TWO-DIMENSIONAL PURIFICATION OF 7-HYDROXYMITRAGYNINE USING CPC AND HPLC

APPLICATION NOTE AN1047

BENEFITS

- 2D purification by CPC and Prep HPLC column with one PLC system
- Increase the purity of your target compound from ~40% to 98% after two steps
- Achieve higher throughput in processing crude samples than conventional HPLC methods

ADDRESSED ISSUES

- Separation of peaks that co-elute under traditional HPLC and CPC methods
- Increasing global usage of kratom extracts demands more extensive pharmacological studies of mitragynine to better understand mitragynine's analgesic properties
- Allows for the recovery of high purity mitragynine that can be used for future medical studies

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INTRODUCTION

7-hydroxymitragynine (Figure 1) is an alkaloid compound found in kratom, an evergreen tree common to Southeast Asia and a relative to the coffee plant. Kratom has been traditionally used as a herbal medicine to treat a variety of ailments from general pain to bacterial infections. While kratom mostly contains the dehydroxylated mitragynine, it has been shown to be metabolized into the more pharmacologically active 7-hydroxymitragynine after ingestion.[1] In recent years, kratom has become an increasingly popular recreational drug around the world; consequently, this increase in popularity is matched by increasing concern over its potential to worsen the current opioid epidemic.[2] As a result, further studies of kratom and its constituent compounds are necessary to better understand the mechanisms of action of these compounds and to evaluate possible therapeutic benefits of mitragynine or 7-hydroxymitragynine, present in kratom. To this end, it is of vital importance that these studies be conducted with highly pure compounds for accurate conclusions.

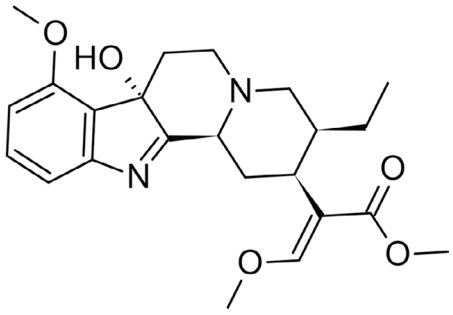

Figure 1

Chemical structure of 7-hydroxymitragynine

In this application note, a two-dimensional separation is conducted on crude extract containing ~40% 7-hydroxymitragynine. Performing a first pass purification using centrifugal partition chromatography (CPC) can increase purity of the 7-hydroxymitragynine sample to over 85%. These fractions, however still contain a few impurities, but these final impurities can be separated out using

high performance liquid chromatography (HPLC) without optimization to yield 98% 7-hydroxymitragynine. This proof-of-concept demonstrates how CPC is a technique that is able to supplement HPLC as an initial first step in large-scale production.[3]

It also shows the capabilities/flexibility of the Gilson PLC Purification System to work with both prep HPLC and CPC columns.

MATERIALS AND METHODS

Crude 7-hydroxymitragynine purification was performed on a CPC 250 Classic (Figure 2). It is comprised of a Gilson CPC 250 column connected to a PLC 2250 Purification System (Compact LC system) which is configured with a 250 mL/min quaternary gradient pump, automatic injection valve, backflush valve, UV/VIS diode array detector, fraction collector, and GLIDER control software. Subsequent HPLC purification was also performed on a Gilson PLC 2250 Purification System attached to a Phenomenex Luna 50 x 21.2, C18, 5 µm column. Analytical analysis was performed on a Shimadzu LC2030C 3D Plus HPLC configured with a Restek Raptor 150 x 4.6, ARC-18, 2.7 µm column.

CPC/HPLC solvent systems:

A two-dimensional separation consisting of CPC separation as the first dimension followed by an HPLC separation using buffered acetonitrile/water solution as the second dimension was applied. CPC runs were conducted in the ascending mode with both the lower and upper phases automatically generated using GLIDER software and the PLC 2250 low pressure gradient valves. HPLC runs were conducted using a simple gradient of acetonitrile buffered with 0.1% formic acid in a 5 mM aqueous ammonium formate solution.

Sample preparation:

500 milligrams of crude 7-hydroxymitragynine, provided by Peli Labs, was dissolved in 10 mL of lower phase and drawn up into a disposable syringe affixed with a 0.45-micron syringe filter. 9 mL were loaded into a 10 mL injection loop on the PLC 2250 and automatically injected onto the CPC 250 using GLIDER software.

The second dimension of purification via HPLC involved concentrating the CPC fractions containing 7-hydroxymitragynine, redissolving in buffered acetonitrile, and then manually injecting onto a Phenomenex Luna C18(2) 100Å column using GLIDER software.

