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Application News

New Derivatization Method for Quantitative Analysis of Δ⁹-THC-COOH in Urine

Mahiro Yoshida[1], Yuki Sakamoto[1], and Maiko Kusano[2]
1 Shimadzu Corporation, 2 Department of Legal Medicine, Showa Medical University School of Medicine

User Benefits

- The risk of limited availability of stable isotope-labeled standards can be avoided by synthesizing internal standards through derivatization.
- The derivatizing agent IPPAH and its corresponding stable isotope IPPAH-d_6 can be synthesized easily and inexpensively from acetone (or acetone-d_6).
- This approach achieves sufficient sensitivity and quantitative accuracy for the determination of Δ⁹-THC-COOH in urine.

■ Introduction

In Japan, the Cannabis Control Act and the Narcotics and Psychotropics Control Act were amended in December 2024. While cannabis was legalized for industrial and medical uses, stricter cannabis regulations have been enacted to prevent harm from its abuse. The primary psychoactive cannabinoid, delta-9-tetrahydrocannabinol (Δ⁹-THC), is extensively metabolized in the body and is excreted mainly in urine and feces as 11-nor-9-carboxy-THC (Δ⁹-THC-COOH) and its glucuronide conjugates. Consequently, Δ⁹-THC-COOH must be analyzed to confirm cannabis use. For LC-MS/MS quantitation, the stable isotope-labeled internal standard Δ⁹-THC-COOH-d_3 is commonly used to correct for matrix effects. However, most suppliers of stable isotope standards are located in the U.S., making these standards difficult to obtain due to import procedures. Furthermore, there may be additional import-related supply risks for stable isotope standards in the future.

This Application News describes a technique for quantitating Δ⁹-THC-COOH using an internal standard that is synthesized easily and inexpensively by derivatization with IPPAH (1-isopropylpiperidine carboxylic acid hydrazide), which characteristically reacts with carboxyl groups, and its corresponding stable isotope IPPAH-d_6.

■ Synthesis of the Derivatizing Agents IPPAH and IPPAH-d_6

Fig. 1 shows the synthesis of the derivatizing agents IPPAH and IPPAH-d_6, and Fig. 2 shows the details of the workflow. Acetone or acetone-d_6 was added to 4-piperidinecarboxylic acid ethyl ester in the presence of a reducing agent. After the reaction, saturated aqueous sodium carbonate solution was added, and the organic phase was separated, vacuum-dried, and reacted with 20 % hydrazine. After solvent extraction and vacuum drying, IPPAH and IPPAH-d_6 were obtained[1]. Thus, the derivatizing agents IPPAH and IPPAH-d_6 can be synthesized from inexpensive starting materials using a simple procedure. IPPAH and IPPAH-d_6 exhibit high reactivity toward carboxyl groups.

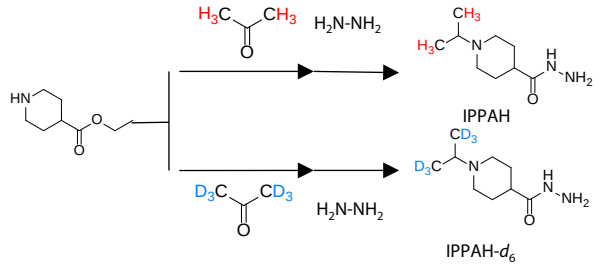

Fig. 1 Reaction Scheme of IPPAH and IPPAH-d_6

4-Piperidine carboxylic acid ethyl ester 1.6 g
100 mL chloroform with 0.5 % acetic acid
5 mL acetone or acetone-d_6
Sodium triacetoxy borohydride 5 g
Stir for 3 days at room temperature
100 mL saturated sodium carbonate
Liquid-liquid extraction
Discard aqueous phase
} x 3
Vacuum concentrate organic phase
20 mL EtOH with 20 % hydrazine
Stir for 20 hours at 70 °C
Vacuum evaporate residual solvent
50 mL chloroform
50 mL saturated sodium carbonate
Liquid-liquid extraction
Discard aqueous phase
} x 3
Vacuum dry organic phase
Synthesis of IPPAH and IPPAH-d_6

