Michael J. Kogan,[1] *Ph.D.; Jamil Al Razi,*[2] *B.S.;*
David J. Pierson,[1] *M.S.; and Nicholas J. Willson,*[1] *M.D.*

Confirmation of Syva Enzyme Multiple Immunoassay Technique (EMIT®) d.a.u. and Roche Abuscreen® Radioimmunoassay (RIA) (^{125}I) Urine Cannabinoid Immunoassays by Gas Chromatographic/Mass Spectrometric (GC/MS) and Bonded-Phase Adsorption/Thin-Layer Chromatographic (BPA-TLC) Methods

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ABSTRACT: Thirty human urines screened positive by the Syva enzyme multiple immunoassay technique (EMIT®) d.a.u. urine cannabinoid assay were also positive for the major marijuana urinary metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) when assayed by gas chromatographic/mass spectrometric (GC/MS) and a noninstrumental qualitative bonded-phase adsorption/thin-layer chromatographic (BPA-TLC) technique. The noninstrumental BPA-TLC procedure was the simpler of the two techniques to perform and interpret. Assay of these same samples by the Roche Abuscreen® radioimmunoassay (RIA) for cannabinoids (^{125}I) revealed that reliance on the 100-ng/mL equivalent positive calibrator yielded a high incidence of false negative results (10 out of 30). The performance of these same 4 assays on 30 true negatives also was evaluated. All samples were negative for cannabinoids by EMIT and RIA, and for THC-COOH by BPA-TLC. GC/MS assay, however, detected spurious low levels of approximately 5-ng/mL THC-COOH in two instances. Because of this, a reliability level of 10 ng/mL was set for the routine quantitative confirmation of THC-COOH by the GC/MS method.

KEYWORDS: toxicology, immunoassay, marijuana, chromatographic analysis

The most widely used, commercially available, immunoassays for detecting cannabinoids (marijuana) in urine are the Syva enzyme multiple immunoassay technique (EMIT®) can-

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[1]Head, Chromatography Section, head, Immunoassay Section, and associate laboratory director, respectively, New York State Psychiatric Institute, Department of Neuropathology and Neurotoxicology, New York, NY.

[2]Head, Analytical Toxicology, Psychiatric Diagnostic Laboratories of America, Inc., South Plainfield, NJ.

nabinoid assays (d.a.u. and less sensitive st) and the Roche Abuscreen® radioimmunoassay for cannabinoids (^{125}I) [*1*]. These assays are rapid, reliable, and sensitive. They detect the metabolites of the major psychoactive cannabinoid of marijuana, Δ^9-tetrahydrocannabinol (THC), by reacting to the primary THC urinary metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and structurally related THC derivatives [*1,2*]. Although it is recommended that positive screening results be confirmed by a nonimmunological (chromatographic) technique [*2,3*], the requirement of confirmation is mandatory when adversary matters are involved [*4*]. Unlike the EMIT and Abuscreen (RIA) immunoassays, chromatographic assays are based exclusively on the detection of a single cannabinoid metabolite THC-COOH [*5–7*]. At present, gas chromatography/mass spectrometry (GC/MS) generally is considered the method of choice for confirming positive immunoassay results [*8*]. In this article we compare the results of the analyses of 60 urine specimens by 4 different methods: (1) the Syva EMIT d.a.u. immunoassay, (2) the Roche Abuscreen urine cannabinoid immunoassay, (3) gas chromatography/mass spectrometry (GC/MS), and (4) qualitative bonded-phase adsorption/thin-layer chromatography (BPA/TLC).

Materials and Methods

EMIT® Cannabinoid Assay

The Syva EMIT d.a.u. urine cannabinoid assay was performed according to the manufacturer's protocol [*9*]. Samples were processed on a Syva AutoLab 6000 system, which consists of a Syva Lab Processor 6000, an AutoCarousel, and a Gilford Stasar S-III spectrophotometer. Calibrators (negative, low, and medium) were assayed in triplicate, and assay calibration values were automatically entered into the system data processor. All clinical samples were run in duplicate.

