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# Validation of a HPLC-UV analytical and bioanalytical method for dexamethasone determination in nanoemulsions, porcine nasal mucosa, and mouse plasma and brain tissue

Luísa Degrandi Sehn[a], Gabriela Fusinatto de Oliveira[a], Melaine Maiara da Silva Lopes[a], Helder Ferreira Teixeira[a], Ursula da Silveira Matte[a,b], Roselena Silvestri Schuh[a,b], Flávia Nathiely Silveira Fachel*[a]

[a] *Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil;* [b] *Células, Tecidos e Genes, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil*
**Corresponding author: flavia.fachel@ufrgs.br*

Dexamethasone (DEX) is a synthetic glucocorticoid widely used in the treatment of inflammatory and autoimmune diseases. However, its clinical efficacy is limited by low water solubility, poor bioavailability, and a high incidence of side effects. To overcome these limitations, nanotechnology-based drug delivery systems, such as nanoemulsions, have emerged as promising alternatives, particularly when combined with nasal administration targeting the treatment of neuroinflammation in the central nervous system. This study describes the validation of an analytical and bioanalytical method using high-performance liquid chromatography with ultraviolet detection (HPLC-UV) for the quantification of DEX active pharmaceutical ingredient (API) in nanoemulsions, porcine nasal mucosa, and mouse plasma and brain tissue. The method was validated according to current regulatory guidelines and was based on a procedure previously described in the 7th edition of the Brazilian Pharmacopoeia (2024). The method demonstrated specificity, with no interference from endogenous components of the matrices. Linearity was confirmed in the range of 0.5 to 10.0 µg $mL^{-1}$ for standard solutions, nanoemulsion, porcine nasal mucosa, and mouse plasma, and from 1.0 to 10.0 µg $mL^{-1}$ for mouse brain samples, with correlation coefficients (r) greater than 0.99. The method has also been shown to be precise, accurate and with low matrix effect within regulatory acceptance criteria. These results confirm the method's reliability for determination of DEX in both nanotechnological and biological matrices. The validated method is intended to support future performance studies of nanotechnology-based formulations for nasal administration of DEX.

**Keywords**: Dexamethasone; HPLC; nanotechnology; biological matrices; nasal; plasma; brain.

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## Introduction

Dexamethasone (DEX) is a synthetic glucocorticoid first synthesized in 1957 and introduced into clinical practice in 1961 (Figure 1). Due to its potent anti-inflammatory, immunosuppressive, analgesic, and antiallergic properties, DEX plays a central role in the treatment of a numerous pathological conditions, including autoimmune diseases, allergic disorders, ocular inflammation, neuroinflammation, cancer, and, more recently, severe cases of COVID-19 (1–11). Despite its clinical relevance, the therapeutic potential of DEX is significantly limited by its low water solubility, which results in poor bioavailability and a high incidence of side effects (1). These limitations have prompted a growing interest in the development of advanced drug delivery systems to improve DEX biodistribution (2,3).

Recent advances in nanotechnology-based drug delivery systems have enabled the development of various nanostructures – such as nanoemulsions, liposomes, polymer-based nanocarriers, lipid nanoparticles, and others – that aim to enhance targeted delivery, reduce systemic toxicity, and optimize therapeutic outcomes (2,3). In addition, recent studies have highlighted the potential of alternative delivery routes for transporting anti-inflammatory therapeutic agents directly to the central nervous system, among these, the nasal route stands out due to its ability to bypass the blood-brain barrier (12,13). Thus, our research group has recently focused on developing DEX-loaded nanoemulsion-based delivery systems for nasal administration. Nevertheless, a critical component of pharmaceutical development is the validation of analytical and bioanalytical methods that enable identification and quantification of drugs in both formulations and biological matrices without interference (14).

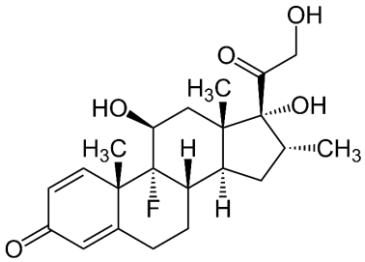


Figure 1. Dexamethasone (DEX) chemical structure (15).

