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High Performance Liquid Chromatography-Immunoassay of Δ^9-Tetrahydrocannabinol and Its Metabolites in Urine

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ABSTRACT: High performance liquid chromatographic-immunoassay (HPLC-IA) profiles of cannabinoid metabolites in urine samples were obtained using four different antisera. The urines were chromatographed on a reverse phase system using a gradient of acetonitrile in water (pH 3.3) and fractions collected every 30 s. Some urine samples were hydrolyzed with methanolic sodium hydroxide before fractionation. Peaks of immunoreactivity were detected at a fraction corresponding to 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (COOH-THC) and at an early eluting fraction; however, the profiles depended upon the specificity of the antisera used.

KEYWORDS: toxicology, chromatographic analysis, immunoassay, tetrahydrocannabinol, urine

A variety of chromatographic techniques have been used to assay Δ^9-tetrahydrocannabinol (Δ^9-THC) and its metabolites in biological fluids, including gas chromatography [*1-3*], gas chromatography-mass spectrometry [*4-6*], thin-layer chromatography [*7,8*], high performance liquid chromatography (HPLC), and combinations of these techniques [*11,12*]. Immunoassay (IA) procedures have been used presumptively to identify Δ^9-THC and its metabolites [*13-18*]. HPLC with immunoassay detection has also been used to assay biological fluids for these cannabinoids [*19-22*].

The major Phase I metabolic route of Δ^9-THC involves hydroxylation of the allylic methyl group followed by further oxidation to 11-nor-9-carboxy-Δ^9-THC (COOH-THC). A major portion of urinary Δ^9-THC metabolites in animals and man are conjugates susceptible to hydrolysis by mixed β-glucuronidase/arylsulphatase enzymes [*23,24*] or with β-glucuronidase alone [*25*]. Evidence for the existence of a glucuronide conjugate of COOH-THC has been provided using methanolic sodium hydroxide for hydrolysis of conjugates [*21*]. The following report describes a study of HPLC-IA profiles of cannabinoid metabolites in human urine using radioimmunoassay (RIA) and enzyme multiple immunoassay technique (EMIT®) procedures. These urine specimens were collected from suspected marihuana smokers.

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Materials

HPLC analyses were performed on a Varian Model 5000 liquid chromatograph equipped with a Varian UV-5 detector set at 215 nm (Varian Instrument Co., Walnut Creek, CA). The column used was a Spherisorb-ODS (5 μm), 10 cm by 4.6 mm (Supelco, Inc., Bellefonte, PA). Fractions were collected using an LKB 2070 fraction collector. All chemicals used were HPLC grade.

Three different RIA procedures were used, two of which are reported to be specific for Δ^9-THC and COOH-THC, and were supplied by Research Triangle Institute through the Research Technology Branch, National Institute on Drug Abuse; the other RIA procedure, an Abuscreen® kit for cannabinoids (Roche Diagnostics Inc., Nutley, NJ), was supplied through the U.S. Navy Laboratory at Portsmouth, VA. All of these procedures use an ^{125}I tracer. The EMIT-d.a.u. assay (Syva, Palo Alto, CA) for cannabinoids was also used.

Methods

Two millilitres of urine are mixed with 2 mL of methanol and 2 mL of a methanol:water pH 5.5 (1:1) solution. After centrifuging, 5 mL of the supernatant are injected via a 5-mL injection loop directly onto the HPLC column. The cannabinoids are eluted by acetonitrile: water (adjusted to pH 3.3 with phosphoric acid). The flow rate is 4 mL per minute and the following gradient is used: 0 to 10 min, 36% acetonitrile; 10 to 20 min, 36 to 70% acetonitrile; and 20 to 25 min, 70% acetonitrile. Fractions were collected every 30 s; the first two fractions were discarded. Each fraction was evaporated to dryness at 40°C under reduced pressure using a Buchler vortex-evaporator attached to a water aspirator. Several urine samples were hydrolyzed by methanolic sodium hydroxide, as described by Williams et al [*19*].

Immunoassays were performed on each of the fractions by dissolving the residue in 400 μL of methanol: 50- and 100-μL aliquots, respectively, are used for RIA analysis of Δ^9-THC and COOH-THC by the Research Triangle Institute (RTI) procedure. After re-evaporation, the residue is dissolved in 300 μL of urine and analyzed using the Abuscreen and EMIT procedures.

The urine samples were also analyzed for "total" and "free" COOH-THC by gas chromatography-chemical ionization mass spectrometry (GC-CIMS) as described by Foltz et al [*6*].