Fig. 2 Synthesis Process for IPPAH and IPPAH-d_6

■ Synthesis of the Δ⁹-THC-COOH-IPPAH-d_6 Internal Standard

Fig. 3 shows the workflow for the synthesis of the Δ⁹-THC-COOH-IPPAH-d_6 internal standard, and Fig. 4 shows the reaction scheme. The coupling reagent HSTU (*N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium hexafluorophosphate) and IPPAH-d_6 were added to Δ⁹-THC-COOH and allowed to react at room temperature for 30 min. The reaction mixture was then purified using a MonoSpin C18 spin column (GL Sciences) to obtain Δ⁹-THC-COOH-IPPAH-d_6.

Δ⁹-THC-COOH in MeOH
40 mg/mL IPPAH-d_6
in MeOH:ACN (2:8) solution with 0.5 % pyridine 40 µL
40 µL 100 mM HSTU (coupling reagent) in ACN
React for 30 min at room temperature
400 µL 0.5 % formic acid
Purify with MonoSpin C18 column (GL Sciences)
Condition with 200 µL ACN and 200 µL 0.1 % formic acid
Add the entire reaction solution
Rinse with 200 µL (7:3) A:B solvent solution*1
Elute with 200 µL (5:5) A:B solvent solution
Dry under a nitrogen gas stream
Reconstitute with 200 µL MeOH (1 µg/mL concentration)
Δ⁹-THC-COOH-IPPAH-d_6

*1 Solvent A: 5 mM ammonium formate (aqueous) with 0.1 % formic acid
Solvent B: 5 mM ammonium formate (90 % acetonitrile) with 0.1 % formic acid

Fig. 3 Synthesis Process for Δ⁹-THC-COOH-IPPAH-d_6

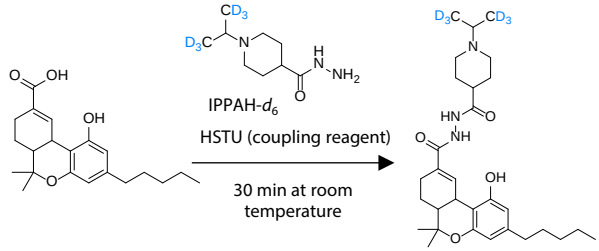

Fig. 4 Reaction Scheme for Δ⁹-THC-COOH-IPPAH-d_6

■ Urine Sample Pretreatment

Fig. 5 shows the workflow for urine sample pretreatment and derivatization, and Fig. 6 shows the reaction scheme for Δ⁹-THC-COOH-IPPAH. Δ⁹-THC-COOH was added to human urine and hydrolyzed with sodium hydroxide. After neutralization with 50 % trifluoroacetic acid, acetonitrile was added for protein precipitation. The resulting supernatant was evaporated to dryness under nitrogen stream and reconstituted in methanol. The prepared Δ⁹-THC-COOH-IPPAH-d_6, the IPPAH derivatizing agent, and the HSTU coupling reagent were then added, and the mixture was allowed to react at room temperature for 30 min. After acidification, the reaction mixture was purified using a MonoSpin C18 spin column and reconstituted in 50 % methanol.

