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A simple and Cost-effective Method to Determine Dexamethasone in Murine Cochlea Using Solvent Extraction and Multiple Reaction Monitoring

Jun Yeop Kim[1†], Ye Bin Shin[1†], Yoon Gyoon Kim[1], Sung Giu Jin[2], Dong-Kee Kim[3], Myung Joo Kang[1]*, and Yong Seok Choi[1]*

[1]College of Pharmacy, Dankook University, Cheonan, Chungnam 31116, South Korea
[2]Department of Pharmaceutical Engineering, Dankook University, Cheonan, Chungnam 31116, South Korea
[3]Department of Otolaryngology, College of Medicine, The Catholic University of Korea, Daejeon, 34943, South Korea†These authors contributed equally to this work.

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Abstract : Dexamethasone (DEX) is a glucocorticoid commonly used to treat idiopathic sudden sensorineural hearing loss (ISSNHL) and inner ear disorders like Meniere's disease. However, systemic administration of DEX is associated with significant side effects, such as hypertension and peptic ulcer, highlighting the need for safer and more effective intratympanic (IT) formulations and reliable methods for their *in vivo* evaluation. However, methods to determine DEX in the cochlea require a tissue lyser, uncommon in laboratories for instrumental analyses, and their analytical performances have not been validated. To address these issues, a simple and cost-effective method to determine DEX in murine cochlear tissue was developed using triamcinolone acetonide as the internal standard (IS), acetonitrile as a single extraction solvent, and LC-MS/MS as an instrumental method. The developed method was successfully validated through selectivity, linearity ($r^2 \geq 0.999$ within 1–500 ng/mL), accuracy (ranging from 86.8% to 100.2%), precision (≤ 5.8%), matrix effect (91.56% to 104.46%), recovery (93.1% to 104.5%) and the lower limit of quantitation (1.0 ng/mL) following FDA guidelines. This method is expected to contribute to the development of novel formulations for IT delivery of DEX for inner ear disorders.

Keywords : dexamethasone, triamcinolone acetonide, murine cochlea, solvent extraction, MRM

Introduction

Dexamethasone (DEX, Figure 1A) is a glucocorticoid widely used in the treatment of inner ear disorders such as idiopathic sudden sensorineural hearing loss (ISSNHL) and Meniere's disease.[1] However, systemic administration of DEX can cause serious side effects such as hypertension, peptic ulcer, hyperglycemia, and fluid-electrolyte imbalance.[2] To address these side effect issues, studies to find an alternative route for DEX administration have been carried out, and intratympanic (IT) administration has been proven to be the safest and most effective.[3] However, since DEX has poor solubility in water (log P = 1.83), the development of optimized DEX formulations for IT delivery has been actively pursued to overcome this issue.[4] Consequently, there is a need for appropriate methods for the *in vivo* evaluation of new DEX formulations. Recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS), especially employing multiple reaction monitoring (LC-MRM), has been preferred for quantifying DEX in biological samples due to its high specificity and sensitivity.[5-7] In terms of extraction and purification (E/P) steps, various approaches such as solvent extraction and solid-phase extraction (SPE) have been employed, but stable isotope-labeled substances are generally accepted as internal standards.[8-11] While stable isotope-labeled ISs may enhance the accuracy of analytical results, their high cost makes them uneconomical. For the determination of DEX in cochlear tissue using LC-MRM, a tissue lyser has been widely used to homogeneously grind the cochlear tissue to increase the extraction efficiency during extraction and purification (E/P) steps.[12-14] However, their analytical performances were

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†These authors contributed equally to this work.
*Reprint requests to Yong Seok Choi
https://orcid.org/0000-0001-6740-3160
E-mail: analysc@dankook.ac.kr
*Reprint requests to Myung Joo Kang
https://orcid.org/0000-0001-8878-2972
E-mail: kangmj@dankook.ac.kr

not proven through validation processes and their E/P steps require a tissue lyser, uncommon in a laboratory for instrumental analyses.

In this study, a simple, efficient, and economical method for determining DEX in murine cochlea by using solvent extraction and LC-MRM was developed and validated. We improved cost-effectiveness by using triamcinolone acetonide as an internal standard (IS) and established simple and efficient E/P steps using 100% acetonitrile. The present method is expected to contribute significantly to the development of novel formulations for IT delivery of DEX for inner ear disorders.

