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Analysis of goldenseal, *Hydrastis canadensis* L., and related alkaloids in urine using HPLC with UV detection

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ABSTRACT

A screening method was developed to extract and detect berberine and hydrastine alkaloids from goldenseal root powder and urine samples using HPLC with UV detection. The isocratic method was developed to detect alkaloids in 5 mL of urine prior to drug screening. Urine samples were spiked with the alkaloids at varying concentrations and extracted twice with 3:1 chloroform:2-propanol ($CHCl_3$:2-propanol). The extracts were combined, concentrated using nitrogen gas and the residue was then reconstituted with a mobile phase of acetonitrile:buffer (32:68). A 17 min isocratic run time was performed with a flow rate of 2.0 mL/min, and UV detection at 230 nm using a C_{18} (250 mm × 4.6 mm) column at room temperature. The method showed good linearity for berberine ($r^2 = 0.9990$) and hydrastine ($r^2 = 0.9983$) over a range of 11.80 ng/mL to 17.64 μg/mL. The LOD for berberine in urine was 12.74 ng/mL and the LOD for hydrastine in urine was 54.48 ng/mL. Urine samples were spiked with goldenseal root powder and liquid extract as part of a blinded study to determine whether berberine and hydrastine alkaloids could also be extracted in vitro from goldenseal and show a presence in urine samples. Out of the 37 blinded urine samples extracted the two spiked samples were correctly identified based on the presence or absence of berberine and hydrastine. The results demonstrated that this method will enable laboratories to test for the herbal supplement in submitted urine samples prior to drug testing, avoiding false negative results.

1. Introduction

With the widespread use of drug testing, there have been many controlled substance users and abusers who are willing to try masking agents or herbal supplements to pass a routine urine drug screen test. Many drug users try to subvert the drug testing process by in vivo adulteration by using herbal products for "flushing" as a means to pass the testing. Today, as in the past, people are under the assumption that goldenseal is one such remedy and can be used as a detoxifying agent to cleanse the body of certain illegal substances. As a result, goldenseal is taken orally with the idea that a person using the herbal supplement can pass a routine urine drug test. There has been conflicting research published stating if ingested goldenseal or other detoxifying agents on the market, actually do mask the illegal drugs that should appear in a routine urine drug test [1,2]. Dietary supplements are also found to contain goldenseal, which may stand alone or be paired with *Echinacea* [3]. The Dietary Supplement Heath and Education Act (DSHEA) does not require the contents of goldenseal alkaloids in dietary supplements to be placed on the label [3]. Due to the use of goldenseal and these other detoxifying agents; toxicology laboratories may see false negative urine samples in routine drug testing methods that are currently employed. The purpose of this project is to create a screening method for toxicology laboratories to utilize in the detection of the major alkaloids, berberine and hydrastine, from goldenseal, *Hydrastis canadensis* L., in urine samples using high performance liquid chromatography (HPLC) with ultra-violet (UV) detection.

Goldenseal, *Hydrastis canadensis* L., is a slow growing perennial herbaceous plant from the buttercup family located in North America, particularly in the areas of southeastern Canada and northeastern United States [4]. It is believed to be an herbal remedy that was traditionally used by Native American Indians. Traditionally, Native Americans used the root of the goldenseal plant as a dye for clothing and then later extended the uses to treat skin disorders, digestive problems, liver conditions, diarrhea, and eye irritations. Today, goldenseal is one of the top selling herbal remedies on the market [4]. People may take the herb to help treat wound healing, bladder infections, fungal infections, common colds and flu's, and sinus and chest congestion [4].

There are three major components in the composition of goldenseal that are assumed to cause bioactivity: berberine, hydrastine, and canadine. The two main components, which were the focus of this study, are isoquinoline alkaloids. These isoquinoline alkaloids are berberine (Fig. 1) and hydrastine (Fig. 2), which both are

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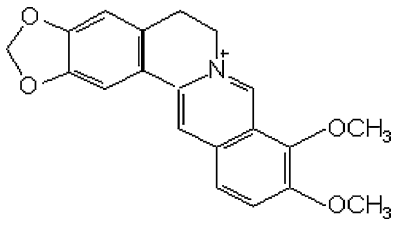

Fig. 1. Structure of berberine.

plant alkaloids. *Hydrastis canadensis* L. has been reported to contain several isoquinoline alkaloids, including 2–4% hydrastine [5] and 2–3% berberine by weight [6]. Canadine was not evaluated in this study due to the low weight percent in goldenseal samples. Canadine is also difficult to obtain in the United States of America. There are also three other minor components of goldenseal that are not widely studied which are berberastine, hydrastinine tetrahydroberberastine, and canalidine [5]. A number of alkaloid containing plants have been substituted for goldenseal, including Chinese goldenthread (*Coptis chinensis*), yellow root (*Xanthorhiza simplicissima*), and Oregon grape (*Mahonia aquifolium*).

