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Determination of Lisdexamfetamine in Human Plasma by LC-MS/MS Method

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Lisdexamfetamine is a prodrug that is converted to d-amphetamine in the body, mainly used to treat attention deficit hyperactivity disorder in children and adults. Its mechanism involves the enhancement of neurotransmitters such as norepinephrine and dopamine, which are critical for attention and behavioral regulation. With the upcoming patent expiration of Vyvanse®, bioanalytical methods for the quantification of lisdexamfetamine in plasma are needed for use in pharmacokinetic studies. In this work, we developed and validated a sensitive bioanalytical method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for lisdexamfetamine quantification in human plasma. The method developed was performed for 4 min *per* sample and using the concentration range of 0.3-100 ng mL^{-1}. Intra-batch, inter-batch and instrument reproducibility, the precision was lower than 10%. The results demonstrated that the method is rapid, sensitive, robust, and suitable for pharmacokinetic studies.

Keywords: bioanalytical method, LC-MS/MS, lisdexamfetamine, pharmacokinetics

Introduction

Lisdexamfetamine (LDX), a prodrug of d-amphetamine, is a central nervous system stimulant that was approved by the Food and Drug Administration (FDA) in 2007.[1] Since its initial approval was for pediatric attention-deficit / hyperactivity disorder (ADHD) management, its indications have expanded to include adult and adolescent ADHD treatment, as well as adult ADHD maintenance therapy.[2] The pathogenesis of ADHD is multifactorial, encompassing a diverse array of genetic and environmental contributors that culminate in a spectrum of neurobiological alterations. This makes it important to understand how LDX medications work aiming to avoid adverse effects.[3]

The pharmacological profile of LDX is characterized by its inactive state until metabolized to d-amphetamine, which exerts the therapeutic effects. d-Amphetamine, a potent non-catecholamine sympathomimetic amine, increases synaptic concentrations of norepinephrine and dopamine primarily by inhibiting their respective membrane transporters.[4]

The active metabolite of LDX modulates central nervous system activity through the inhibition of dopamine transporter (DAT), norepinephrine transporter (NET), trace amine-associated receptor 1 (TAAR1), and vesicular monoamine transporter 2 (SLC18A2), among other targets.[5] This modulation is crucial for regulating catecholamine reuptake and release within the synaptic cleft. Upon administration, LDX is enzymatically cleaved by erythrocytes, releasing the active d-amphetamine from its lysine moiety. Subsequent analyses focused on d-amphetamine, as the parent compound, LDX, lacks biological activity before its conversion.[6]

Anticipating the Vyvanse® patent expiration stimulated the development of pharmacokinetic (PK) quantification assays such as methodologies based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The expected popularization of generic products requires rapid and simple methods for drug monitoring to facilitate effective therapeutic drug and toxicokinetic evaluations.[7]

The LC-MS/MS technique is a powerful analytical tool widely used in the analysis of drugs and their metabolites in

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human plasma.[8] The importance of this technique lies on its ability to provide a highly sensitive and specific detection of chemical compounds in complex biological matrices.[9] In clinical pharmacology, the high-throughput nature of this technique and the precision of quantification it provides make it indispensable for ensuring the safety and efficacy of pharmacokinetics studies.[10-13]

The advancement in bioanalytical methods for the quantification of LDX in human plasma marks a significant improvement in PK studies. There are only a few studies based on the development and validation of LDX.[14-29] Traditional LC-MS methods, while effective, have limitations such as longer analysis times and lower sensitivity, with a typical lower limit of quantification (LLOQ) around 1 ng mL^{-1}.[15,16,30] The new method presented here addresses these issues, reducing the analysis time and achieving a more sensitive LLOQ of 0.3 ng mL^{-1}.

This work also improves the knowledge of LDX PK parameters as it contains assays for sample stability and method interference by other usual medications. As a result, the advantages of this study lie on a rapid and sensitive LC-MS/MS method with a simple sample preparation procedure and short analysis time for the quantification of LDX in plasma, offering a promising tool for pharmacokinetic studies.