Experimental

Chemicals and Reagents

Dexamethasone (≥ 99.0%), triamcinolone acetonide (the IS, ≥ 99.0%, Figure 1B), and formic acid (LC-MS-grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) and ultrapure water were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Preparation of Standard Solutions

Stock solutions of DEX and IS were prepared by dissolving them in acetonitrile at a concentration of 1 mg/mL and stored at -27°C until use. For the DEX working solution, the DEX stock solution was diluted with acetonitrile to prepare 1000 ng/mL. In addition, the extraction solvent was prepared by diluting the IS working solution to 200 ng/mL with acetonitrile. All solutions were stored at -27°C prior to use.

Sample Collection

Murine cochlear tissues were collected in accordance with the National Research Council guidelines for the care and use of laboratory animals and with the approval of the Animal Experiment Ethics Committee of the Catholic University of Korea Daejeon St. Mary's Hospital. (Approval No: CMCDJ-AP-2023-004, April 5, 2023). Eight-week-old male BALB/c mice (Orient Bio, Seoul, South Korea) weighing 20–23 g were anesthetized via intraperitoneal injection of 30 mg/kg tiletamine/zolazepam (Zoletil®, Virbac, Carros, France) and 10 mg/kg xylazine (Rompun®, Bayer, Leverkusen, Germany). The animals were placed on a temperature-controlled heating pad, and a midline incision was made to expose the middle ear for the collection of cochlear samples (approximately 14 mg). The samples were rinsed with isotonic phosphate-buffered saline (PBS) to remove blood from their surfaces. The cochlear tissues were stored at -80°C until sample preparation.

Sample Preparation

Approximately 14 mg of frozen murine cochlear tissue was pulverized using a mortar and pestle while wrapped in weighing paper. The resulting powdered tissue was then mixed with 400 μL of extraction solvent containing IS and shaken for five minutes (min). The mixture was centrifuged at 1,200 × g for 10 min, and the supernatant was analyzed using LC-MS/MS. A matrix-matched standard (MMS) and a standard-spiked sample (SSS) were prepared by adding an appropriate volume of a DEX working solution to the prepared blank murine cochlea extract and to blank murine cochlea prior to sample preparation steps, respectively. Calibration curves based on standard solutions were prepared for concentrations ranging from 1 to 500 ng/mL. SSSs were employed as quality control (QC) samples at specific concentrations (1, 3, 250, and 500 ng/mL). In all solutions analyzed by LC-MS/MS, the concentration of IS was maintained at 100 ng/mL.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

The LC-MRM analysis was performed using a Shimadzu Nexera UPLC system (Tokyo, Japan) and a Shimadzu LCMS 8050 triple quadrupole mass spectrometer with electrospray ionization (ESI) in positive ion mode. Separation was achieved using a Phenomenex Gemini NX-C18 column (2.1 × 150 mm, 3 μm, Torrance, CA, USA) under gradient conditions, with 0.1% formic acid in water (A) and 100% acetonitrile (B) as the mobile phases. It was maintained at 40% (*v/v*) of mobile phase B for the initial 0.50 min and then increased to 80% (*v/v*) of mobile phase B until 1.50 min. This mobile phase condition was maintained until 3.00 min. Then, the portion of mobile phase B was reduced again to 40% (*v/v*) until 3.50 min, where it was maintained until the end of the separation (5.00 min). Autosampler and column oven temperatures were maintained at 4°C and 40°C, respectively. The mass spectrometer was operated with the following settings: nebulizing gas flow rate of 3 L/min, heating gas flow rate of 10 L/min, dry gas flow rate of 10 L/min, interface temperature of 300°C, DL temperature of 250°C and heating block temperature at 400°C. Among the MS/MS scan modes, MRM was selected for the selective and sensitive determination of DEX, a total of three MRM transitions per DEX or IS were monitored. In the case of the DEX, 393.3 *m/z* (precursor ion) / 373.2 *m/z* (product ion) / -10 V (collision energy) was set for the screening transition, and 393.3 *m/z* / 355.1 *m/z* / -10 V, and 393.3 *m/z* / 337.1 *m/z* / -28 V were used as confirmatory transitions. For IS, 435.1 *m/z* / 415.2 *m/z* / -25 V was used as