In this context, the present study aims to validate an analytical and bioanalytical method using high-performance liquid chromatography with ultraviolet

detection (HPLC-UV), based on the procedure described in the 7th edition of the Brazilian Pharmacopoeia (2024) for the quantification of the active pharmaceutical ingredient (API) DEX (15). Since the pharmacopoeial method was originally developed for the analysis of DEX in its pure and base form, validation is required to confirm its suitability for more complex matrices, such as nanoemulsions and biological samples, including porcine nasal mucosa, mouse plasma, and brain tissue.

## Experimental section

### Chemicals and reagents

The reference standard of dexamethasone (DEX), with a purity between 97.0% and 102.0%, was purchased from Sigma-Aldrich Ltd. (St. Louis, US). HPLC-grade methanol was obtained from Merck (Darmstadt, DE). Ultra-pure water used in HPLC analyses was produced using a Milli-Q apparatus from Millipore® (Billerica, US). Dexamethasone base and medium chain triglycerides (MCT) were acquired from Delaware (Porto Alegre, BR). Egg-lecithin (Lipoid E-80®), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Lipoid GmbH (Ludwigshafen, DE).

### Instrumentation and chromatographic conditions

The DEX analyses were performed using a High-Performance Liquid Chromatography (Shimadzu, Kyoto, Japan), equipped with a temperature controlled automatic injector, an UV/VIS detector and an LC LabSolutions software (Shimadzu, Kyoto, Japan) for data processing. Chromatographic separation was carried out in a Shimadzu Shim-pack GIST C18 column (250 × 4.6 mm i.d.; particle size, 5.0 µm; Shimadzu, Kyoto, Japan) guarded by a pre-column filter in-line Shim-pack GIST (Shimadzu, Kyoto, Japan) at room temperature. The mobile phase was composed of methanol and water (75:25, v/v), filtered through a 0.22 µm porous nylon membrane and degassed under vacuum, set in isocratic mode up to 8 min. The samples were kept at 4 °C in the auto-sampler, and a volume of 10 µL was injected. The eluent system was pumped at a flow rate of 1.0 mL·min$^{-1}$ and detection wavelength was set to 254 nm (15).

### Standard and matrices solutions preparation

*Standard solutions*

A DEX stock solution (1 mg·mL$^{-1}$) was prepared by accurately weighing 10 mg of DEX into a 10 mL calibrated volumetric flask and dissolving it in methanol. Standard solutions (SS) were obtained by diluting appropriate aliquots of the stock solution in a methanol/water mixture (75:25, v/v) and filtering through a 0.22 µm nylon syringe filter. Both stock and diluted standard solutions were stored at 4 ± 2 °C.

*Nanoemulsion matrix*

Blank nanoemulsions ($NE_B$) were prepared via microfluidization using LV-1 microfluidizer (Microfluidics, Ottawa, CAN). The formulations consisted of MCT, egg lecithin, DOPE, DSPE-PEG, and ultrapure water. Appropriate aliquots of the nanoemulsion were diluted in methanol/water mixture (75:25; v:v) and filtered using a 0.22 µm nylon syringe filter.

*Porcine nasal mucosa matrix*

Porcine nasal mucosa (PNM) was carefully excised from the nasal turbinates following an incision along the nasal septum of the porcine heads, as previously described by Barbi et al. (16). The tissue was stored at −20 °C until use. For extraction procedure, six PNM slices (~1.80 cm$^2$ each) were placed in 24 mL of methanol and kept in an ultrasound bath for 45 min. Appropriate aliquots of the resulting solution were filtered through 0.22 µm nylon syringe filter prior to analysis.

*Mouse plasma matrix*

Mouse plasma (MP) matrix was obtained from C57BL/6 mice (n=3) and proteins were precipitated with 5% (w/v) trichloroacetic acid. Plasma homogenates were centrifuged at 1,000 g for 10 minutes, and the supernatants were collected, diluted 1:1 in methanol/water mixture (75:25; v:v), filtered using a 0.22 µm nylon syringe filter and analyzed.

