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Potassium Nitrite Reaction with 11-Nor-Delta9-Tetrahydrocannabinol-9-Carboxylic Acid in Urine in Relation to the Drug Screening Analysis*

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ABSTRACT: Recently potassium nitrite has been used as an adulterant to interfere with the analysis of 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in urine. A comprehensive study of the THC-COOH and nitrite reaction chemistry and stability under various conditions is presented. Reverse phase high performance liquid chromatography (HPLC) and negative electrospray mass spectrometry (ESMS) results are given to substantiate the derived reaction mechanism and properties leading to reaction termination. The addition of potassium carbonate as a buffering agent prior to or following sample void as a means of preventing the formation of a nitroso-complexed form of the 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid is evaluated.

KEYWORDS: forensic science, forensic toxicology, drug screening, urine, marijuana, 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid, potassium nitrite, adulterant

A contractual agreement for the submission of an initial drug screening sample, and even periodic submissions for specific job categories, is not an inconceivable prerequisite prior to employment. Once an employee consents to a mandatory drug-screening program, the temptation to adulterate a sample exists for those individuals having just cause for concern with the positive identification of a psychoactive constituent in their urine. To prevent the adulterating substance from interfering in the analysis of illicit drugs or drug metabolites in a biological specimen, it is essential to understand the conditions and mechanisms leading to false negative or non-reportable results. Once the chemical reactions are identified, steps may be taken to minimize or eliminate the interference.

Conformation of biological specimens screened positive, usually through immunoassay, for THC-COOH is predominately accomplished by means of gas chromatography/mass spectrometry (GC/MS) (1), although alternative methods are employed such as thin layer chromatography (TLC) (2), reversed-phase high-performance liquid chromatography (HPLC) (3), and micellar electrokinetic capillary chromatography (MEC) (4,5). A recent increase in the lack of conformation on samples screened positive for cannabinoids, in addition to low GC/MS internal standard recovery on the non-confirmed samples, initiated the work by ElSohly et al. to determine the source of the problem (6). They identified a commercially available drug-testing adulterant sold under the product name Klear, potassium nitrite, as the interferent.

Due to the low limit of detection required for positive identification of THC-COOH (~1.0 ng/mL), GC/MS methods generally require extraction, derivatization, and concentration steps prior to a mass analysis in selected ion monitoring mode as a means of achieving maximum sensitivity for the m/z values observed. During the process of preparing the sample for the analysis, residual adulterant in the urine could potentially react with any material having properties similar to THC-COOH, such as the internal standard, leading to low yields in addition to a non-detected analyte. ElSohly et al. found that the addition of sodium bisulfite prior to the analysis of a laboratory prepared THC-COOH standard containing an appropriate internal standard (THC-COOH-d_6) lead to a conformation of the spiked drug and recovery of the internal standard. However, in a study to determine sources and concentrations of nitrite in urine and to distinguish between natural sources and adulteration, Urry et al. (7) indicated that the bisulfite step reversed the nitrite interference in approximately half of their samples that initially screened positive for cannabinoids. The remaining cases could not be confirmed positive. In order to help prevent erroneous drug screening or unconfirmed results caused by the addition of potassium nitrite, one must understand the chemical mechanisms that takes place in the urine sample and stability of the reaction products. With this knowledge, the reaction may either be prevented or reaction products monitored.

In this study, reverse phase high performance liquid chromatography (HPLC) was employed to monitor the effects of potassium nitrite addition to a sample containing THC-COOH, assist in the identification of the separation products, measure reaction times, and determine stability. HPLC solute band fractions were collected

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and positively identified using negative ion electrospray tandem mass spectrometry (ES/MS/MS).