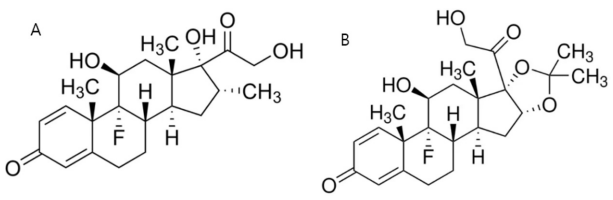

Figure 1. Chemical structures of dexamethasone (A) and triamcinolone acetonide (B).

Table 1. Properties of dexamethasone (DEX) and triamcinolone acetonide (IS).

Compound	Exact mass (amu)	logP	Retention time (minutes)	MRM transitions		
				Precursor ion (*m/z*)	[a]Product ion (*m/z*)	[b]CE (V)
Dexamethasone (DEX)	392.5	1.83	2.87	393.3	373.2	-10.0
					355.1	-10.0
					337.1	-28.0
Triamcinolone acetonide (IS)	434.2	2.50	3.05	435.1	415.2	-25.0
					339.1	-13.0
					321.0	-16.0

[a]The product ion of a screening transition; the product ion of a confirmatory transition
[b]Collision energy; the CE of a screening transition; the CE of a confirmatory transition

the screening transition, and 435.1 *m/z* / 339.1 *m/z* / -13 V, and 435.1 *m/z* / 321.0 *m/z* / -16 V were employed as the first and second confirmation transitions, respectively (Table 1). All LC-MS/MS data were processed using Shimadzu Lab Solutions software version 5.93. The screening transition peak area ratios of DEX versus IS were used for quantification if the following conditions were met: retention times for all three transitions were consistent, the signal-to-noise ratio (S/N) for the screening transition peak was greater than 10, and the S/N for the confirmation transition was greater than 3.

Results and Discussion

Liquid Chromatography and Multiple Reaction Monitoring

As mentioned above, many studies on determining DEX in biological samples have employed stable isotope-labeled DEXs as internal standards for high accuracy, but they are uneconomical.[8-11] Since we have successfully replaced stable isotope-labeled ISs with other non-labeled compounds which have similarities in chemical structures and logP values in previous studies, triamcinolone acetonide was selected as the IS of the present study due to its similarity to DEX in the chemical structure (Figure 1) and the logP value (1.83 and 2.50 for DEX and the IS, respectively.[4,15-17] While a couple of studies to determine DEX in dried blood and skin samples employed triamcinolone acetonide as the IS, the present study is its first use as the IS for the analysis of DEX in cochlear tissue.[18,19] For MRM analysis in positive ion mode, $[M+H]^+$ ions were selected as precursor ions (393.2 *m/z* and 435.1 *m/z* for DEX and the IS, respectively). Product ions of transitions were decided based on the results from product ion scans (PIS) of the precursor ions: For screening transitions, the most intense fragment ion in each PIS spectrum was chosen (373.2 *m/z* and 415.2 *m/z* for DEX and the IS, respectively), the second and third most intense fragment ions were selected for confirmatory transitions (DEX: 355.1 *m/z* and 337.1 *m/z*; IS: 339.1 *m/z* and 321.0 *m/z*) (Table 1). Screening transitions were used for quantification, while confirmatory transitions were utilized to verify analyte identity. For the separation of DEX, the C18 column and mobile phases with formic acid were used, because DEX mainly exists in the singly protonated form, enabling stable separation on the C18 column and detection at positive ion mode of a mass spectrometer, at low pH (approximately 3) of the mobile phase.[20,21]