Experimental

Chemicals and reagents

Reference standards for LDX dimesylate (CAS No. 608137-33-3) were obtained from the Ind-Swift Ltd. (Punjab, India) (Figure 1a) and amphetamine-d_8 hydrochloride (CAS No. 145225-00-9) were acquired from LGC Standards (Luckenwalde, Germany) (Figure 1b), utilized as an internal standard (IS). Along with concomitant medications, 4-methyl-amino-antipyrine (4-MAA) (CAS No. 519-98-2) and scopolamine butyl bromide (CAS No. 149-64-4), were acquired from Purity Grade Standards Labs (San Francisco, USA). Additional medications such as dimenhydrinate (CAS No. 523-87-5), pyridoxine hydrochloride (CAS No. 58-56-0), and caffeine (CAS No. 58-08-2) were sourced from U.S. Pharmacopeia (Rockville, USA). Paracetamol (CAS No. 103-90-2) and metoclopramide hydrochloride (CAS No. 7232-21-5) were acquired from the Oswaldo Cruz Foundation-Fiocruz (Rio de Janeiro, Brazil), in contrast, ondansetron hydrochloride (CAS No. 99614-01-4) was obtained from the European Pharmacopoeia (Strasbourg, France). For the preparation of ultrapure water (H_2O) with a resistivity of 18.2 MOhm cm, a Milli-Q Water Purification System from Merck KGaA (Darmstadt, Germany) was employed. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were procured from J.T. Baker (Radnor, USA), formic acid and ammonium acetate were purchased from Merck KGaA (Darmstadt, Germany).

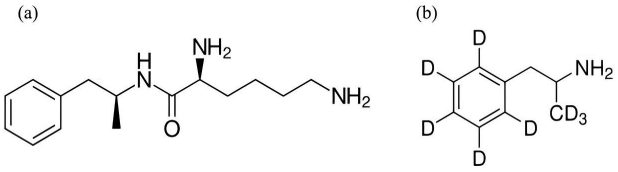

Figure 1. Chemical structures of (a) LDX and (b) IS.

Preparation of standards

LDX stock solution (108.5 μg mL^{-1}) was prepared in water (H_2O), and diluted with H_2O to obtain the corrected stock solution (100 μg mL^{-1}), for the spiking calibration curve and quality controls (QC) samples. QC samples were prepared in the following levels: low, 0.9 ng mL^{-1} (LQC); medium, 50 ng mL^{-1} (MQC), high, 75 ng mL^{-1} (HQC), and dilution 42,5 ng mL^{-1} (DQC) (upper limit of quantification (ULOQ, 100 ng mL^{-1}) added of 70% (dilution factor = 4)). Calibration curves in plasma were prepared to the final concentration range of 0.3, 1, 5, 20, 40, 60, 80, and 100 ng mL^{-1}. IS was prepared in methanol.

Concomitant medication

Concomitant medication stock solutions (4-MAA hydrochloride, dimenhydrinate, paracetamol, metoclopramide, scopolamine butyl bromide, pyridoxine, caffeine, and ondansetron) were prepared and diluted in plasma to be equal or superior to the expected maximum plasma concentration of these drugs. Concomitant drugs were selected as they were occasionally prescribed or used by some volunteers during the clinical trial.[31]

Sample preparation

Approval from the Institutional Review Board (IRB) was obtained (protocol number 0343018.4.0000.5514). All study participants signed a consent form and were free to withdraw from the study at any time. Venous blood (5 mL) was collected for development and validation analysis using tubes containing ethylenediaminetetraacetic acid (EDTA). After collection, the samples were centrifuged at 1,500 rpm for 10 min. Subsequently, the plasma was carefully collected and stored at –80 °C for later analysis. An aliquot of the plasma (200 μL) was combined with 50 μL of IS solution in acetonitrile (ACN), and 400 μL of ACN the mixture was vigorously shaken in Finemixer table shaker

(Gyeonggi-do, South Korea) for 5 min. The sample was then centrifuged by Centrifuge 5418 R purchased of Eppendorf, (Hamburg, Germany) at 13,200 rpm at 4 °C for 10 min, post-centrifugation, 50 µL of the supernatant was carefully transferred and 200 µL of H_2O with 0.1% formic acid was added. This mixture was shaken for an additional 2 min and then transferred the solution into a vial and injected into the LC-MS/MS system for analysis.