The main objective of the project was to create a screening method for toxicology laboratories to detect goldenseal, and related alkaloids, in urine samples using HPLC. In this article, we describe an isocratic HPLC method with UV detection adapted from Weber et al. [7] and Chen and Chang [8] to determine the weight percent of the two major isoquinoline alkaloids found in goldenseal, berberine and hydrastine, and to identify and quantify these alkaloids in human urine samples.

2. Experimental

2.1. Chemicals and reagents

Berberine chloride, (1R,9S)-(−)-β-Hydrastine, and phenolphthalein were obtained from Sigma–Aldrich (St. Louis, MO, USA). Goldenseal root powder was purchased from GNC under the brand name Nature's Fingerprint (Cambridge, MA, USA). Goldenseal root liquid extract was purchased from GNC under the brand name General Nutrition Corporation (Pittsburgh, PA, USA). Ammonium acetate, phosphoric acid (ACS reagent), isopropyl alcohol (ACS reagent), and chloroform (ACS reagent) were purchased from Sigma (St. Louis, MO, USA). Triethylamine (TEA) was purchased from Aldrich (Milwaukee, WI, USA). Glacial acetic acid is a Baker Analyzed Reagent from J.T. Baker Chemicals (Phillipsburg, NJ, USA). HPLC and ACS reagent grade acetonitrile and water were purchased from Burdick and Jackson (Morristown, NJ, USA). Nitrogen gas and helium gas were purchased from Air Products (Allentown, PA, USA).

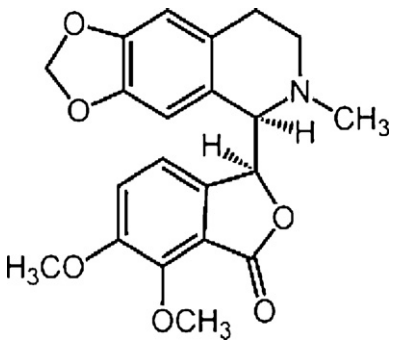

Fig. 2. Structure of hydrastine.

2.2. Instrumentation and chromatography

All analysis was performed using a HPLC Lab Alliance Series II system (State College, PA, USA) paired with a Millipore Waters Lambda Max Model 480 LC Spectrophotometer (Waters Corp., Milford, MA, USA), which was controlled by Peak Simple Chromatography Data System software (version 2.83) from Buck Scientific (East Norwalk, CT, USA). Separation was achieved on an Apollo C_{18}, 5 μm particle size, 250 mm × 4.6 mm HPLC column (All-Tech, Deerfield, IL, USA) operated with 100% mobile phase.

The LC operating conditions were an isocratic flow rate of 2.0 mL/min over a 17 min run time with a 10 μL injection volume. The UV detection was performed at 230 nm. The UV wavelength of 230 nm was chosen based on work that was performed by Li and Fitzloff [9] that determined both hydrastine and berberine absorb strongly between 220 nm and 240 nm. LC mobile phase buffer consisted of 2.3 g ammonium acetate and 2 mL triethylamine (TEA) per 1 L of HPLC grade water. The pH of this solution was adjusted to 4.85 using glacial acetic acid. The LC mobile phase consisted of buffer:acetonitrile (68:32, v/v) degassed using helium gas.

True™ syringe filters 0.2 μm were purchased from AllTech (Deerfield, IL, USA). The sonicator was an Ultrasonic Device from Heat Systems Ultrasonics, Inc. (Plainview, NY, USA) and the mini vortex and hotplate:stirrer were purchased from VWR (West Chester, PA, USA). The incubator shaker was purchased from New Brunswick Scientific (Edison, NJ, USA), model 12400, and was operated at 30 °C and 17 rotations per minute (RPM's).

The mixed standards, standard stock, and goldenseal extracts were all run under the HPLC protocol.