Results

Figure 1 shows a HPLC-ultraviolet (UV) chromatogram of 11-hydroxy-THC, COOH-THC, and Δ^9-THC. Using the gradient system described above, the retention volumes were 56.0, 57.2, and 73.6 mL, respectively. To determine recovery of COOH-THC, a cannabinoid-free urine sample was spiked with ^3H-COOH-THC at a concentration of 100 ng/mL, fractionated and evaporated as described in the Methods section. Ninety-four percent of the radioactivity was recovered in the fraction collected at the retention volume of COOH-THC. Blank urine was also fractionated and analyzed by the various immunoassay procedures; no significant immunoreactivity was observed in any fraction.

Figures 2 and 3 illustrate HPLC-RIA chromatograms for two urine samples (56 and 117), unhydrolyzed and hydrolyzed. The RTI procedure for COOH-THC was used as the method of detection. For both unhydrolyzed samples significant (greater than 10% of the total) amounts of immunoreactivity were observed for fractions eluting between 6 and 16 mL (Fractions 1 to 6) and 58 and 64 mL (Fractions 27 to 29) of eluent. This latter peak corresponded to the retention volume of COOH-THC. In addition, there was a significant amount of immunoreactivity in the fraction eluting between 6 and 36 mL (Fractions 8 to 16) for Sample 56. When these urine samples were hydrolyzed, fractionated, and chromatographed, the HPLC-RIA profiles were similar for each urine. For Urine 117 there was a decrease in the amount of immunoreactivity eluting in the early fractions, but an increase in

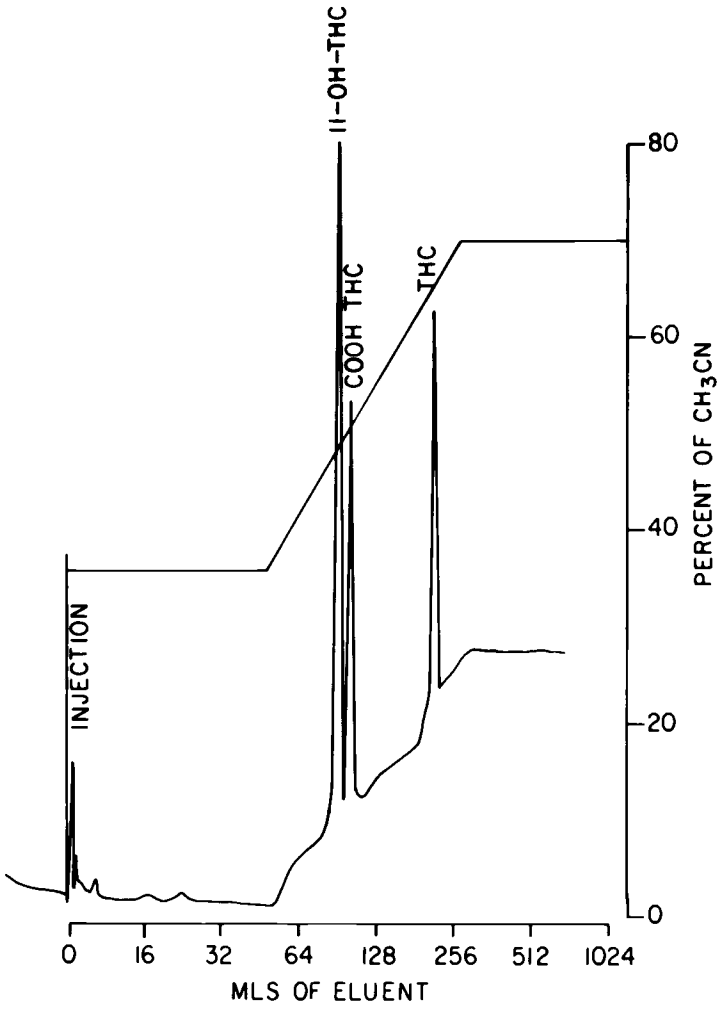

FIG. 1—*HPLC-UV chromatogram of 11-hydroxy-THC, COOH-THC, and Δ^9-THC.*

that eluting between 32 and 36 mL (Fractions 14 and 15) of eluent. No change was observed in the early eluting fraction for Sample 56; however, immunoreactivity was observed between 32 and 36 mL (Fractions 14 and 15) of eluent. As with both unhydrolyzed samples a peak was observed with an identical retention volume to COOH-THC. The percentages of total immunoreactivity eluting in these various fractions are tabulated in Table 1 together with the results of the GC-CIMS analysis on the nonfractionated urine.