Experimental

Fresh urine specimens were collected for each experiment. 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH-d_3) was spiked into a 5.0 mL urine sample to give a final THC-COOH-d_3 concentration of 50.0 ng/mL for the HPLC studies and 100.0 μg/mL for the ES/MS/MS identification and adulterant inhibition experiments (Radian International LLC, Austin, TX). The HPLC separations were conducted using an Acuflow series IV Pump and an Acutect 500 UV/Vis detector (Fisher Scientific, Pittsburgh, PA) equipped with a Hypersil ODS 5 μm (250 mm × 4.6 mm) column (Supelco Inc., Bellefonte, PA). UV/Vis data were collected and stored using EZChrom software (Scientific Software Inc., San Ramon, CA). 18 MΩ purified water was used throughout the research project (Millipore Milli-Q purification system, Marlborough, MA). The HPLC mobile phase was composed of an acetonitrile: 0.01 M potassium phosphate (60:40) mixture and maintained at a flow rate of 1.0 mL/min (J. T. Baker, Phillipsburg, NJ). Each compound separated by HPLC for the ES/MS/MS identification was monitored at an appropriate wavelength, and the fractions (based on peak width and flow rate) were collected, placed on ice to impede decomposition, and analyzed by ES/MS/MS. The ES/MS/MS experiments were performed using a VG Quattro II triple quadrupole electrospray mass spectrometer (Micromass Inc., Beverly, MA). A Model 22 Harvard syringe pump was used for sample introduction in the infusion experiments (Harvard Apparatus, South Natick, MA). Samples analyzed by ES/MS/MS were adjusted to a pH of 8.5 using triethylamine and sprayed with a solution of acetonitrile:water (1:1) (J. T. Baker, Phillipsburg, NJ).

During the adulterant experiments, potassium nitrite crystals (≈ 0.125 g, J. T. Baker, Phillipsburg, NJ) were added to 5.0 mL urine samples spiked with THC-COOH-d_3 (100 μg/mL). In order to evaluate the inhibition of a nitroso-THC-COOH-d_3 complex formation, potassium carbonate (J. T. Baker, Phillipsburg, NJ) was added to urine samples containing THC-COOH-d_3 for pH adjustment to approximately 7.0 prior to the addition of potassium nitrite.

Results and Discussion

Initially, a urine sample spiked with THC-COOH-d_3 (50 ng/mL) was directly injected onto a HPLC column to ensure that THC-COOH-d_3 was detectable at the 214 and 254 nm wavelengths. The THC-COOH-d_3 chromatogram was then compared to that of a blank urine sample, and found to be detectable above an unspiked urine background signal. In each of the HPLC studies, absorbance was monitored at one or more wavelengths depending upon the experiment. A carboxylic acid chromophore absorbed at 214 nm, an aromatic ring absorbed at 254 nm, and a nitroso chromophore absorbed at 300 nm. THC-COOH-d_3 was detected at both the 214 and 254 nm wavelengths, and was found to have a retention time of 2.7 min. Potassium nitrite was added to the sample containing THC-COOH-d_3, and the resulting chromatogram was evaluated for adulterant effects on the THC-COOH-d_3 signal and ingrowth of any reaction product. Approximately 5 min after the addition of potassium nitrite to the spiked sample, HPLC analysis yielded the chromatogram shown in Fig. 1. Both the THC-COOH-d_3 peak and an unknown second peak were observed. The retention time of the reaction product was approximately 4.9 min. As shown in Table 1, timed studies revealed that while the level of the second peak (solute band B) increased, the level of the THC-COOH-d_3 peak (solute band A) decreased. During this evaluation, potassium nitrite was added to a THC-COOH-d_3 spiked urine sample, and the sample was allowed to react for approximately 5 min before the initial analysis. The time elapsed between potassium nitrite addition and initial HPLC separation is recorded in Table 1. Blank samples were analyzed between each analysis to ensure that analytes were flushed through the system. The THC-COOH-d_3 and unknown peak areas were integrated, and percentages were calculated (Table 1). Upon evaluation of the data presented, the stability of the unknown reaction product was questioned. After one hour, it was evident that the THC-COOH-d_3 analyte was almost completely converted to an unknown; however, the reaction product appeared unstable since the summed peak areas did not remain constant over the 4134 s time period. HPLC detection of the separated species at the 214 and the 254 nm wavelengths revealed that both peaks continued to absorb at these energy regions, indicating that the carboxylic acid chromaphore and the aromatic ring remained intact in the reaction product species. In addition, at the 300 nm wavelength, only one large peak was observed at a retention time characteristic of the unknown. From this characterization data, the unknown species appeared to be a nitroso-THC-COOH-d_3 complex.