CPC/HPLC separation method:

All methods were programmed using the hardware's respective provided software. The column was first loaded in ascending mode with the aqueous mobile phase solvent system at 50 mL/min and 500 rpm. The organic mobile phase of the solvent system was pumped at 12 mL/min and 2000 rpm.

After injection, elution with the mobile phase was performed for the duration of 25 minutes, then an extrusion step was applied to recover any remaining compounds from the sample in the stationary phase. During this last step, the fresh aqueous phase was pumped at 50 mL/min and 500 rpm.

The second HPLC step was conducted using a linear gradient of 0.1% formate buffered acetonitrile in aqueous 5 mM ammonium formate over the course of 13 min at 12 mL/min, and then acetonitrile was ramped up to 70% for another 3 minutes.

Both the CPC and HPLC effluent were monitored by UV detection at 254 nm and 280 nm, and in scan mode from 200 to 600 nm. Variable fraction volumes, determined by peak shape, were collected for both CPC and HPLC purification steps. Analytical HPLC analysis was used to determine the recovery and purity of the obtained solutes after each purification step.

Figure 2

VERITY® PLC/CPC/HPLC setup

RESULTS AND DISCUSSION

CPC purification of the 7-hydroxymitragynine crude initially showed great resolution with one main peak during the elution phase followed by a collection of smaller impurity peaks during the extrusion phase about 15 minutes later, as shown in Figure 3. The main peak containing the 7-hydroxymitragynine was then concentrated down and redissolved in acetonitrile and reinjected onto the PLC 2250, this time with a reverse phase HPLC column in the flow path instead of the CPC 250. HPLC as a secondary step was able to further remove minor impurities as shown in Figure 4. HPLC analysis of the CPC fractions however showed that while the purity of 7-hydroxymitragynine had been increased significantly, there remained some minor impurities still coeluting with the target compound, as shown in Figure 5. Interestingly, further analysis of the HPLC chromatograms shows that while there were still some impurities in with the 7-hydroxymitragynine, CPC purification as a first pass was able to separate compounds that would have normally co-eluted under HPLC conditions with superb resolution. This orthogonality in separation techniques may become extremely useful in the future in making complex HPLC methods using multiple columns and steps more efficient by providing an avenue to easily separate compounds that normally coelute using HPLC.

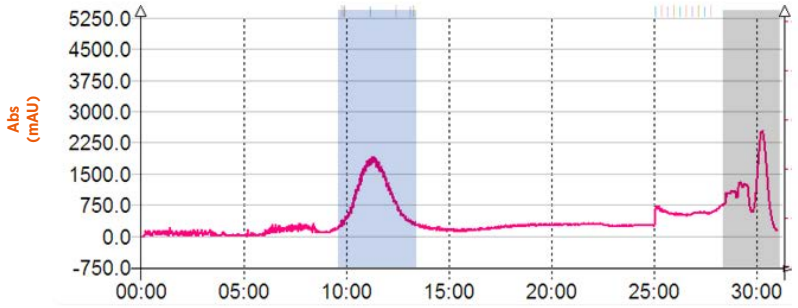

Figure 3

CPC chromatogram of the Arizona L ascending separation applied to a ~40% purity sample of 7-hydroxymitragynine using Gilson GLIDER software. 7-hydroxymitragynine containing peak (blue) was collected along with other impurities.

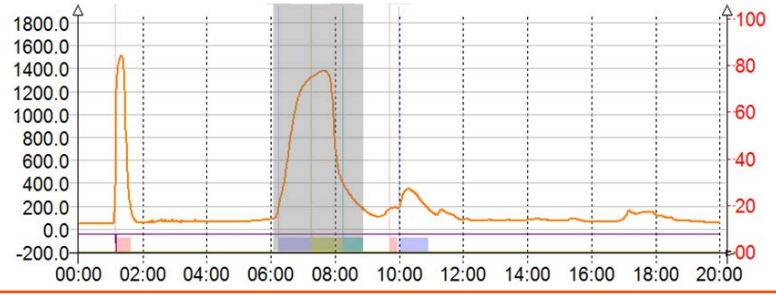

Figure 4

Preparative HPLC chromatogram of 7-hydroxymitragynine containing fraction from CPC purification using Gilson GLIDER software. 7-hydroxymitragynine peak highlighted. (UV detection at 254/280 nm).