LC-MS/MS analysis

Chromatographic separation was performed using a Phenomenex Synergi 4 µm Fusion (Torrance, USA) reverse phase 80 Å column (150 mm, 2 mm) at a controlled temperature of 22 °C. The separation utilized a dual mobile phase system. Phase A consisting of 10 mM ammonium acetate in H_2O with 0.1% formic acid, and phase B composed of ACN with 0.1% formic acid, in a 9:1 (A:B) ratio, the mobile phases were filtered and degassed. The flow rate was maintained at 0.6 mL min^{-1} in the first 1.2 min, and in the next 1.8 min, the flow was 0.4 mL min^{-1} and returned to the initial conditions of 0.6 mL min^{-1} by the end of the analysis, totalizing a 4 min run, using 10 µL as the injection volume. Mass spectrometric analysis was conducted using a Waters Xevo TQ-S (Newcastle, United Kingdom) tandem quadrupole mass spectrometer, equipped with an electrospray ionization source in the positive mode. Nitrogen was used as the ion source gas, with argon as the collisional gas. Optimized settings included a capillary voltage of 2.0 kV, cone voltages of 30 V for LDX and 20 V for IS, and collision energies of 20 V for LDX and 10 V for IS. The source and desolvation temperatures were set to 130 and 600 °C, respectively. Multiple reaction monitoring (MRM) tracked the *m/z* transitions: 264.18 > 84.09 for LDX and 144.08 > 97.02 for the IS. Data processing was performed with the MassLynx 4.1 software from Waters (Newcastle, United Kingdom).

Method validation

The analytical method was validated according to Agência Nacional de Vigilância Sanitária (ANVISA) Resolution No. 27/2012,[32] with the following parameters: selectivity, concomitant selectivity, carryover assay, matrix effect, concomitant matrix effect, calibration curve, accuracy, precision, and stability. In addition, we tested the LC-MS/MS system reproducibility with a second instrument.

Selectivity

Selectivity was ascertained by analyzing blank human plasma samples from six individuals (four normal, one lipemic and one hemolyzed), comparing the chromatograms with chromatograms from blank human plasma spiked with LDX in the lower limit of quantification (LLOQ) concentration (0.3 ng mL^{-1}) and IS. Selectivity was also tested in the presence of concomitant medications.

Carryover

Carryover was ascertained by analyzing three injections of the same blank sample, one sample in the LLOQ concentration (0.3 ng mL^{-1}), and one sample ULOQ (100 ng mL^{-1}) chromatograms. In this sequence: LLOQ sample, blank sample, ULOQ samples, and two blank samples.

Matrix effect

The matrix effect was ascertained by spiking eight different extracted blank human plasma samples (four normal, two lipemic, and two hemolyzed) with LDX at QC (LQC and HQC) concentrations and the IS. Peak areas of extracted spiked samples were compared to those of standard solutions. The results will be evaluated based on the calculation of the normalized matrix factor, where the value of the individual response is divided by the average of the solution response. Matrix effect was also tested in the presence of concomitant medications.

Calibration curve/linearity

The LLOQ was determined based on the data obtained in the literature and should preferably be within the limits of 1 to 5% of the expected maximum concentration (C_{max}). To evaluate the sensitivity, precision, and accuracy of the method, samples with the LLOQ value were also included in the validation procedure. The ULOQ is usually defined based on the observed mean C_{max} value plus at least 80 to 100% of this value. The other points of the calibration curve must be appropriately distributed along the curve, taking into account the interval between the LLOQ and the ULOQ.

The calibration curve was prepared using a blank sample (plasma free of drug standard and IS), a zero sample (plasma with the addition of IS), and seven levels ranging from 0.3, 5, 20, 40, 60, 80, and 100 ng mL^{-1}. Analyte concentrations in samples were calculated by linear regression equation, typically described by equation $y = ax + b$ where *y* corresponds to the analyte/IS peak area ratio and *x* corresponds to the ratio of LDX to IS concentration. Due to the range of the calibration curve and the lower value of the sum of the relative errors of the nominal values of the calibration *versus* its values obtained by the curve equation, the weighting factor of reciprocal concentration squared ($1/x^2$) was applied.