Figures 4 and 5 show the HPLC-EMIT chromatograms for the same two urine specimens. The profiles were similar to those illustrated in Figs. 2 and 3 with the major amount of immunoreactivity present in an early eluting fraction and a fraction corresponding to COOH-THC. Alkaline hydrolysis did not alter the HPLC-IA profile. In particular, no significant immunoreactivity was observed between 32 and 36 mL (Fractions 14 and 15) of eluent.

Figure 6 shows a HPLC-RIA profile of a hydrolyzed urine sample (56) using the Abuscreen cannabinoid detection kit. The HPLC-IA profile was similar to those obtained by

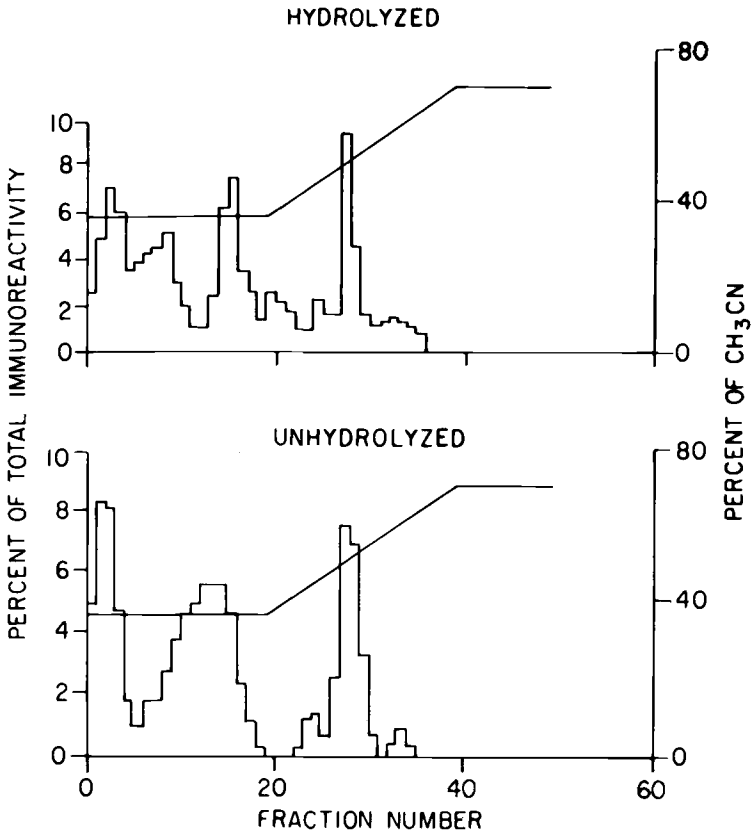

FIG. 2—*HPLC-RIA chromatograms of urine sample (56) using the RTI procedure for COOH-THC.*

HPLC-EMIT. Significant immunoreactivity was observed in an early eluting fraction and in a fraction corresponding to COOH-THC.

Figures 7 and 8 illustrate the HPLC-RIA profiles obtained using the RTI-RIA procedure for Δ^9-THC. No immunoreactivity was observed at the retention volume corresponding to Δ^9-THC (74 mL); however, peaks of immunoreactivity were observed between 6 and 18 mL (Fractions 1 to 7) and 58 to 62 mL (Fractions 27 and 28) of eluent.

Although the urine samples illustrated in Figs. 2 to 8 contained appreciable concentrations of COOH-THC, similar profiles have been obtained from a urine sample containing 37 ng/mL of "free" and 46 ng/mL of "total" COOH-THC.

Discussion

The various immunoassay procedures used in this study have differing cross-reactivities to COOH-THC and other urinary metabolites of Δ^9-THC, depending upon the antisera used. These are shown in Table 2 for the EMIT and Roche antisera. The EMIT assay uses an antisera derived from an antigen based on 11-nor-9-oxo-hexahydrocannabinol coupled via its 9-oxime. The Roche antigen is conjugated to 11-nor-Δ^8-carboxy-Δ^9-tetrahydrocannabinol. The RTI-RIA for COOH-THC is the only one based on an antigen in which a free 9-carboxyl group is present in the cannabinoid molecule. COOH-THC is coupled to the car-