Sample Preparation

In the present study, DEX was extracted from the murine

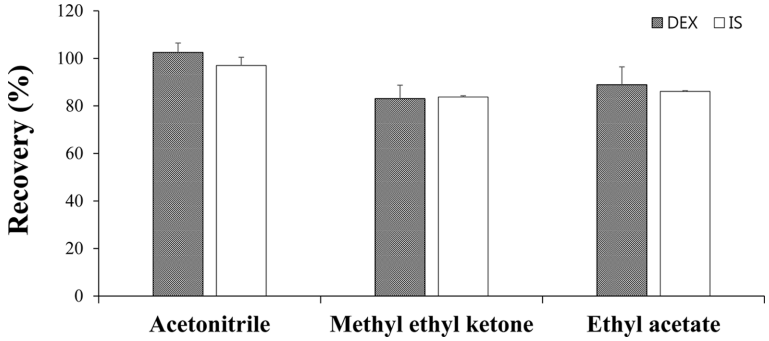

Figure 2. Effect of different extraction solvents on the recovery of dexamethasone (DEX, 100 ng/mL) and Triamcinolone acetonide (IS, 100 ng/mL) in murine cochlea ($n = 3$).

cochlea simply and rapidly using a solvent extraction method. In previous studies, methanol was used to extract DEX from the murine cochlea, but its volatility can cause concentration fluctuations during the E/P and analyses.[8-10] This probable issue was not reported in the studies, and it must have been related to the fact that no validation of their analytical methods was carried out. To address this issue, three organic solvents with a similar polarity index to that of methanol (5.1) but a higher boiling point (BP) than that of methanol (64°C) were selected for their comparative experiments: acetonitrile (the polarity index of 5.8 and the BP of 82°C), methyl ethyl ketone (the polarity index of 4.7 and the BP of 80°C), and ethyl acetate (the polarity index of 4.4 and the BP of 77°C).[22-27] As shown in Figure 2, only acetonitrile experiments produced recovery values close to 100% (102.50 ± 3.97% and 97.01 ± 3.46% for DEX and the IS, respectively) from the recovery test (100 ng/mL for DEX and the IS). Thus, acetonitrile was selected as the optimal extraction solvent. Additionally, no contamination on the surface of the mass spectrometer curtain plate due to substances derived from cochlear tissue was confirmed from continuous runs of QC samples. Another notable feature of the present E/P method is its reliance on a simple solvent, acetonitrile, rather than a tissue lyser. The simple solvent extraction during E/P also reduced time for E/P.

Method Validation

This method was validated according to FDA guidance, specifically 'M10 Bioanalytical Method Validation and Research Sample Analysis'.[28] The validation assessed specificity, linearity, sensitivity (the lower limit of quantification, LLOQ), accuracy, precision, matrix effect, and recovery. The results of the evaluated parameters should have an accuracy of 80–120% and a precision (coefficient of variation, CV) of 20% or less at the LLOQ level and an accuracy of 85–115% and a precision (CV) of 15% or less at other levels. Specificity was confirmed by comparing the chromatograms from the blank murine cochlea extract with those from LLOQ QC samples (Figure 3). In the LLOQ chromatogram, the DEX and IS peaks were observed at approximately 2.8 and 3.0 min, respectively, whereas these peaks were not observed in the blank murine cochlea chromatogram. We verified the linearity of the method over the selected concentration range (1, 10, 50, 100, 250, and

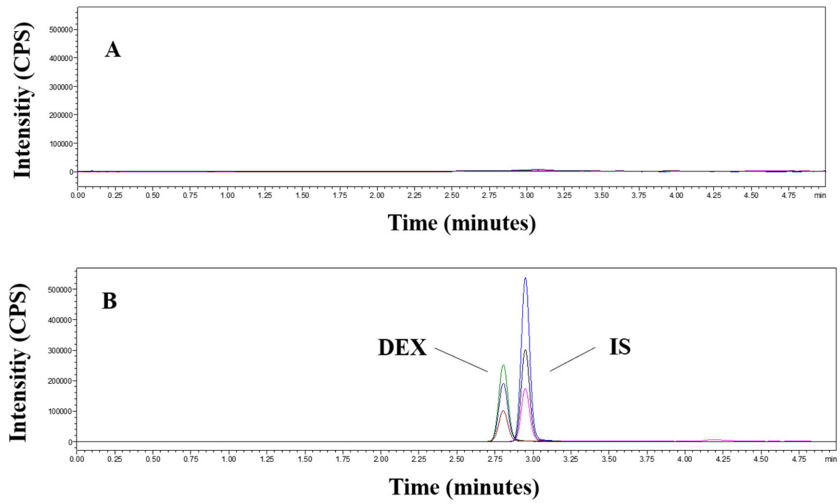

Figure 3. Multiple reaction monitoring chromatograms of blank murine cochlea (A) and murine cochlea including 100 ng/mL of DEX and IS (B). DEX and IS stand for dexamethasone and triamcinolone acetonide, respectively.

