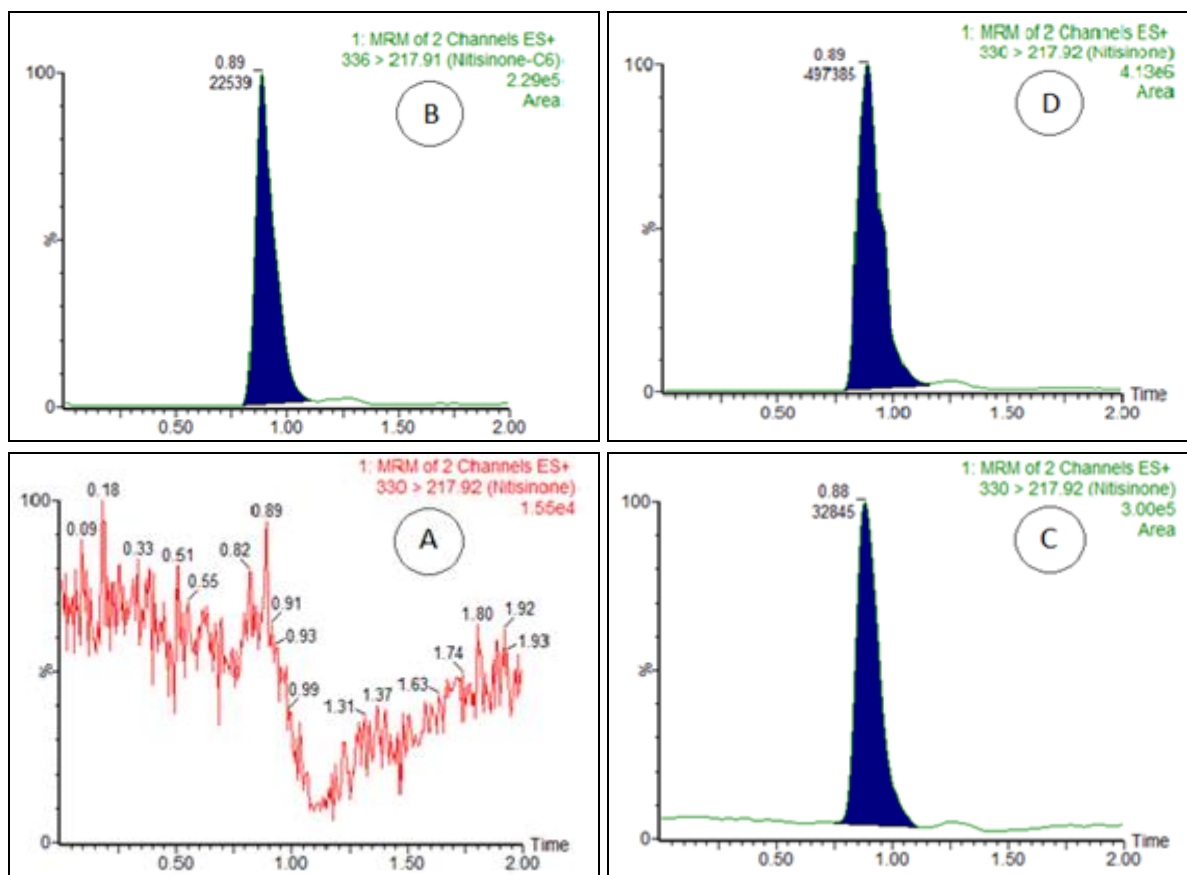
FIG. 2: MULTIPLE REACTION CHROMATOGRAM OF 20 µL EXTRACTS OF HUMAN PLASMA A) DRUG FREE-PLASMA B) IS-SPIKED PLASMA C) NTBC-PLASMA-SPIKED (6 µG/ML) AND D) NTBC-PLASMA-SPIKED (90 µG/ML)

Linearity, Range and Limit of Detection Limit and Quantification: By analyzing standard containing nitisinone in human plasma at seven different concentrations in range of (2–100 µg/ml), we were able to assess the assay's linearity. Regression analysis was implemented to examine the peak-area ratio and concentrations that corresponded. The mean regression equation (n=10) was Y= 0.2947 x + 0.0125, (R^2 = 0.9994). Limits of detection and quantification for nitisinone were 0.1 µg/ml and 0.3 µg/ml, respectively. **Table 1** represents the data of calibration curves. All back-calculated concentrations were well within the acceptable limits.