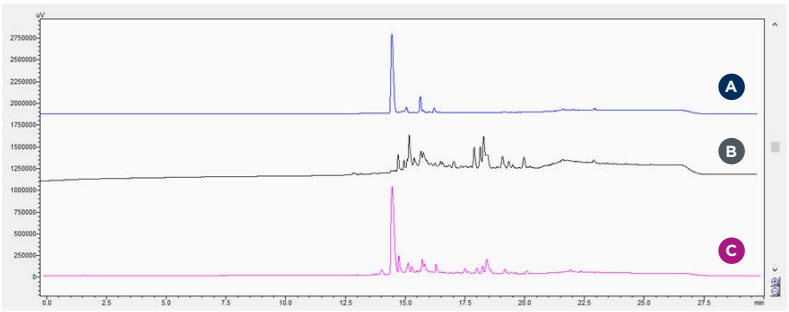

Figure 5

Analytical HPLC chromatograms of recovered (a) 7-hydroxymitragynine after CPC-only purification and (b) main impurity CPC fractions plotted in a stack alongside (c) starting crude mixture.

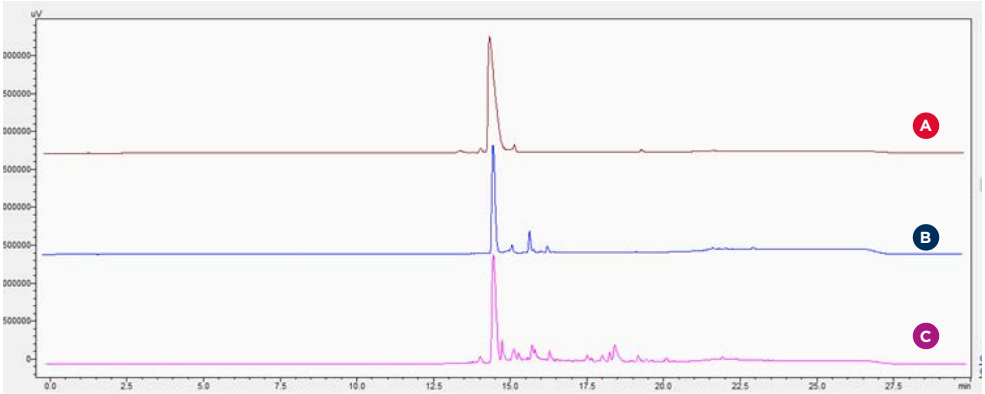

Figure 6
Analytical HPLC chromatograms of recovered 7-hydroxymitragynine fractions after (a) CPC/HPLC purification and (b) CPC-only purification plotted in a stack alongside (c) starting crude mixture.

The collected fractions from the CPC purification containing 7-hydroxymitragynine were then pooled together, concentrated down, and then further purified using HPLC. This second step of HPLC was able to easily remove the remaining impurities and increase the purity of 7-hydroxymitragynine to a final purity of 98% (Figure 6).

CONCLUSIONS AND BENEFITS

The Gilson PLC Purification Systems can be equipped with both CPC and HPLC capabilities, allowing the system to perform a two-dimensional CPC/HPLC purification. In this study, using the two-step procedure achieved three goals:

- The extraction of 7-hydroxymitragynine in high purity. This two-step procedure was able to yield 7-hydroxymitragynine of 98% purity from crude extract.
- By using CPC, a silica-free liquid-liquid chromatography technology, as a first step before HPLC, throughput can be increased significantly as CPC is able to handle larger, dirtier mixtures than HPLC.
- Final purification using traditional HPLC can further increase 7-hydroxymitragynine purity. The semi-purified 7-hydroxymitragynine after CPC is now in a state that can be further "refined" much more efficiently by HPLC with less chance for mechanical malfunctions due to particulates/ impurities.

The use of CPC before HPLC in this purification of 7-hydroxymitragynine not only demonstrates the purification of our target molecule, but also showcases the synergy between both techniques when used in tandem. CPC can handle larger injections with lower resolution with no sample loss due to its lack of a solid phase. On the other hand, HPLC is capable of high resolution at the cost of robustness and efficiency. Because CPC and HPLC separation work differently on a molecular level, separating peaks that coelute with one technique may be more easily separated with the other. The use of both CPC and HPLC when developing purification protocols allows for both high resolution and throughput, as CPC shows to be capable of addressing the limitations of HPLC and vice-versa. As the Gilson PLC Purification system can run both methods, this eliminates the need for additional equipment to perform these 2-D purifications, allowing for improved laboratory efficiency.

With the global rise in kratom use and its reported opioid-like properties, research into its mechanism of action will no doubt also see a rise in the near future to better understand kratom and guide potential future regulation regarding its use by the masses. These studies will require mitragynine, and its active metabolite 7-hydroxymitragynine, in high purity for accurate conclusions. This two-step protocol can not only provide the purity, but also at a higher throughput and efficiency than could be achieved through HPLC alone.

REFERENCES

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Acknowledgments

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