Abuscreen RIA Cannabinoid Assay

The Roche Abuscreen radioimmunoassay for cannabinoids (^{125}I) was performed according to the manufacturer's protocol [*10*]. Radioactivity was measured using a Beckman Model 4000 Gamma Counter interfaced to a Beckman DP 5000 data processor. Calibrators (negative and positive) were run in triplicate, counts averaged, and the means compared to each sample's counts to determine results. All clinical samples were run in duplicate.

TLC Assay

A modification of a previously described bonded-phase adsorption/thin-layer chromatographic (BPA-TLC) assay for the qualitative detection of THC-COOH [*11*] was used. After developing the chromatogram, the plate is sprayed with concentrated ammonium hydroxide. The base intensifies the reaction of the visualizing fast blue RR spray and makes identification of THC-COOH instantaneous. The TLC assay was deemed qualitatively positive if a scarlet color reaction was observed at the R_f corresponding to that of a hydrolyzed and extracted THC-COOH urine control, or negative if no scarlet color was observed at this R_f. Urinary THC metabolites other than THC-COOH are either hydroxysubstituted monocarboxylic acids or dicarboxylic acids [*12*]. Being more polar, these metabolites would most probably migrate differently and thus would not be identified as THC-COOH. The sensitivity of the BPA-TLC method is 20-ng/mL THC-COOH when using a 10-mL sample [*11*].

GC/MS Assay

THC-COOH was extracted from 4 mL of base-hydrolyzed urine onto an ion exchange column with a Prep I® Automated Sample Processor (DuPont Instrument Co., Wilmington,

DE). A deuterated analogue of THC-COOH (Research Triangle Institute, Research Triangle, NC) was added before extraction as an internal standard. Following removal of the solvent, THC-COOH was converted to its methylester [7]. Analysis was performed on a Hewlett Packard 5995 electron impact mass spectrometer. Two ions at 314 and 317 AMU were monitored for THC-COOH presence as well as three additional ions for confirmation. Separation was performed on a capillary OV-17 column. The concentration of THC-COOH was determined by comparing the abundance ratio of an unknown to that of the standards ($N = 7$). The standard curve was linear over the range of 5 to 200-ng/mL THC-COOH. Samples were assayed using a reduced aliquot to determine concentrations above 200 ng/mL.

Samples

Human urine samples, submitted to the N.Y.S. Central Reference Laboratory for routine drugs of abuse testing, were analyzed for cannabinoids by the EMIT method. Thirty randomly selected urines from the group screened positive by EMIT were retained for further analyses. Cannabinoid-free (negative) urines were collected in house from six persons known to be nonusers of marijuana. Each of these urines was divided into five aliquots giving a total thirty negative urines. All specimens were collected in polypropylene containers, stored in the absence of light under refrigeration (maximum three days), and then frozen at -15°C. Frozen samples assayed by RIA and BPA-TLC were thawed, equilibrated to room temperature, and centrifuged to separate sediment before analysis. A frozen aliquot was transported to another facility (Psychiatric Diagnostic Laboratories of America, Inc.) for GC/MS analysis.

Results

Table 1 presents the results of assays performed on 30 different clinical urine samples which were screened positive for cannabinoids by EMIT. Immunoassay positive results are expressed qualitatively with respect to the nearest EMIT or RIA calibrator response level exceeded by the sample. The values shown for the GC/MS assays are the concentrations of THC-COOH measured in each sample. The presence of THC-COOH was confirmed (+) in all immunoassay positive samples by the BPA-TLC assay.

Table 2 presents the results of the above four assays performed blind on six true negative urines. Each group of five sample numbers represents the replicate assay results on aliquots of an individual urine. All 30 sample aliquots were negative for cannabinoids by EMIT and RIA immunoassays. The THC-COOH metabolite was not detected (. . .) in any sample aliquot by the BPA-TLC assay. The GC/MS assay, however, reported spurious low levels of THC-COOH in two instances.