*Mouse brain matrix*

Mouse brain (MB) was collected from C57BL/6 mice (n=3). For extraction, the brain (0.4072g) was reduced to tiny pieces and placed in a falcon tube with 5 mL of methanol followed by sonication in an ultrasonic bath for 30 minutes. The homogenates were centrifuged at 12,000 rpm for 10 minutes. The supernatant was collected, diluted in 20 mL methanol, filtered through a 0.22 µm nylon syringes filter, and then analyzed.

### HPLC method validation

The HPLC-UV detection method previously described in the 7th edition of the Brazilian Pharmacopoeia (2024) (15) for the analysis of DEX API was validated in terms of specificity, linearity, matrix effect, precision, and accuracy, according to the official guidelines (17–20). Prior to method validation, key chromatographic parameters including retention time, number of theoretical plates, and tailing factor for DEX were measured and evaluated to verify system suitability (21).

*Specificity*

Specificity was assessed by comparing chromatographic profiles, evaluating the retention time of a pure DEX standard solution against those observed in blank matrix samples ($NE_B$, PNM, MP, MB) prepared as described previously. This comparative analysis was employed to ensure that the analytical method could clearly distinguish

DEX from any potential compounds or interfering substances naturally present in the biological matrices.

*Linearity and matrix effect*

Three standard curves were obtained by plotting the measured peak area versus the concentration of DEX standard (0.5, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$), $NE_B$ (0.5, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$), PNM (0.5, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$), MP (0.5, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$), and MB (1.0, 2.0, 4.0, 6.0, 8.0, and 10.0$\mu g \cdot mL^{-1}$), by three replicates per concentration across three consecutive days. Linearity was evaluated by regression analysis using the least square method, and the curves were described in the form of y=ax+b. Analysis of variance (ANOVA) was performed using Microsoft Excel® with a significance level of α=0.05. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated from the slope and the standard deviation of the response of the calibration curves.
The matrix effect was assessed by comparing slopes of standard curves obtained in non-biological and biological DEX-spiked matrices with that of the pure DEX standard curve obtained during linearity assay.

*Precision*

Precision was expressed as the relative standard deviations (% RSD) for each level of DEX and evaluated as both intra-day precision (repeatability) and inter-day precision (intermediate precision). Repeatability and intermediate precision were obtained by analyzing the levels 0.5, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$ of DEX in the presence of the matrices: SS, $NE_B$, PNM and MP, in nine replicates of each level. And for the MB matrix, repeatability and intermediate precision were obtained by analyzing the levels 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$ of DEX, in nine replicates of each level.

*Accuracy*

Accuracy was determined by adding known amounts of DEX standard at levels 0.5, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$ to the matrices: SS, $NE_B$, PNM and MP, in nine replicates of each level. Moreover, for the MB matrix, the accuracy was determined by adding known amounts of DEX standard at levels 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu g \cdot mL^{-1}$, in nine replicates of each level. The determination was calculated as follows: Accuracy % = (mean experimental concentration x 100/ mean theoretical concentration).

*Ethical committee approval*

All animal procedures were conducted with prior approval from the Animal Use Ethics Committee of the Hospital de Clínicas de Porto Alegre, Brazil, under protocol number 20220602. The care and use of animals were in accordance with the Brazilian Guidelines for the Care and Use of Animals in Scientific Research (DBCA) and the National Council for the Control of Animal Experimentation (CONCEA).

## Results and Discussion

### HPLC method validation

The HPLC-UV method for quantification of the DEX API, based on the 7th edition of the Brazilian Pharmacopoeia, was validated according to the parameters established by the official guidelines to demonstrate that the method is selective for detection of the DEX in the complex matrices tested (15). The overall system suitability results demonstrated that the HPLC-UV detection method is appropriate for the analysis of DEX in $NE_B$, PNM, MP, and MB. The performance of the evaluated parameters was considered satisfactory, with approximate mean values and corresponding relative standard deviations (RSD, %) as follows: retention time of approximately 4.68 ± 0.05 minutes, number of plates of about 6684.81 ± 0.08, and a tailing factor close to 1.13 ± 0.06 (17).
Several HPLC-UV methods for dexamethasone quantification have been reported in the literature, including application to microemulsions (22) biological tissues (23), drug-loaded microspheres (24), and medical devices such as drug-eluting stents (25). While these methods show good performance, many involve complex sample preparation, longer run times, or limited applicability to specific matrices. In contrast, the method proposed in the present study offers simple and reproducible sample preparation protocol, adaptability to multiple biological matrices, faster runtime and simple mobile phase. Furthermore, by being based on a compendial method, it benefits from a solid foundation of reliability, ensuring reproducibility and easy implementation in routine.