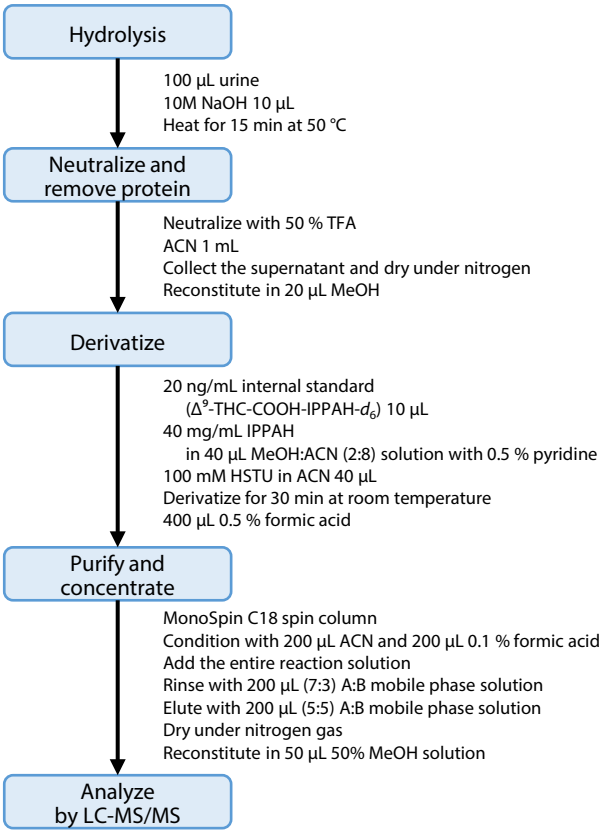

Fig. 5 Urine Sample Pretreatment Flowchart

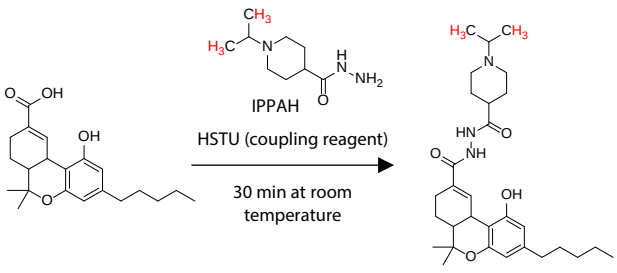

Fig. 6 Reaction Scheme for Δ⁹-THC-COOH-IPPAH

■ Analysis Conditions

LC-MS/MS analysis conditions are shown in Table 1. The Nexera™ XR and LCMS-8050 systems were used.

Table 1 Analysis Conditions

LC System:	Nexera XR
Column:	Raptor ARC-18 (100 mm × 3.0 mm I.D., 1.8 µm P/N: 931421E, Restek)
Column Temp.:	40 °C
Injection Volume:	2 µL
Mobile Phase A:	10 mM ammonium formate + 0.1 % formic acid in water
Mobile Phase B:	10 mM ammonium formate + 0.1 % formic acid in MeOH
Flowrate:	0.4 mL/min
Time Program (%B):	75 % (0 to 9 min) → 85 % (12 to 18 min) → 30 % (18-23 min)
MS System:	LCMS-8050 (positive ESI)
Nebulizer Gas:	3 L/min
Drying Gas:	5 L/min
Heating Gas:	15 L/min
DL Temp.:	150 °C
Heat Block Temp:	300 °C
Interface Temp.:	400 °C

Compound	Quantitation Ion	Reference Ion
Δ⁹-THC-COOH-IPPAH	512.15 > 154.15 (CE -31 V)	512.15 > 193.10 (CE -31 V) 512.15 > 299.20 (CE -45 V)
Δ⁹-THC-COOH-IPPAH-d_6	518.00 > 160.10 (CE -32 V)	518.00 > 193.20 (CE -47 V) 518.00 > 299.20 (CE -33 V)

■ Analytical Results

The calibration curve for Δ⁹-THC-COOH-IPPAH was prepared using urine samples containing Δ⁹-THC-COOH at concentrations of 1, 5, 10, 50, 100, and 500 ng/mL. The calibration curve and MRM chromatograms obtained at 5 ng/mL are shown in Fig. 7. Linearity was demonstrated with a correlation coefficient (R) of 0.998 or higher, and clear detection was achieved even at the low concentration of 5 ng/mL.

Table 2 shows the intra-day precision results (n = 5) at 15 ng/mL (low concentration; cut-off value) and 200 ng/mL (high concentration) in urine. Accuracy was 87.7 % at the low concentration and 100 % at the high concentration, with repeatability (%RSD) of 5.7 % and 6.4 %, respectively.