Discussion

The detection of the cannabinoid biotransformation product THC-COOH in urine is evidence of cannabis intake [1]. Since GC/MS and BPA-TLC assays identified this metabolite in all samples screened positive by EMIT (Table 1), both assays provided a qualitative confirmation of the EMIT results. The GC/MS assay adds an additional quantitative element by reporting the actual THC-COOH concentration. When qualitative confirmation is the only requirement of an alternate assay, either the GC/MS or BPA-TLC technique could be used.

The advantage of BPA-TLC over GC/MS is that it is a simpler, noninstrumental technique. The BPA-TLC assay is based on a visual color reaction and is easier to interpret than a quantitative GC/MS spectral analysis. The measurement of cannabinoids by GC/MS is affected by the biologic background and errors in calibration [13]. Also, spontaneous changes in cannabinoid concentration with time contribute to the uncertainty inherent in replicate GC/MS determinations [14].

TABLE 1—*Results of assays performed on clinical urines screened positive for cannabinoids by EMIT d.a.u.*

Sample	EMIT[a]	RIA[b] Abuscreen	GC/MS THC-COOH, ng/mL	BPA-TLC THC-COOH
1	L	GN	17	+
2	L	GN	25	+
3	L	GN	29	+
4	L	GN	74	+
5	L	P	243	+
6	M	GN	16	+
7	M	GN	16	+
8	M	P	29	+
9	M	P	39	+
10	M	P	41	+
11	M	GN	71	+
12	M	GN	78	+
13	M	P	79	+
14	M	P	80	+
15	M	P	95	+
16	M	GN	123	+
17	M	P	131	+
18	M	P	154	+
19	M	P	158	+
20	M	GN	161	+
21	M	P	186	+
22	M	P	208	+
23	M	P	230	+
24	M	P	250	+
25	M	P	266	+
26	M	P	420	+
27	M	P	455	+
28	M	P	575	+
29	M	P	685	+
30	M	P	1717	+

[a]L = positive response greater than low calibrator (20-ng/mL 11-nor-Δ^8-THC-9-carboxylic acid) (Δ^8THC-COOH); M = positive response greater than medium calibrator (75-ng/mL Δ^8THC-COOH) [9].

[b]GN = response greater than normal urine calibrator (0-ng/mL cannabinoid); P = positive response greater than positive calibrator (Δ^8THC-COOH equivalent to 100-ng/mL THC-COOH) [10].

Direct comparison of the two immunoassays, EMIT d.a.u. and Abuscreen RIA, is difficult if one strictly adheres to the manufacturer-suggested positive calibrators. The manufacturer of the Abuscreen RIA assay [10] indicates an assay sensitivity of 5 ng/mL but selects 70 ng of Δ^8 THC-COOH (equivalent to 100 ng of THC-COOH) as its nominal positive calibrator [15]. This level was set because of guidelines of the Department of Defense (DOD), a major user of the assay [16]. The use of a 100-ng/mL cutoff assured DOD that greater than 90% of the RIA screening results could be confirmed by their approved gas chromatographic method of assay [7,16]. Strict reliance on a 100-ng/mL positive cutoff increases the number of false negatives. The ten samples responding at the (GN) level (Table 1) would be negative by strictly interpreted Abuscreen RIA criterion. However, these samples were positive by EMIT, GC/MS, and BPA-TLC, and in this report they are considered to be positive.

EMIT and RIA results were in qualitative agreement (as expected) in all cases [14,17]. However, the magnitude of the positive immunoassay responses did not always parallel each other (Table 1). This lack of a consistent relationship between the two immunoassays agrees with

TABLE 2—*Results of assays performed on cannabinoid-free urines.*

Sample	EMIT[a]	RIA[b] Abuscreen	GC/MS THC-COOH, ng/mL	BPA-TLC THC-COOH
1	N	LN	0	...
2	N	LN	0	...
3	N	LN	0	...
4	N	LN	0	...
5	N	LN	0	...
6	N	LN	4	...
7	N	LN	0	...
8	N	LN	0	...
9	N	LN	0	...
10	N	LN	0	...
11	N	LN	0	...
12	N	LN	0	...
13	N	LN	0	...
14	N	LN	0	...
15	N	LN	0	...
16	N	LN	0	...
17	N	LN	0	...
18	N	LN	0	...
19	N	LN	5	...
20	N	LN	0	...
21	N	LN	0	...
22	N	LN	0	...
23	N	LN	0	...
24	N	LN	0	...
25	N	LN	0	...
26	N	LN	0	...
27	N	LN	0	...
28	N	LN	0	...
29	N	LN	0	...
30	N	LN	0	...