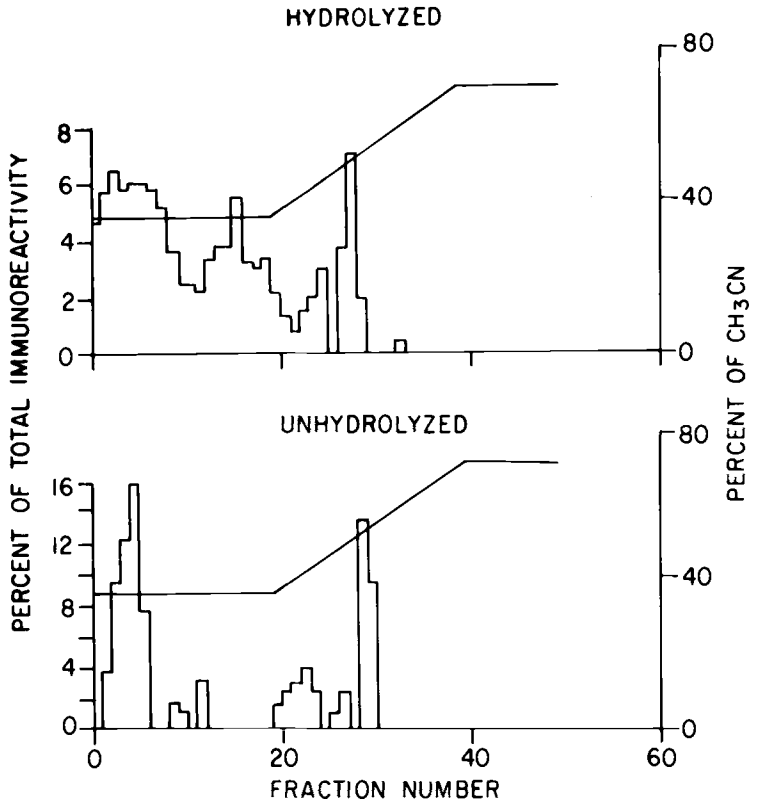

FIG. 3—*HPLC-RIA chromatograms of urine sample (117) using the RTI procedure for COOH-THC.*

TABLE 1—*Percentages of immunoreactivity detected in various fractions by the RTI-RIA procedure for COOH-THC.*

	Fraction Number	% of Immunoreactivity	Urine Concentration of COOH-THC by GC-MS, ng/mL
		UNHYDROLYZED	
Sample 117	1 to 6	50.8	204
	27 to 29	23.4	
Sample 56	1 to 6	24.8	263
	8 to 16	54.1	
	27 to 29	17.3	
		HYDROLYZED	
Sample 117	1 to 6	30.2	295
	14 and 15	11.7	
	27 to 29	8.6	
Sample 56	1 to 6	23.6	396
	14 and 15	8.4	
	27 to 29	15.4	