Precision, reproducibility and accuracy

The study evaluated the reproducibility within the same batch (intra-batch), across different batches (inter-batch), and using another LC-MS/MS system under the same analysis conditions. The inter-batch precision assays were assessed with at least a 48-h difference. The evaluation was conducted at five different levels: LLOQ, LQC, DQC, MQC, and HQC (0.3, 0.9, 42.5, 50, 75 ng mL^{-1}), QC samples were analyzed in quintuplicate (five replicates) within three different batches. The accuracy was evaluated by the relative error (RE) for accuracy should not exceed ±20% for the LLOQ, and for other QC samples not exceed ±15%. The coefficient of variation (CV) is used to assess precision. The CV should follow the same quality parameter: ±20% for the LLOQ and ±15% for other QC samples. The RE and CV values play a crucial role in assessing the method's reliability and consistency.

Drug stability in plasma

All stability assays were performed to cover the conditions anticipated for handling real samples: freshly prepared, post-processing, short-term, freeze-thaw, and long-term were evaluated at concentrations of 0.9 ng mL^{-1} (LQC) and 75 ng mL^{-1} (HQC). Post-processing, the samples were left in the autosampler and on the bench at room temperature (RT, ca. 22 °C) for ca. 35 h. For short-term stability, experimental samples were kept at RT for ca. 24 h. For freeze-thaw stability, 3 freeze-thaw cycles were performed and samples were at −20 °C freezer and each at −70 °C freezer. For long-term stability, experimental samples were kept at −20 or −70 °C for 167 days. RE and CV were used to check possible variations.

Results and Discussion

Method validation

The chromatograms depicted in Figure 2 illustrate the analysis of LDX, with a retention time of 1.41 min, and the IS, at 2.68 min. Selectivity assay for LDX showed that interference peak areas of all the blank samples were found to be lower than 20 and 5% when compared with the LLOQ and IS, respectively. The monitoring of specific production ions for each compound (analyte and IS) ensured a highly selective method. As shown in Figure 2, LDX and IS presented no interfering peaks from the endogenous components in blank plasma. Selectivity for concomitant medication was tested, and all interferences were lower than the evaluation criteria for the analyte and IS, not interfering with quantifications.

We performed carryover assays and no residual area was observed in the chromatograms of the blank samples for the analyte and its IS, evidencing the absence of a carryover effect. Assessing matrix effects for co-elution with any other plasma molecules is essential to maintain consistent ionization efficiency and avoid any potential signal suppression. In this assay, the coefficient of variation of the value of the normalized matrix factor was 2.16%, demonstrating that our method had no matrix effects and can be used to quantify the analyte in samples of normal,