*Specificity*

No co-eluting substances were detected in the blank matrices at the same retention time as dexamethasone (DEX) (Figure 2), indicating the absence of interfering peaks. This result confirms that the method is specific for the determination of DEX, even in the presence of complex matrices such as NEB, PNM, MP, and MB.

*Linearity*

The linearity of the method was evaluated through the analysis of DEX standard curves. As shown in Table 1, the correlation coefficients indicated that the method was linear for the determination of DEX across all matrices within the tested range (17). The calculated LOD and LOQ are also presented in Table 1. The lower limit of quantification (LLOQ) was established at 0.5 $\mu g \cdot mL^{-1}$ for the SS, NEB, PNM and MP matrices, and at 1.0 $\mu g \cdot mL^{-1}$ for the MB matrix. These values represent the lowest concentrations of DEX that could be quantified with acceptable precision and accuracy. Table 2 presents the linear regression results and demonstrates the absence of deviation from linearity, as confirmed by ANOVA for regression significance and confidence intervals of the intercepts.

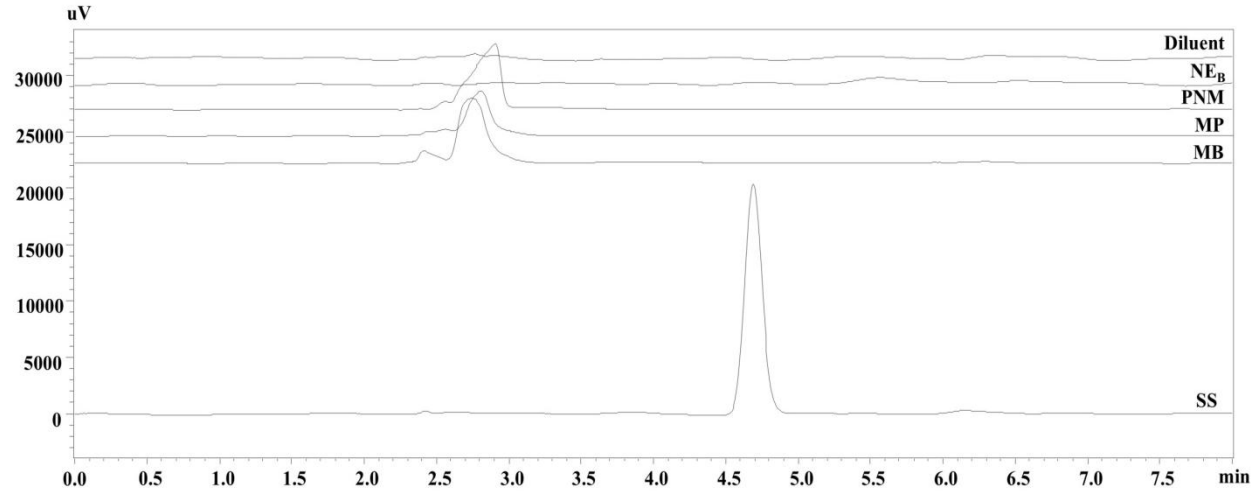


Figure 2. DEX chromatographic profile standard at 10 $\mu g \cdot mL^{-1}$ and blank samples (specificity in the different matrices). SS: DEX standard solution; $NE_B$: blank nanoemulsion; PNM: porcine nasal mucosa; MP: mouse plasma; MB: mouse brain.