2.3. Analytical procedure

2.3.1. Preparation of stock and working standard solutions

Approximately 0.0500 g, weighed on an electronic analytical balance, of phenolphthalein was added to 10 mL of HPLC grade methanol. The solution was agitated until phenolphthalein, the internal standard, was completely dissolved. 100 μL of the phenolphthalein and methanol solution, the internal standard mixture, was placed in 5 mL of the prepared berberine and hydrastine standard stock solutions respectively. Phenolphthalein was selected as the internal standard because it is not expected to be in human urine and is well retained from berberine and hydrastine. Stock solutions were prepared for each of the alkaloids in 30:70 acetonitrile:HPLC grade water with concentrations of 1.718 mM for berberine and 1.5098 mM for hydrastine based on the methods described by Weber et al. [7]. A serial dilution of each of the alkaloids was performed to prepare working standards until the LOD for each alkaloid was determined.

Each standard solution was filtered as a precautionary step to ensure that for all samples tested, the solution was homogenous and did not contain particulates. Each sample was then injected in triplicate. This was the standard protocol for each sample injection. By filtering the samples this allowed for any residue powder that was not dissolved and remained from the berberine, hydrastine, or goldenseal root samples to be removed before injecting onto the column and possibly blocking or building up on the column or causing carryover between sample injections.

2.3.2. Samples preparation

All samples were diluted with acetonitrile:HPLC grade water (30:70, v:v).

Goldenseal root powder was weighed to 0.3022 g and 100 mL of extraction solvent or 100 mL of HPLC grade boiling water was added. The sample was sonicated for 5 min and placed on the incubator shaker for 10 min at 30 °C and 17 RPM. The sample was diluted with diluent in three separate sample vials with concentrations of

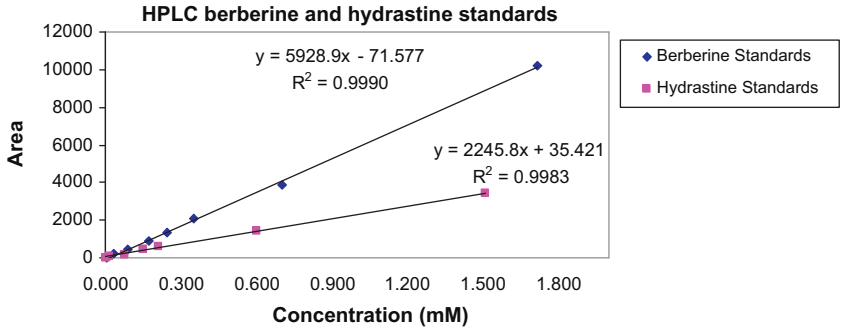

Fig. 3. Calibration curves for berberine and hydrastine working standards.

75%, 50%, and 25% of the original concentration. The samples were diluted to monitor the response of berberine and hydrastine in less concentrated samples.

To test for the presence of goldenseal alkaloids in a liquid solution, goldenseal root liquid was extracted using 50 mL of extraction solvent or 50 mL of HPLC grade boiling water, 1 mL of goldenseal root liquid was added, and stirred continuously for 15 min. The sample was diluted with diluent in three separate sample vials with concentrations of 75%, 50%, and 25% of the original concentration to monitor the alkaloid response in less concentrated samples.

2.3.3. Spiked urine sample preparation

The urine extraction procedure was modified from Chen and Chang [8]. Urine samples were spiked with 100 μL of each standard at different concentrations. The modified method used 5 mL of a 3:1 chloroform:2-propanol ($CHCl_3$:2-propanol) extraction solvent agitated with 5 mL of urine and the organic layer was removed. This process was repeated a second time with 5 mL of urine. The 3:1 $CHCl_3$:2-propanol solutions were then combined and concentrated using a stream of nitrogen gas. The dried extract was then reconstituted with 100 μL of mobile phase and 10 μL of the reconstituted sample was injected onto the HPLC column. The procedure was performed for berberine, hydrastine, and 100% goldenseal root powder and liquid extract.

2.3.4. Blind study urine samples preparation

A blind study was conducted to test human urine samples donated from a private toxicology drug testing laboratory. Marijuana is one of the most commonly detected drugs of interest at the donating laboratory and is one of the drugs that goldenseal is believed to mask in a routine urine drug screen. Tetrahydrocannabinol (THC) is the main metabolite of marijuana and is screened for in the donating laboratory as a screen for the presence of marijuana. Using the method developed for spiked urine samples, four different categories of human urines were analyzed by HPLC–UV. Samples containing low creatinine were targeted for this study as goldenseal is a diuretic and if consumed may cause the creatinine levels in urine of a subject to be much lower than expected in a normal healthy individual.