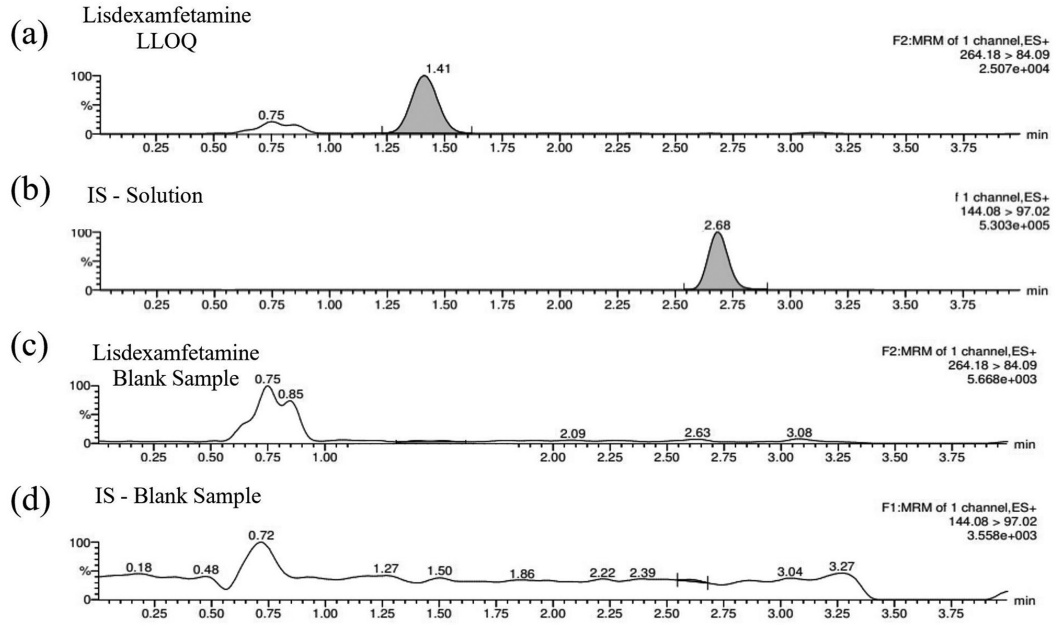

Figure 2. Chromatographic analysis demonstrating method selectivity. Chromatogram of the sample infused with LDX detection channel (a) and IS (b). Chromatogram of the blank sample for both the LDX and IS detection channels (c-d).

lipemic and hemolyzed plasma, with a degree of hemolysis up to four. Furthermore, it was confirmed that concomitant medications did not interfere with quantification (2.72%), reinforcing the consistency and reliability of the method.

The calibration curves showed good linearity in the range of 0.3-100 ng mL^{-1}. The individual regression equations and coefficient of determination (r^2) are as follows in Figure 3, where *y* is the ratio of analyte peak area to IS peak area and *x* is the corresponding plasma concentration. The RE values for each point on the calibration curve were satisfactory.

Accuracy and precision results are summarized in Table 1. All QC concentrations presented RE values within the threshold of ±15%. Intra-, inter-batch, and other LC-MS/MS system precision was lower than 10%, demonstrating the closeness of measurements of the same concentration. Also, the precision values of diluted samples are evidence that samples could be accurately and precisely diluted four times from their original concentration.

Concomitant medications have been tested for precision and accuracy. The CV (%) was –7.467 (LLOQ), 1.867 (LQC), –1.314 (MQC), 4.181 (DCQ), and –5.246 (HQC). Thus, none of the concomitant medications interfered in the LDX and IS quantification.

The stability results of plasma samples after different storage conditions are summarized in Table 2. All assays presented precision and accuracy values lower than 15%, demonstrating stability during the period tested. Moreover,

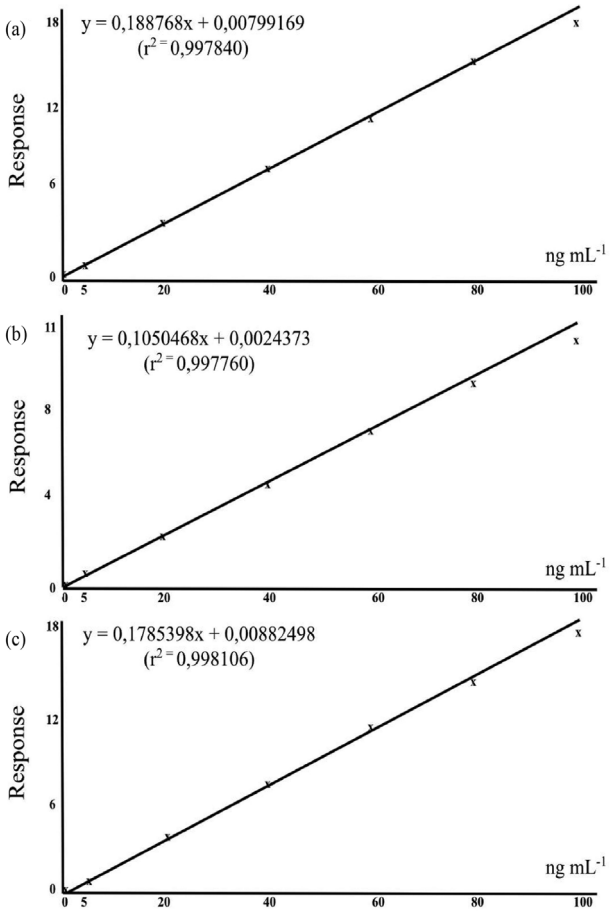

Figure 3. The regression equations and coefficient of determination (r^2) for calibration curves.