TABLE 1—*THC-COOH-d_3 adulterant kinetics study.*

Time(s)	THC-COOH-d_3 (Area)	THC-COOH-d_3 (%)	Unknown (Area)	Unknown (%)
304	4890321	92.9	372281	7.1
811	3429869	61.1	2182944	38.9
1321	1764603	38.9	2773601	61.1
1901	604584	17.3	2892329	82.7
2596	321659	10.3	2796028	89.7
3160	94092	3.2	2811503	96.8
3673	97628	3.7	2550064	96.3
4134	15304	1.0	1457615	99.0

In order to confirm the identity of the reaction product, potassium nitrite was added to a THC-COOH-d_3 spiked urine sample (100 μg/mL), the solution was allowed to react approximately 5 min, and solute bands A and B were HPLC separated and collected for analysis by negative electrospray mass spectrometry. A reference product spectrum was obtained by directly adding 100 μg THC-COOH-d_3 to 1.0 mL buffer solution and analyzing by ES/MS/MS. When the *m/z* 347 negative molecular parent ion was monitored from the reference solution, the product spectrum shown in Fig. 2 was obtained. Several characteristic peaks were observed including the *m/z* 347 M^- and *m/z* 346 M-H peaks. The loss of a CO_2 group is typical of substituted acids, and was noted at *m/z* 303 and 302 from the M^- and M-H species, respectively. Solute band A (retention time 2.7 min) yielded a product spectrum from the *m/z* 347 negative molecular parent ion similar to Fig. 2, corresponding to the presence of THC-COOH-d_3. The solute band B (retention time 4.9 min) showed a negative molecular ion at *m/z* 376. The presence of a molecular peak 29 amu higher than the reference compound confirms the presence of a NO group attached to the aromatic ring. The product spectrum of the *m/z* 376 negative molecular ion parent is given in Fig. 3. The *m/z* 376 M^- and *m/z* 375 M-H peaks were present in the solute B product spectrum. In addition, peaks at *m/z* 331 and 332 from the M^- and M-H species, respectively, were observed due to the loss of a CO_2 group.

Due to the especially high reactivity of the phenol ring, Morrison and Boyd state that ring nitrosation reactions (electrophilic substitution) occurs upon addition of nitrous acid (8). After con-