The samples were further separated into four distinct categories by the donating laboratory following gas chromatography–mass spectrometry (GC–MS) analysis prior to the samples arriving onsite for goldenseal screening. The four different sample types were separated based on their GC–MS results for the presence/absence of THC and creatinine levels. The first set of samples that were analyzed by the donating laboratory screened positive for THC and showed interference by GC–MS, the preferred screening method at the donating laboratory. The interference observed at the donating laboratory was caused by a loss of internal standard, co-eluting peaks, or the detection of an oxidizing agent. The other three categories were samples that screened positive for THC with dilute creatinine levels, screened negative for THC (however, THC was detected in low quantities, and the samples had dilute creatinine levels), and samples that did not have THC detected during the screening and had dilute creatinine levels. The samples were analyzed using the method described for urine samples to determine if the goldenseal alkaloids, berberine and hydrastine, could be detected in any of the spiked samples that may have contributed to the results initially observed by the donating laboratory.

2.4. Method validation

The evaluation of the method included the determination of specificity, selectivity, linearity, lowest limit of detection, precision, accuracy, extraction recovery, stability, and system suitability testing.

The specificity and selectivity of the method were evaluated by comparing chromatograms from sources of clean blank urine collected from individuals that did not ingest goldenseal, blank urine samples spiked with berberine and hydrastine standards, mobile phase, buffer, and extraction solvents.

To evaluate the linearity, urine calibration curves were prepared and determined in triplicate on three separate days. Calibration curves were calculated by the peak area ratio of the analyte over the internal standard versus analyte concentration. The lower limit of quantification (LLOQ) was defined as the lowest quantifiable alkaloid concentration on the calibration curve that had a signal-to-noise greater than at least five times the baseline noise.

To evaluate the precision and accuracy, urine samples were analyzed in triplicate and the standard deviation and percent coefficient of variance were determined at a concentration range of 12.7 ng/mL to 1.78 μg/mL for berberine and 54.5 ng/mL to 17.6 μg/mL for hydrastine. Inter day retention time imprecision was determined for both alkaloids.

The stability of the alkaloids was assessed by monitoring the relative retention time of berberine and hydrastine across intra and inter day analysis. The standard deviation was calculated for the reproducibility of the alkaloids.

To evaluate system suitability, a blind study was conducted to test human urine samples donated from a private toxicology drug testing laboratory. Thirty seven urine samples were tested using the developed method.

The extraction recovery of berberine and hydrastine was determined by calculating the weight percent of each alkaloid in goldenseal root powder and liquid extract.

3. Results and discussion

3.1. HPLC method development

The main goal was to develop a HPLC method to detect goldenseal isoquinoline alkaloids, berberine and hydrastine, in human

Table 1
Goldenseal root alkaloid weight percent content determined with the extraction solvent.

Goldenseal root	Berberine	Hydrastine
Wt. % powder	2.44% (w/w)	1.78% (w/w)
Wt. % liquid extract	7.86% (w/v)	3.64% (w/v)

urine samples. During the development of the HPLC analysis method, a series of standard concentrations were tested to determine if berberine and hydrastine could be detected before spiking urine samples. Using a HPLC method adapted from Weber et al. [7], it was possible to differentiate the two alkaloids from one another and to also differentiate the internal standard, phenolphthalein, added to the samples. The method showed good linearity for berberine and hydrastine working standards. The calibration curve for berberine was linear over the range of 3.50 μM to 1.72 mM, using a 10 μL sample injection, and could be expressed by the equation $y = 5928.9x - 71.577$ ($r^2 = 0.9990$) (Fig. 3). The calibration curve for hydrastine was linear over the range of 1.51 μM to 1.51 mM, using a 10 μL sample, and could be expressed by the equation $y = 2245.8x + 35.421$ ($r^2 = 0.9983$) (Fig. 3).