Table 1. Precision and accuracy of lisdexamfetamine in human plasma

Intra-batch (n = 15)				Inter-batch (n = 5)				Other LC-MS/MS system (n = 5)		
Sample (No. replicate)	Concentration (mean ± SD) / (ng mL^{-1})	RE / %	CV / %	Sample	Concentration (mean ± SD) / (ng mL^{-1})	RE / %	CV / %	Concentration (mean ± SD) / (ng mL^{-1})	RE / %	CV / %
LLOQ (1)	0.277 (0.012)	–7.667	4.407							
LLOQ (2)	0.307 (0.006)	2.400	1.999	LLOQ	0.296 (0.016)	–1.178	5.517	0.302 (0.024)	0.533	7.884
LLOQ (3)	0.305 (0.006)	1.733	1.985							
LQC (1)	0.937 (0.040)	4.089	4.234							
LQC (2)	0.951 (0.010)	5.667	1.083	LQC	0.964 (0.040)	7.141	4.107	0.876 (0.034)	–2.659	3.870
LQC (3)	1.005 (0.024)	11.667	2.383							
DQC (1)	38.715 (0.409)	–8.905	1.056							
DQC (2)	45.309 (0.341)	6.609	0.753	DQC	43.395 (3.500)	2.106	8.065	41.466 (0.535)	–2.433	1.289
DQC (3)	46.161 (1.035)	8.615	2.243							
MQC (1)	50.310 (0.939)	0.621	1.866							
MQC (2)	49.016 (0.746)	–1.969	1.521	MQC	51.515 (2.853)	3.029	5.538	48.857 (1.347)	–2.287	2.758
MQC (3)	55.218 (0.533)	10.436	0.965							
HQC (1)	73.131 (1.061)	–2.492	1.450							
HQC (2)	74.306 (1.348)	–0.925	1.813	HQC	75.480 (2.926)	0.640	3.876	72.450 (1.206)	–3.400	1.526
HQC (3)	79.002 (1.700)	5.337	2.151							

SD: standard deviation; RE: relative error; CV: coefficient of variation; LLOQ: lower limit of quantification (0.3 ng mL^{-1}); LQC: low-quality control (0.9 ng mL^{-1}); DQC: dilution quality control (42.5 ng mL^{-1}); MQC: medium quality control (50 ng mL^{-1}); HQC: high-quality control (75 ng mL^{-1}).

long-term stability results demonstrated that LDX in EDTA plasma samples can be stored at 70 °C for 167 days without degradation.

Finally, we have conducted a comprehensive analysis of the existing quantification techniques for LDX as documented in the literature (Table 3), focusing on PK studies. This examination aimed to delineate the principal attributes of these methods and to underscore the superior aspects of our proposed technique.

Table 3 illustrates the development of various LDX quantification methods in plasma. It is important to note the omission of substantial data from several of these studies making methodology comparisons a challenge. The LC-MS/MS methodology is predominantly evidenced in PK studies, is attributed to the detection of the drug's low concentration levels in biological fluids, and the LLOQ proposed by our study (0.3 ng mL^{-1}) is the lowest of all.[14-29] Protein precipitation is the favored technique for plasma sample preparation but varies in plasma volume and organic solvent composition.