*Matrix effect*

Matrix effect assessment is critical during method validation, particularly when analyzing biological or complex samples, to ensure accurate quantification of the analyte regardless of matrix components (26,27). According to Niessen et al. (28), matrix effects are considered low for the response in the range of -20% to +20%. The results obtained for the matrix effects (Table 1) in $NE_B$, PNM, MP and MB were all below 16.46% in absolute value, indicating a low matrix effect in the quantification of DEX.

Table 1. Linearity of DEX standard and matrices.

| Matrix | Range | Equation | r | LOD | LOQ | ME |
|---|---|---|---|---|---|---|
| | ($\mu g \cdot mL^{-1}$) | | | ($\mu g \cdot mL^{-1}$) | | (%) |
| SS | 0.5 – 10.0 | y = 17586x – 911.59 | 0.9986 | 0.103 | 0.313 | - |
| $NE_B$ | 0.5 – 10.0 | y = 17496x + 128.39 | 0.9984 | 0.111 | 0.336 | -0.51 |
| PNM | 0.5 – 10.0 | y = 21052x – 1287.1 | 0.9939 | 0.220 | 0.670 | 16.46 |
| MP | 0.5 – 10.0 | y = 20750x + 629.35 | 0.9988 | 0.099 | 0.303 | 15.25 |
| MB | 1.0 – 10.0 | y = 16644x + 1705.3 | 0.9938 | 0.230 | 0.690 | -5.66 |

SS: DEX standard solution; $NE_B$: blank nanoemulsion; PNM: porcine nasal mucosa; MP: mouse plasma; MB: mouse brain; r= correlation coefficient; LOD= limit of detection; LOQ= limit of quantification; ME= matrix effect.

Table 2. Summary of the output of the ANOVA for the evaluation of linearity.

| | *Regression* | *Intercept* | | |
|---|---|---|---|---|
| | Significance F | p-value | Lower 95% | Upper 95% |
| SS | 5.69.E-76 [a] | 0.104 [b] | -194.34 | 2017.53 |
| $NE_B$ | 1.99E-74 [a] | 0.828 [b] | -1049.80 | 1306.58 |
| PNM | 3.36E-59 [a] | 0.363 [b] | -4163.64 | 1529.36 |
| MP | 2.32E-77 [a] | 0.321 [b] | -632.26 | 1890.95 |
| MB | 5.32E-59 [a] | 0.147 [b] | -623.23 | 4033.73 |

SS: DEX standard solution; $NE_B$: blank nanoemulsion; PNM: porcine nasal mucosa; MP: mouse plasma; MB: mouse brain. [a] 95% confidence level = significant linear regression; [b] 95% confidence level = no significant linearity deviation.

*Precision and accuracy*

The results for repeatability (intra-day precision), intermediate precision (inter-day precision), and accuracy of the DEX assay in the tested matrices are presented in Table 3. The matrices were spiked with DEX at concentrations of 0.5 $\mu g \cdot mL^{-1}$ or 1.0 $\mu g \cdot mL^{-1}$ (LLOQ), 2.0 $\mu g \cdot mL^{-1}$, 4.0 $\mu g \cdot mL^{-1}$, 6.0 $\mu g \cdot mL^{-1}$, 8.0 $\mu g \cdot mL^{-1}$, and 10.0 $\mu g \cdot mL^{-1}$.

Intra-day precision, expressed as the relative standard deviation (RSD, %), was below 3.7% for analytical assays (SS and NEB) and below 15.0% for bioanalytical assays (PNM, MP and MB), except at the LLOQ level. Inter-day precision showed RSD values lower than 3.7% for analytical assays, also except for the LLOQ. For bioanalytical assays, inter-day RSD values remained below 15.0% (29).

The accuracy results for the analytical assays ranged from 97.77% to 100.83%, meeting the acceptance criteria of ±5% (i.e., within the range of 95–105%). For the bioanalytical assays, accuracy values ranged from 93.30% to 109.68%, which are within the acceptable limits of ±15% (85–115%) and ±20% at the LLOQ level. Based on these results, the method was considered precise and accurate for the determination of dexamethasone (DEX) in standard solutions, nanoemulsions, porcine nasal mucosa, mouse plasma, and mouse brain matrices, in accordance with official validation guidelines (15,17–20).