TABLE 1: MEAN MEASURED LEVELS OF NITISINONE IN CALIBRATION CURVE

Nominal Level (µg/ml)	Measured Level (n=10)	SD	CV (%)	Bias (%)
2	2.045	0.171	8.3	2.2
5	4.851	0.170	3.5	3.0
10	10.120	1.075	10.6	1.2
15	14.707	0.542	3.7	2.0
30	29.391	1.663	5.7	2.0
60	59.021	1.444	2.4	1.6
100	100.540	0.654	0.7	0.5

Precision and Inaccuracy: The inter-day precision and inaccuracy were determined using three quality control concentration (2, 50, and 90 µg/ml). The intra-day (n=10) and inter-day (n=20) precision

were $\leq$ 4.5% and $\leq$ 6.2%, respectively. The intra-day and inter-day bias were in the range of 2.7 to 4.5% and 2.4 to 3.1% respectively. The results are summarized in **Table 2**. The results indicate that the method was reliable within the studied concentration range.

TABLE 2: INTRA-DAY AND INTER-DAY PRECISION AND INACCURACY OF THE ASSAY

Nominal Level (µg/ml)	Intra-day (n=10)			Inter-day (n=20)		
	Mean (SD) measured level	CV (%)	Bias (%)	Mean (SD) measured level	CV (%)	Bias (%)
50	52.27 (1.00)	1.9	4.5	51.40 (1.67)	3.2	2.8
90	92.42 (4.18)	4.5	2.7	92.75 (5.74)	6.2	3.1

SD is standard deviation. CV is coefficient of variation (SD/mean measured level multiplied by 100). Inaccuracy= measured level - nominal level / nominal level multiplied by 100.

Extraction Recovery: The extraction recovery of nitisinone was assessed by comparing (5 duplicates) the peak area of nitisinone at one of three concentrations (2, 50, and 90 µg/ml) spiked on unextracted blank plasma (and then extracted according to the described method) with the peak area of equivalent nitisinone concentrations spiked on pre-extracted blank plasma.

The recovery of the IS was assessed similarity at (100 µg/ml). The mean extraction recovery of nitisinone and the IS was $\geq$ 90% and 99%, respectively.

Stability: Stability of analyte is an important pre-analytical variable. We assessed the stability of nitisinone under common laboratory conditions. Nitisinone stability in processed and unprocessed plasma samples was investigated. Nitisinone in processed samples (2, 50 and 90 µg/ml) was found to be stable for 24 hours at room temperature ($\geq$ 100%). Nitisinone in unprocessed plasma samples was stable for at least 24 hours at room temperature ($\geq$ 95%), eight weeks at -20ºC ($\geq$ 91%) and after three freeze-and thaw cycles ($\geq$ 94%). Data are summarized in **Table 3.**

TABLE 3: STABILITY OF NITISINONE IN VARIOUS LABORATORY CONDITIONS

	Processed		Unprocessed		Freeze & Thaw		
	Zero Time	24 hr (RT)	24 hr (RT)	8 wks (- 20 ºC)	Cycle-1	Cycle-2	Cycle-3
Measured level Mean (µg/ml)	6.32	6.34	6.01	5.79	6.08	5.80	5.94
SD	0.13	0.15	0.20	0.11	0.23	0.22	0.50
Stability (%)	-	100	95	91	96	92	94
Measured level Mean (µg/ml)	51.22	51.70	50.30	49.73	51.70	50.82	53.57
SD	2.59	0.19	1.49	1.05	0.19	2.37	2.12
Stability (%)	-	101	98	97	101	99	105
Measured level Mean (µg/ml)	94.78	99.30	92.36	90.05	92.96	93.18	93.25
SD	4.25	2.47	1.96	3.42	5.65	4.23	1.36
Stability (%)	-	105	97	95	98	98	98

Mean measured (n=5), Room temperature (RT), Hours (hr), Weeks (wks), Freeze-Thaw (FT). Stability (%) equals to average measured level (n=5) at specified time/ average measured level (n=5) at baseline x 100.

CONCLUSION: A simple and rapid validated UPLC-MS/MS assay for measurement of nitisinone levels in human plasma is reported here. The assay is based on one-step precipitation extraction procedure and it was used to determine stability of nitisinone under common clinical laboratory conditions. Assay could be utilized for monitoring nitisinone levels in patient samples.

CONFLICTS OF INTREST: None declared.

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