[a]N = negative response below low calibrator (positive cutoff).
[b]LN = negative response less than or equal to normal human urine calibrator (0-ng/mL cannabinoid).

other studies that indicate no correlation between EMIT d.a.u. semiquantitative values and Abuscreen RIA [*14,16*].

The level of positive response by EMIT or RIA did not always correlate with the THC-COOH concentrations quantitated by GC/MS (Table 1). The lack of a quantitative parallel between THC-COOH concentration and EMIT or RIA immunoassay response may be due to immunoassay reactivity with other cannabinoids, variable reactivity to the glucuronide fraction, actual variations in the amount of THC-COOH, or any combination of these factors. The THC-COOH metabolite reportedly accounts for only 27% of all acidic urinary metabolites [*12*] and exhibits wide individual differences in the pattern of glucuronide elimination [*18*].

The BPA-TLC assay correctly identified the absence and presence of THC-COOH in all true negative and positive EMIT screened samples (Tables 1 and 2). This finding is in agreement with an earlier study which reported BPA-TLC capable of consistently confirming EMIT d.a.u. screening results [*6*]. The GC/MS and BPA-TLC results were in qualitative agreement for all EMIT positive screened samples (Table 1). However, BPA-TLC did show an advantage over GC/MS in correctly identifying 30 out of 30 true negative samples which GC/MS correctly identified 28 out of 30 (Table 2). In four other instances the GC/MS assay of these two urines yielded negative results.

GC/MS cannabinoid assays are known occasionally to measure low levels of cannabinoids in blank quality control urine [*19*] and blood samples [*13*]. Because of the qualitative emphasis placed on a positive confirming assay result, a study of assay performance on true negative urines is valuable. Such a study gives an indication of the likelihood of a false positive responses.

Analyses of true negative urines (Table 2) by GC/MS found spurious levels of 4 and 5 ng/mL THC-COOH in two different samples. Four replicate analyses by GC/MS assays of these same urines were negative. The origin of this phenomenon may be an instrumental contribution to background as a result of increased electron multiplier (EM) intensity. At the 5-ng/mL level, quantitation often requires a higher EM setting. The increased voltage may produce a higher background which can be interpreted as positive. These two low levels nevertheless were statistically different (Mann Whitney U. Test, $p < 0.01$) and distinguishable from THC-COOH concentrations measured in clinical samples screened positive by EMIT and confirmed positive by BPA-TLC. As a result of these findings, a quantitative cutoff for routine confirmation of immunoassay positive screened urines has been set in our laboratory at 10-ng/mL THC-COOH. This cutoff value is consistent with other reports using electron impact GC/MS for detection of THC-COOH in urines screened positive for cannabinoids by immunoassay [*19*].

We found that all EMIT d.a.u. results for true negative urines were corroborated by the Abuscreen RIA immunoassay. Also, EMIT positive results (response greater than 20-ng/mL cutoff) were corroborated by the Abuscreen RIA immunoassay when RIA responses greater than the assay's normal urine calibrator were interpreted as positive. Furthermore, we observed that strict reliance on the RIA assay's 100-ng/mL positive calibrator gives a high incidence (10 out of 30) of false negatives. Both GC/MS and BPA-TLC proved to be suitable alternative techniques for qualitatively confirming THC-COOH presence in samples screened positive for cannabinoids by the immunoassays. The noninstrumental BPA-TLC assay was the simpler of the two alternate chromatographic methods to perform and interpret. An assay reliability cutoff of 10 ng/mL was used with the routine quantitative determination of THC-COOH by the GC/MS method.

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Address requests for reprints or additional information to
Michael J. Kogan
Department of Neuropathology and Neurotoxicology
722 W. 168th St.
New York, NY 10032