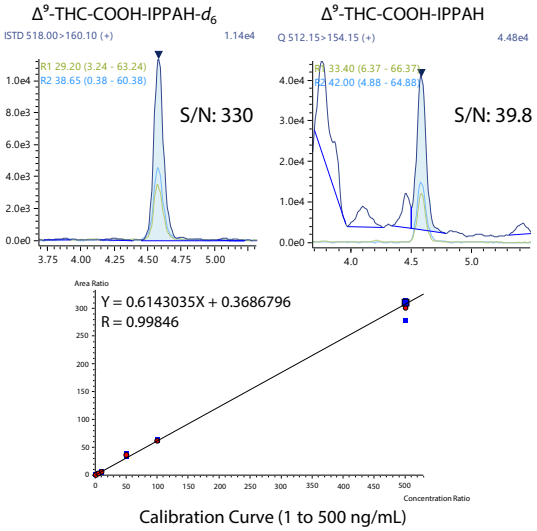

Fig. 7 MRM Chromatograms at 5 ng/mL and Calibration Curve

Table 2 Intra-Day Repeatability (n = 5)

15 ng/mL (Low)	Data 1	Data 2	Data 3	Data 4	Data 5	Avg (ng/mL)	Accuracy (%)	SD	%RSD
Δ⁹-THC-COOH-IPPAH	12.1	13.6	14.0	13.0	13.1	13.2	87.7	0.75	5.7

200 ng/mL (High)	Data 1	Data 2	Data 3	Data 4	Data 5	Avg (ng/mL)	Accuracy (%)	SD	%RSD
Δ⁹-THC-COOH-IPPAH	206.5	192.5	181.1	207.4	212.5	200	100	12.9	6.4

■ Derivatization Reaction Efficiency

Fig. 8 shows the MRM chromatograms of Δ^9-THC-COOH-IPPAH and unreacted Δ^9-THC-COOH obtained from the highest calibration level (500 ng/mL). The concentration of unreacted Δ^9-THC-COOH was below the lower limit of quantitation (1 ng/mL), indicating a derivatization efficiency of at least 99 %.

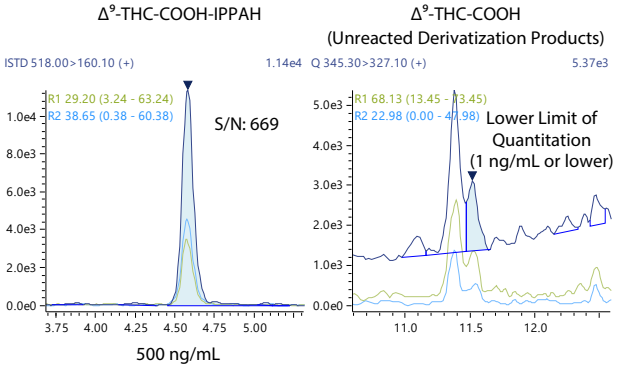

Fig. 8 MRM Chromatograms of Δ^9-THC-COOH-IPPAH and Unreacted Δ^9-THC-COOH at 500 ng/mL

■ Conclusion

Derivatization with IPPAH and its stable isotope analog, IPPAH-d_6, enabled the simple and inexpensive preparation of an internal standard. This approach provides sufficient sensitivity and accuracy for the determination of Δ^9-THC-COOH in urine.

Most stable isotope-labeled standards are supplied by foreign manufacturers, creating a risk of delays or difficulties in procurement due to import/export regulations. By synthesizing the internal standard through derivatization, this risk can be minimized.

Acknowledgments

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References

1) Kawasue S., Kuniyoshi K., et al, (2026) Novel isotope-coded derivatization of carboxylic acids provides accurate quantification of Azaspiracids in shellfish by liquid chromatography–tandem mass spectrometry, Journal of Chromatography B, 1267, 124826.

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