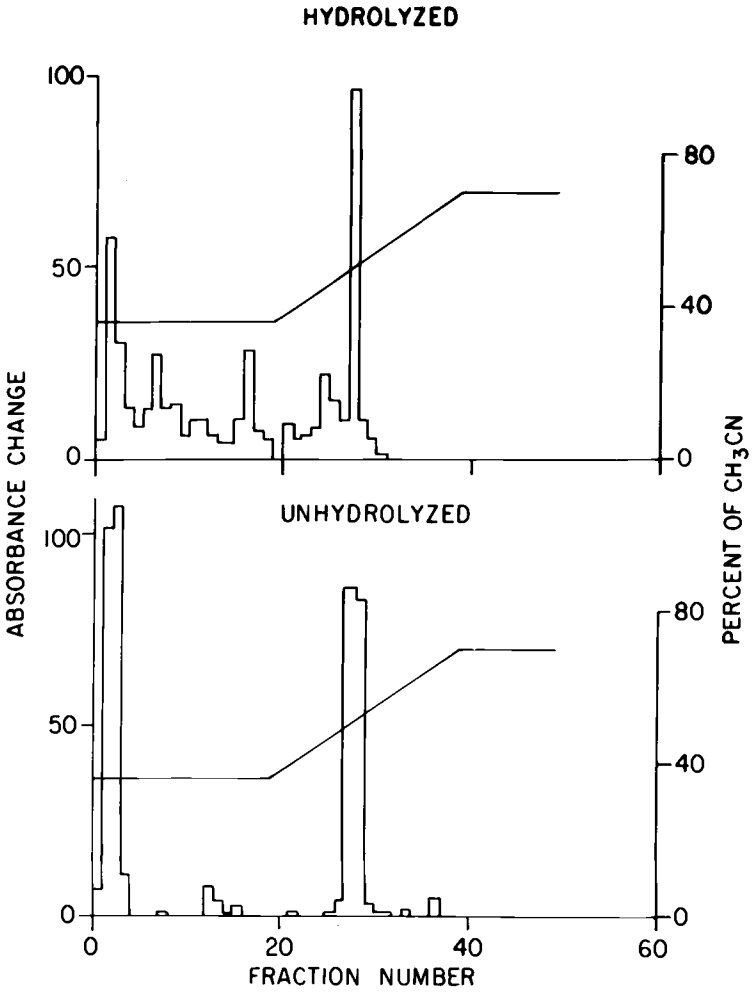

FIG. 4—*HPLC-EMIT chromatograms of urine sample (56).*

rier via the 5′-methyl position. Thus, the RTI antisera would be expected to have greater affinity for the free acid than its glucuronide ester.

With the RTI-RIA procedure for COOH-THC the percentages of total immunoreactivity in the fraction corresponding to COOH-THC did not increase dramatically after alkaline hydrolysis (Table 1), a treatment that might be expected to increase the amount of COOH-THC. Williams et al [*19*] have previously shown that COOH-THC-glucuronide hydrolysis with a 50% aqueous methanolic solution of sodium hydroxide results in the formation of a methyl ester of COOH-THC. Under the HPLC conditions used in this study COOH-THC-glucuronide would elute in the first fraction, (6 to 16 mL). It might be expected that the methyl ester of COOH-THC would have a retention volume beyond COOH-THC. In fact, after alkaline hydrolysis, immunoreactivity occurs between 32 and 38 mL of eluent using the RTI-RIA procedure for COOH-THC. Although this peak was present in the HPLC-EMIT profile, it was less than 10% of total immunoreactivity and was absent from the HPLC-RIA profile obtained using the Roche antisera.