Table 3. Repeatability, intermediate precision, accuracy and stability evaluation of DEX standard and matrices.

| Matrix | Level (µg/mL) | Precision (RSD) | | | | Accuracy (%) |
|---|---|---|---|---|---|---|
| | | First day [a] | Second day [a] | Third day [a] | Inter-day [b] | |
| SS | 0.5 | 3.58 | 1.43 | 1.61 | 3.49 | 99.16 |
| | 2.0 | 0.14 | 0.13 | 0.69 | 1.02 | 100.83 |
| | 4.0 | 0.47 | 0.67 | 0.50 | 1.92 | 99.56 |
| | 6.0 | 0.73 | 1.10 | 0.33 | 2.28 | 100.04 |
| | 8.0 | 0.59 | 0.37 | 0.87 | 2.00 | 100.02 |
| | 10.0 | 3.07 | 2.26 | 2.04 | 2.22 | 100.01 |
| $NE_B$ | 0.5 | 1.42 | 1.44 | 0.75 | 4.38 | 97.77 |
| | 2.0 | 0.83 | 0.59 | 0.33 | 1.30 | 100.16 |
| | 4.0 | 1.05 | 0.63 | 1.10 | 2.27 | 99.87 |
| | 6.0 | 0.91 | 1.14 | 0.82 | 1.94 | 100.56 |
| | 8.0 | 1.39 | 0.51 | 0.70 | 1.92 | 100.11 |
| | 10.0 | 0.65 | 1.56 | 0.71 | 2.61 | 99.75 |
| PNM | 0.5 | 3.98 | 1.72 | 3.38 | 5.22 | 109.68 |
| | 2.0 | 2.43 | 0.98 | 2.44 | 3.22 | 100.23 |
| | 4.0 | 0.98 | 1.55 | 3.74 | 3.94 | 99.26 |
| | 6.0 | 2.39 | 4.21 | 1.54 | 4.30 | 98.68 |
| | 8.0 | 1.61 | 0.82 | 1.55 | 4.06 | 99.99 |
| | 10.0 | 0.89 | 0.13 | 0.35 | 4.93 | 100.57 |
| MP | 0.5 | 15.69 | 6.88 | 1.74 | 11.95 | 105.21 |
| | 2.0 | 3.92 | 1.18 | 2.33 | 2.44 | 99.03 |
| | 4.0 | 0.91 | 1.20 | 0.41 | 0.95 | 101.54 |
| | 6.0 | 1.38 | 1.06 | 1.05 | 1.37 | 97.49 |
| | 8.0 | 1.62 | 1.29 | 0.16 | 2.27 | 100.87 |
| | 10.0 | 0.40 | 0.25 | 0.18 | 0.71 | 100.12 |
| MB | 1.0 | 1.22 | 2.47 | 1.11 | 5.69 | 93.30 |
| | 2.0 | 0.29 | 2.34 | 0.49 | 2.89 | 96.66 |
| | 4.0 | 3.42 | 0.68 | 2.15 | 3.91 | 102.89 |
| | 6.0 | 0.78 | 1.76 | 1.58 | 3.94 | 102.19 |
| | 8.0 | 5.57 | 0.62 | 2.22 | 5.02 | 99.49 |
| | 10.0 | 2.15 | 0.90 | 2.28 | 3.49 | 99.28 |

SS: DEX standard solution; $NE_B$: blank nanoemulsion; PNM: porcine nasal mucosa; MP: mouse plasma; MB: mouse brain. [a] three replicates per day; [b] n = 3 days; RSD = relative standard deviation (%).

## Conclusions

In the present study, an HPLC-UV method previously described in the 7th edition of the Brazilian Pharmacopoeia (2024) was successfully validated for the determination of DEX in nanoemulsions, porcine nasal mucosa, and mouse plasma and brain tissue. The method proved to be specific, linear, precise, and accurate within regulatory acceptance criteria, and exhibited minimal matrix effect in various complex matrices—both biological and non-biological—under the established analytical conditions. As a result, this validation is intended to support future performance studies and contribute to the development of effective nanotechnology-based formulations for the nasal administration of DEX.

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## Conflict of interest

The authors declare no conflicts of interest.

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