3.2. Extraction method for goldenseal root

The extraction of goldenseal was tested in several different solutions, which included the extraction solvent (acetonitrile:HPLC grade water:phosphoric acid (70:30:0.1, v:v:v)), HPLC grade water, HPLC grade boiling water, methanol, chloroform, and 2-propanol, to determine the best way to extract the alkaloids from goldenseal root powder and liquid extract. The extraction of berberine and hydrastine from goldenseal was first created using a modified version of the established method by Weber et al. [7] and the listed extraction solvents. To determine the best method to extract the alkaloids from goldenseal root powder, approximately 0.30 g aliquots of goldenseal root powder were combined with 100 mL of extraction solvent (acetonitrile:HPLC grade water:phosphoric acid (70:30:0.1, v:v:v)) [7] or HPLC grade boiling water. The extraction methods were analyzed and the only method that allowed both alkaloids to be recovered was the acetonitrile:HPLC grade water:phosphoric acid (70:30:0.1, v:v:v).

Results indicate that the best method of extracting the alkaloids from goldenseal root powder is with the Weber et al. [7] extraction solvent. Boiling water extractions revealed peaks for berberine in goldenseal root powder; however, the method did not show any recovery for hydrastine. This was to be expected, because hydrastine does not readily dissolve in water in a powder form. The goldenseal root liquid had results for both alkaloids with the extraction solvent and boiling water. The other extraction solvents attempted did not dissolve the goldenseal root powder or liquid extract; therefore, these methods were abandoned and no further analysis was performed with these solvents. The weight percent content for the goldenseal alkaloids, berberine and hydrastine, in the extraction solvent are located in Table 1. The weight percent results for goldenseal root powder are within the expected range of berberine and hydrastine alkaloids based on previous research; however, the goldenseal root liquid extract results are much higher than expected [3].

The weight percent of berberine and hydrastine in goldenseal root powder and liquid extract was calculated in a three step method. Step one was a calculation of the sum of each calibration standard. The calculation determines the average area count response at each concentration. Seven calibration standards for berberine and hydrastine were averaged and this was equivalent to the average area count divided by the concentration. The average area count for the seven berberine standards was 5661.25 AC/mM and for hydrastine 2354.94 AC/mM.

Step two was a calculation of the concentration of berberine and hydrastine in the goldenseal root powder and goldenseal liquid extract samples. The average area response of berberine and hydrastine in goldenseal root powder and liquid extract was calculated and divided by the results of Step one. The berberine concentration response was 0.1964 mM and the concentration response of hydrastine was 0.1395 mM in 100% goldenseal root powder extraction.

Step three was the actual weight percent calculation for berberine and hydrastine in the goldenseal samples. Berberine weight percent was calculated as follows:

$$\left[\frac{(\text{concentration of berberine (Step two)} \times \text{molecular weight of berberine (371.8022 g/mol))}}{(\text{concentration of goldenseal root powder (g/L)} \times 1000 \text{ mg/g})}\right] \times 100$$

The 100% goldenseal root powder berberine weight percent was equal to 2.44%.

Hydrastine weight percent was calculated as follows:

$$\left[\frac{(\text{concentration of hydrastine (Step two)} \times \text{molecular weight of hydrastine (397.3959 g/mol))}}{(\text{concentration of goldenseal root powder (g/L)} \times 1000 \text{ mg/g})}\right] \times 100.$$

The 100% goldenseal root powder hydrastine weight percent was equal to 1.78%.

The calculations were repeated for all of the dilutions of goldenseal root powder and liquid extract.

Berberine and hydrastine were detected in goldenseal root powder and liquid extract in serial dilutions of the original concentration of goldenseal in each extraction. The weight percent of berberine and hydrastine in 100% goldenseal root powder and 100% goldenseal liquid extract extracted in Weber's [5] extraction solvent is located in Table 1. The weight percent of berberine in 100% goldenseal root liquid extracted in HPLC grade boiling water was 7.10% weight/volume and hydrastine was 3.57% weight/volume. Hydrastine was not extracted from goldenseal root powder using the HPLC grade boiling water; therefore, the weight percent of berberine and hydrastine was not calculated for the goldenseal root powder in HPLC grade boiling water.

3.3. Extraction method for urine

Berberine and hydrastine were extracted from urine using a developed method adapted from Chen and Chang [8]. The developed method showed good linearity for berberine and hydrastine over a range of 12.74 ng/mL to 12.52 μg/mL. The sensitivity of this presented method allowed for berberine concentrations of at least 12.7 ng/mL to be detected in 5 mL of urine. The calibration curve for berberine is linear over the range of 12.74 ng/mL to 1.784 μg/mL, using a 10 μL sample injection, and could be expressed by the equation $y = 167738x + 33.275$ ($r^2 = 0.9969$) (Fig. 4). The sensitivity of the method presented allowed for hydrastine concentrations of at least 54.5 ng/mL to be detected in 5 mL of urine. The calibration curve for hydrastine was linear over the range of 54.5 ng/mL to 17.64 μg/mL, using 10 μL sample injection, and could be expressed by the equation $y = 54980x + 63.977$ ($r^2 = 0.9953$) (Fig. 5).