Considering the sensitivity (0.3 ng mL^{-1}), the proposed method aligns with that of other LC-MS/MS techniques used in plasma analysis. A significant advantage of our method is its reduced run time of 4 min, which is particularly beneficial for extensive batch analysis in PK studies. For the applicability in pharmacokinetic studies, assessing the influence of common medications is crucial, especially during bioequivalence assessments. However, only two studies have explicitly considered this variable in its validation phase.[28,30] Additionally, the stability of

Table 2. Stability of lisdexamfetamine in human plasma

Analysis description	Conditions	Sample	Concentration in plasma (mean ± SD) / (ng mL^{-1})	RE / %	CV / %
Freshly prepared	0:00 h	LQC	0.998 (0.032)	10.889	3.248
		HQC	76.450 (2.963)	1.934	3.876
Post processing (auto-injector)	34:29 h. RT	LQC	0.979 (0.029)	8.815	2.925
		HQC	69.748 (1.285)	–7.003	1.843
Short-term	22:02 h. RT	LQC	0.989 (0.016)	9.889	1.665
		HQC	77.694 (0.740)	3.592	0.953
Freeze-thaw (3 cycles)	thawing at –20 °C	LQC	0.938 (0.016)	4.222	1.755
		HQC	72.315 (1.672)	–3.580	2.312
	thawing at –70 °C	LQC	0.938 (0.007)	4.185	0.710
		HQC	73.114 (0.973)	–2.514	1.331
Long term stability	167 days at –20 °C	LQC	0.983 (0.036)	9.222	3.615
		HQC	74.674 (5.254)	–0.434	7.036
	167 days at –70 °C	LQC	0.974 (0.023)	8.185	2.392
		HQC	75.297 (0.921)	0.396	1.223

RE: relative error; CV: coefficient of variation; LQC: low-quality control (0.9 ng mL^{-1}); HQC: high-quality control (75 ng mL^{-1}); SD: standard deviation; RT: room temperature.

Table 3. A review of bioanalytical approaches for LDX in plasma using LC-MS/MS as reported in PK studies

Extraction	Criteria (regulatory agencies)	Run time / min	LLOQ / (ng mL^{-1})	Concomitant medications	Frozen storage stability / days	Reference
–	–	–	1	no	–	14,29
Protein precipitation	FDA	12	1	no	30	15,16
Protein precipitation	EKNZ/Swissmedic	1.5	0.78	no	7	17
Protein precipitation	FDA/EMA	6.5	–	yes	38	30
Protein precipitation	ICH and/or FDA	–	1	no	–	18-20
–	ICH and/or FDA	–	1	no	–	21-25
Protein precipitation	–	–	1	no	–	26,27
Protein precipitation	–	–	1	yes	–	28

FDA: U.S. Food and Drug Administration; EKNZ: Ethics Committee northwest/central Switzerland; Swissmedic: Swiss Agency for Therapeutic Products; EMA: European Medicines Agency; ICH: International Conference on Harmonisation Guidelines; LLOQ: lower limit of quantification; –: not shown or not reported.

sample storage-crucial for mitigating potential issues-was assessed in three of the reviewed methods, revealing a stability period of 7 days,[17] 30 days,[15,16] and 38 days[30] in contrast to the 167 days stability documented by the method being proposed.

Conclusions

The article describes a robust bioanalytical method for the quantification of LDX in human plasma using LC-MS/MS. The method incorporates protein precipitation extraction to minimize chromatographic interferences and ensure reliable retention times for the analyte and IS. The described measurement process is both precise and accurate, providing a linear calibration curve over a range of concentrations and demonstrating excellent reproducibility, even when applied to different LC-MS/MS systems. In addition, the stability of LDX under different conditions within the biological matrix is confirmed, which is crucial for the integrity of pharmacokinetic data. In conclusion, the sensitivity and suitability of the method for future pharmacokinetic studies with LDX is highlighted. This indicates its potential to contribute significantly to the understanding of the drug.

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Author Contributions

Alex A. R. Silva was responsible for investigation and methodology, writing original draft, review and editing; Lilia L. D. Coelho for conceptualization, data curation, formal analysis, writing original draft; Karen Y. S. Luquez for writing original draft, review and editing; Pedro H. D. Garcia for investigation and methodology, data curation, formal analysis; Renata Boldin for conceptualization, investigation and methodology, validation and visualization, data curation, project administration; Anne M. R. Silveira for investigation and methodology, writing-review and editing; Marcia A. Antonio for writing-review and editing, resources, project administration; Andreia M. Porcari for writing original draft, review and editing, supervision, project administration; Patrícia O. Carvalho for conceptualization, funding acquisition, project administration, resources, writing-review and editing.

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