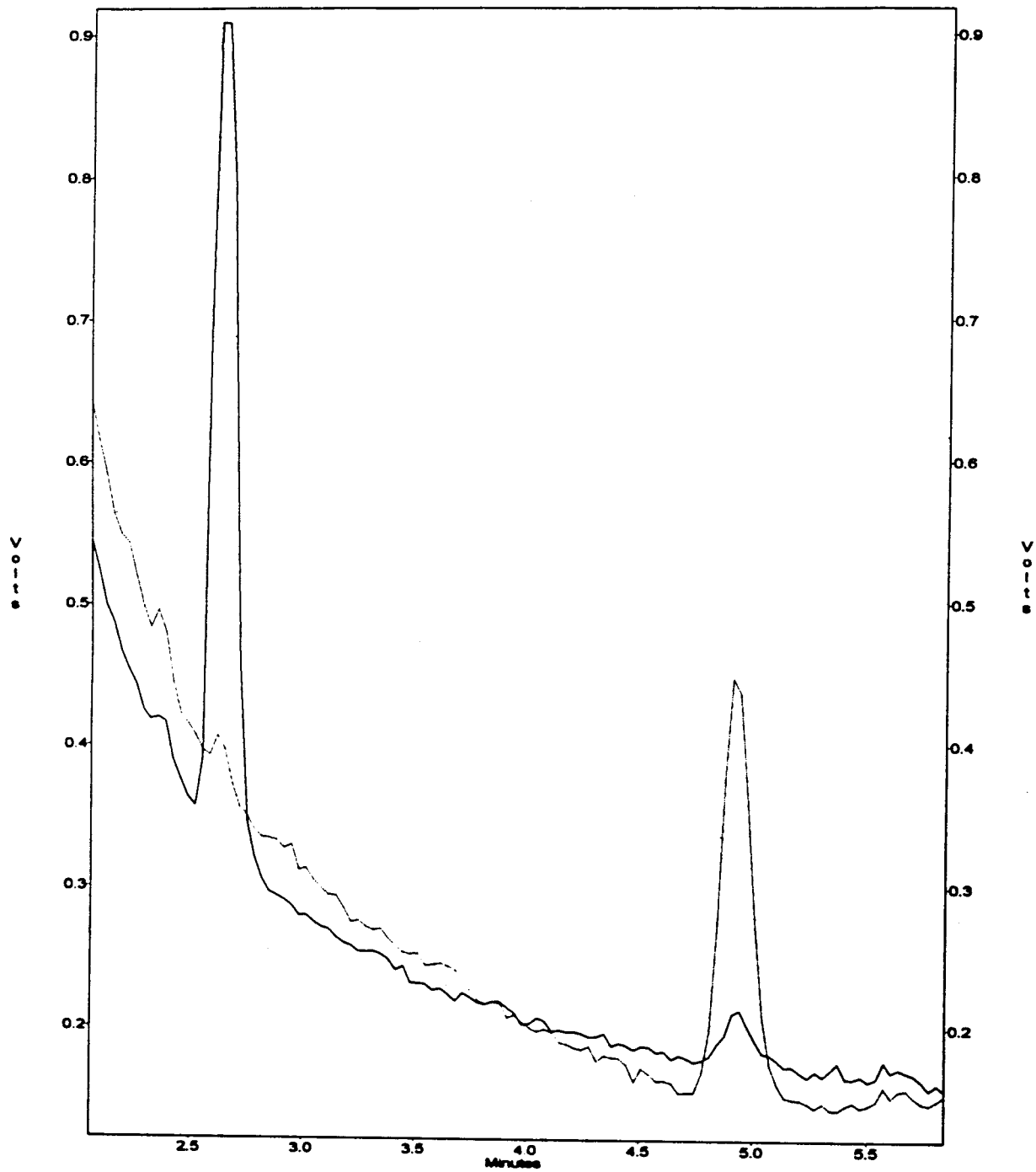

FIG. 1—*Chromatogram of a urine sample spiked with 50.0 ng/mL THC-COOH-d_3 5 min (—) and 53 min (-----) after the addition of potassium nitrite obtained at 250 nm.*

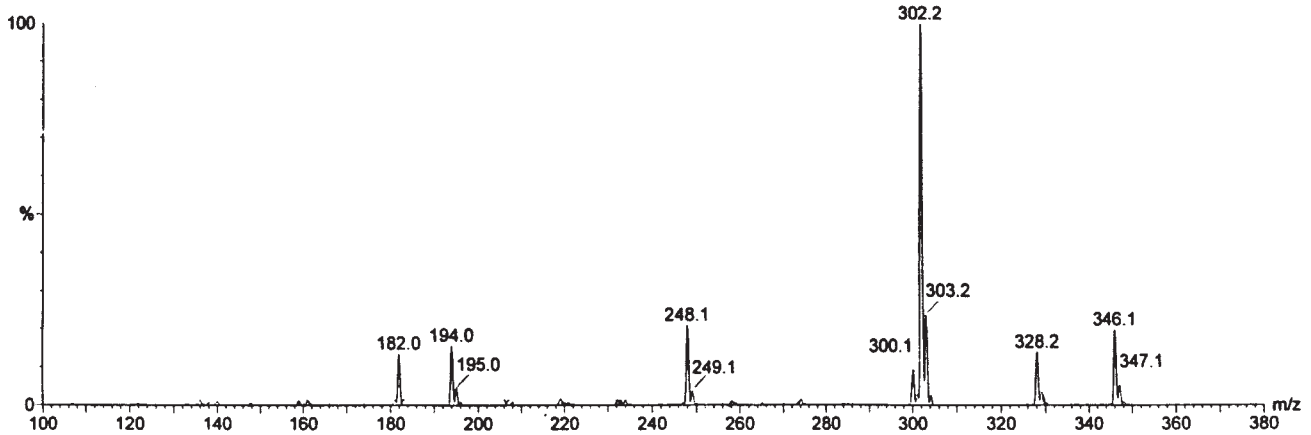

FIG. 2—*ES/MS/MS product spectrum of the THC-COOH-d_3 negative molecular parent ion (*m/z *347) separated from the 100 μg/mL reference solution.*