Table 2. Back-calculated accuracy (%) and its coefficient of variation (CV, %) of seven dexamethasone (DEX) calibrators (n = 6). SD stands for standard deviation.

Nominal concentration of DEX (ng/mL)	Calculated concentration of DEX (Mean ± SD, ng/mL)	Accuracy (%)	CV (%)
1	1.18 ± 0.10	118.25	8.47
10	11.39 ± 0.27	113.94	2.37
50	44.78 ± 0.50	89.57	1.11
100	100.48 ± 1.64	100.48	1.63
250	255.04 ± 3.58	102.01	1.40
500	497.87 ± 8.80	99.57	1.76

Table 3. Accuracy and precision (coefficient of variation of accuracy) assessed from dexamethasone (DEX) quality control samples. SD stands for standard deviation.

Types	Nominal concentration of DEX (ng/mL)	Calculated concentration of DEX (Mean ± SD, ng/mL)	Accuracy (%)	Precision (%)
Intra-day (n = 6)	1	0.93 ± 0.04	93.34	4.30
	3	2.60 ± 0.15	86.78	5.76
	250	250.61 ± 9.60	100.24	3.83
	500	477.06 ± 6.71	95.41	1.40
Inter-day (three days, n = 6 for each day)	1	0.93 ± 0.05	93.32	5.37
	3	2.61 ± 0.11	87.12	4.21
	250	250.08 ± 4.72	100.03	1.89
	500	472.96 ± 5.27	94.59	1.11

Table 4. Recovery and matrix effect of dexamethasone (DEX) in murine cochlea (n=3). SD and CV stand for standard deviation and coefficient of variation, respectively.

Nominal concentration of DEX (ng/mL)	Recovery (Mean ± SD, %)	Matrix effect	
		Mean ± SD (%)	CV (%)
1	104.52 ± 4.67	91.56 ± 4.69	5.12
3	93.05 ± 2.30	97.43 ± 1.72	1.77
250	98.76 ± 0.70	104.46 ± 1.71	1.64
500	98.17 ± 0.73	97.75 ± 0.13	0.13

500 ng/mL) through six calibration curves to support new DEX formulation studies currently being conducted by our research team. The back-calculation accuracy values of the calibrators were within the range of 89.6% to 118.3% (CV values less than 8.5%), meeting the guideline criteria (Table 2). In addition, the mean R^2 of the six calibration curves was 0.999. Accuracy and precision (the CV of accuracy) across all QC samples were within FDA guidelines. Intra-day accuracy ranged from 86.8% to 100.2%, while inter-day accuracy ranged from 87.1% to 100.0%. Intra-day precision did not exceed 5.8%, and inter-day precision did not exceed 5.4% (n = 6, Table 3). Matrix effect (ME) was assessed by comparing the MMS results at all QC sample concentrations with those of the corresponding standard solutions (n = 6, Table 4), and the average ME was between 91.6% and 104.5% and their CV values were less than 5.1%, meeting the guidelines. Recovery was determined by comparing the SSS results with the average MMS results and showed excellent recovery (93.1% to 104.5%), close to 100% for all QC effects (Table 4). Based on these validation results, the excellent analytical performance of the present method to determine DEX in murine cochlea tissue was confirmed.

Conclusions

A simple and cost-effective method to determine DEX in murine cochlea using solvent extraction and LC-MRM was developed. The use of triamcinolone acetonide as the internal standard improved cost-effectiveness, and the use of 100% acetonitrile as the extraction solvent made the E/P process simpler and more efficient. The method was successfully validated according to FDA guidelines, and various parameters such as specificity, linearity, sensitivity, accuracy, precision, recovery, and matrix effect were evaluated. Therefore, the present method is expected to contribute significantly to the development of novel formulations for IT delivery of DEX to help patients suffering from inner ear disorders such as ISSNHL and Meniere's disease.

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