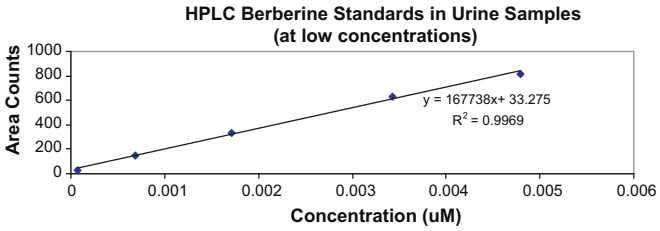

Fig. 4. Calibration curve for berberine standards in spiked urine samples at the lower concentration values.

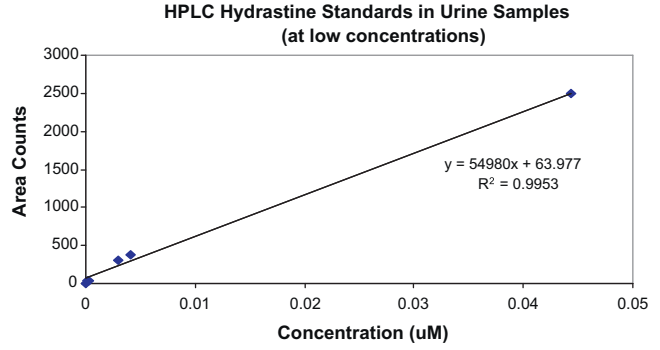

Fig. 5. Calibration curve for hydrastine standards in spiked urine samples at the lower concentration values.

Table 2
Figures of merit for berberine and hydrastine in spiked urine samples.

Figures of merit	Berberine samples	Hydrastine samples
LOD (ng/mL)	12.70 ± 0.09	54.50 ± 0.03
Correlation coefficient (r^2)	0.9969	0.9953
LDR[a]	12.74–1781 ng/mL	54.50–17,640 ng/mL
Reproducibility: $n = 3$ (standard deviation)	0.095	0.031
%CV[b]	6.48%	2.03%
Retention time (min)	7.5 ± 0.2	3.6 ± 0.1

[a] LDR, linear dynamic range.
[b] %CV, percent coefficient of variance.

3.4. Figures of merit

The figures of merit for berberine and hydrastine in spiked urine samples are located in Table 2.

The limit of detection (see Table 2) of berberine and hydrastine was lower than the LOD presented in the previous study [7]. The LDR for berberine and hydrastine in human urine was broader than the previous studies [6,7]. The improved method required less volume of urine sample (5 mL compared to 10 mL) than previous studies [8].

3.5. Blind study samples

A blind study was conducted to test human urine samples donated from a private toxicology drug testing laboratory. Thirty-seven urine samples separated into four different categories by the donating laboratory based on GC–MS toxicology screening for drugs of abuse were tested with the developed HPLC–UV method. The samples were categorized as samples with a positive screen for THC with interference on the GC–MS (8 samples), positive screen for THC with dilute creatinine levels (9 samples), negative screen for THC with low levels of THC detected with dilute creatinine levels (10 samples), and samples with no THC detected and dilute creatinine levels (10 samples). There were two samples spiked by the donating laboratory with goldenseal out of the original 37 samples that were tested for the presence of berberine and hydrastine alkaloids. Both samples screened positive for THC with interference on the gas chromatograph–mass spectrometer by the donating laboratory. These two samples were positively identified as containing both berberine and hydrastine alkaloids using the HPLC–UV urine screening method presented here. A third sample was also identified and may in fact be a positive goldenseal urine sample submitted into the toxicology testing laboratory from a donor.

4. Conclusions

A rapid and sensitive screening method was developed to extract and detect berberine and hydrastine alkaloids from goldenseal root powder, goldenseal liquid extract, and urine samples using HPLC with UV detection. The results show this method will enable laboratories to screen for the herbal supplement in submitted urine samples before proceeding through drug testing, ultimately identifying samples leading to false negative results.

5. Disclosure

The work performed on this project was done in the Chemical and Physical Sciences Department at Cedar Crest College. The authors do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work.

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