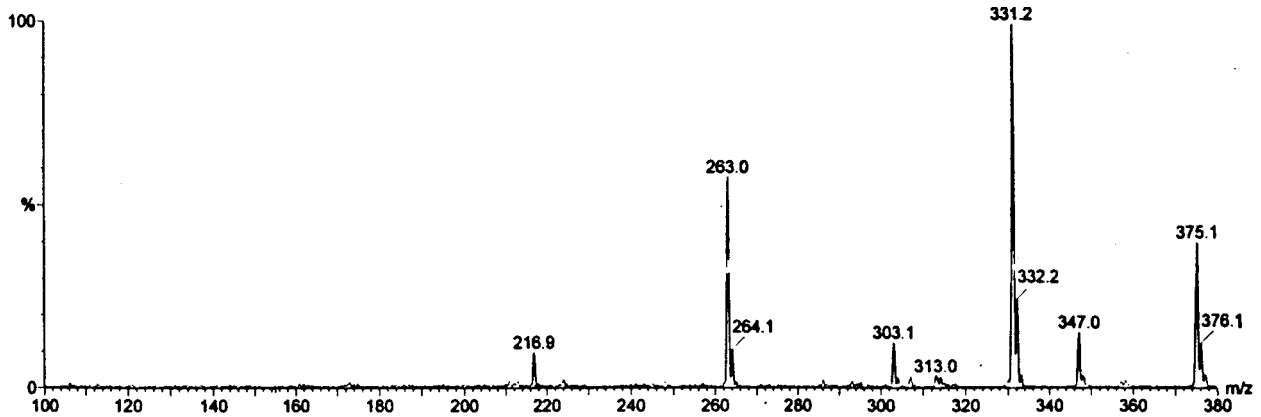

FIG. 3—*ES/MS/MS product spectrum of the nitroso-THC-COOH-d_3 negative molecular parent ion (*m/z *376).*

sidering this statement, and reviewing the HPLC and ES/MS/MS results, the following reactions were proposed:

$$1)\ 2\underset{\text{acid}}{HNO_2} \leftrightarrow N_2O_3 + H_2O$$

$$2)\ \text{THC-COOH} + N_2O_3 \rightarrow \text{NO-THC-COOH} + HNO_2$$

Once the above reactions were derived, the possibility of preventing nitrosation by maintaining a neutral to basic pH to inhibit the formation of nitrous acid was evaluated. The inhibition study was carried out through the immediate addition of a basic buffer, potassium carbonate, to urine samples spiked with both THC-COOH-d_3 and potassium nitrite. Twenty-four hours after the potassium carbonate was added to the spiked urine, HPLC results showed neither evidence of a decrease in area counts for the THC-COOH-d_3 peak nor the presence of any reaction product in the spiked sample chromatograms.

Conclusion

Data presented in this study demonstrated that potassium nitrite added to urine containing THC-COOH was very reactive toward the phenolic compound. At room temperature, the reaction was essentially complete in approximately 70 min. Without pre- or post-treatment, studies at 4°C showed that the reaction was slowed, but complete within 24 h. In addition, the reaction product, nitroso-THC-COOH, was found to be unstable, converting into components neither detectable by UV or ES/MS. Drug adulteration does appear possible for other illicit drugs having similar phenolic structures susceptible to electrophilic substitution. The impact of this reaction process on drug analysis would be to mask the chromatographic and mass characteristics of the parent compound, thus leading to false negative results.

Considering the nitroso-THC-COOH rate of formation, the addition of sodium bisulfite to an nitrite adulterated urine specimen prior to conformation analysis would appear to serve as a means of obtaining a sufficient yield; however, if the analysis was conducted over 70 min past the collection time, any THC-COOH initially present in the urine would either be masked or converted. In addition, Mendiara et al. (9) reported that below a pH of 5.8 the Raschig mechanism between nitrous acid and bisulfite leads to the formation of a nitrene, HNO: a diratical intermediate of N (+1). In the presence of dissolved oxygen, the nitrene could, upon rearrangement, form nitric acid. Dilute nitric acid is known to react with phenolic compounds to form nitrophenols (8). Other substances known to react with the phenonic group include acids, acid chlorides, anhydrides, diazonium salts, sodium hydroxide, and bromine (8). Water-soluble forms of these compounds may be potential adulterants to the THC-COOH analysis. In order to determine if a successful confirmation of the presence of THC-COOH in an adulterated sample is possible, the reaction chemistry, reaction conditions, and rates should be considered.

Termination of nitroso-THC-COOH formation in any nitrite-adulterated drug-screening specimen containing THC-COOH appears possible though either sample pre- or post-treatment with a basic buffer to prevent the N_2O_3 formation. The authors did not evaluate the effect of potassium carbonate on any of the available immunoassay-based screening products. However, should the use of a basic substance, such as potassium carbonate, interfere with the immunoassay test, either the screening could be conducted immediately (considering the 70 min reaction time) prior to the addition of the base, or the sample may be split, and the base added only to the confirmation sample. Recently, the Department of Health and Human Services (HHS) issued guidance to laboratories conducting validity test by defining criteria for diluted, substituted, or adulterated specimen (10). Presently, adulterated samples are reported under the "Test Not Performed" category. Should the chemical prevention of adulteration become an acceptable means of dealing with tampered samples, the authors suggest that positive/adulterated confirmations become an acceptable reporting alternative.

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