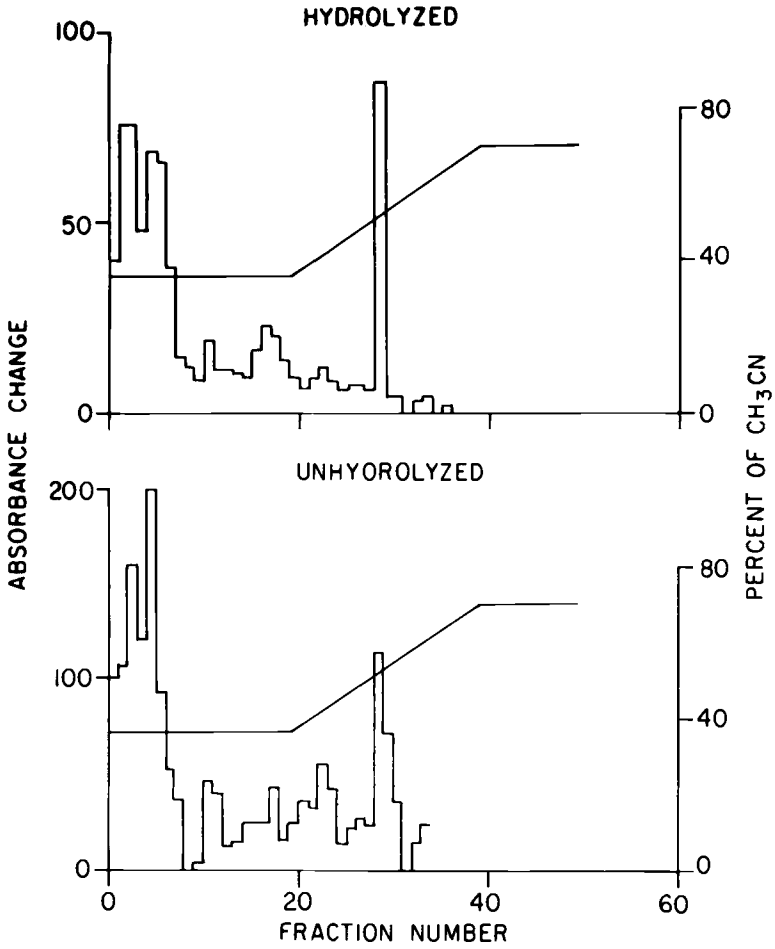

FIG. 5—*HPLC-EMIT chromatograms of urine sample (117).*

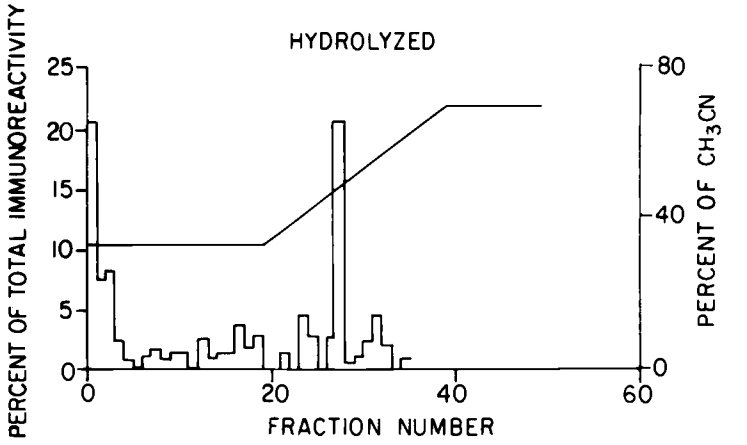

FIG. 6—*HPLC-RIA chromatogram of a hydrolyzed urine sample (56) using the Abuscreen RIA procedure.*

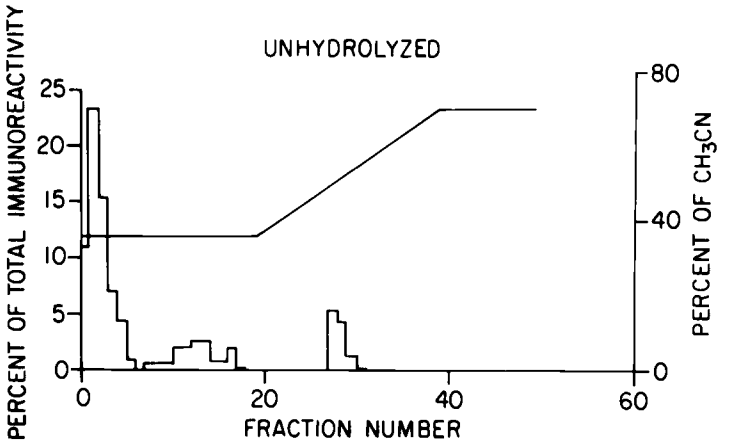

FIG. 7—*HPLC-RIA chromatogram of an unhydrolyzed urine sample (56) using the RTI procedure for* Δ^9*-THC.*

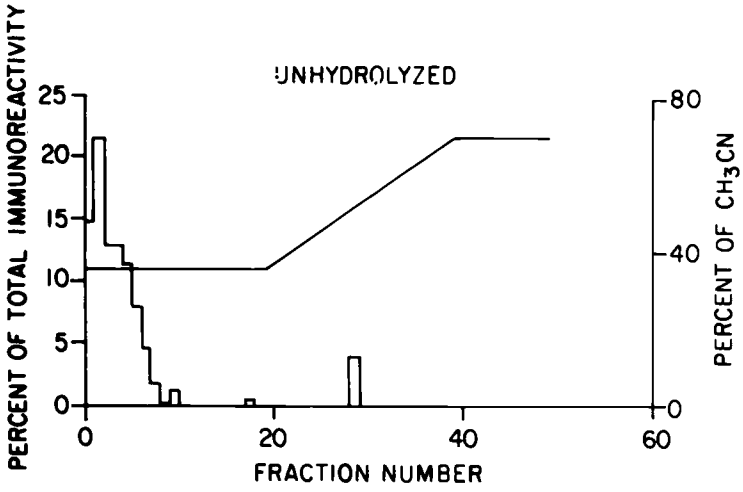

FIG. 8—*HPLC-RIA chromatogram of an unhydrolyzed urine sample (117) using the RTI procedure for* Δ^9*-THC.*

TABLE 2—*Cross-reactivities (as percentage of response to* Δ^9*-COOH-THC) of the EMIT and Roche antisera.*

	EMIT	Roche Abuscreen
Δ^9-COOH-THC	100	100
Δ^8-COOH-THC	89	143
Δ^9-THC	30	42
11-OH-THC	105	80
8-α-OH-THC	...	89
8-β-11-$(OH)_2$-THC	53	20

The HPLC profiles obtained using the EMIT and Roche antisera were similar. Before hydrolysis there were peaks of immunoreactivity at retention volumes corresponding to COOH-THC and COOH-THC-glucuronide. Presumably, the latter peak also contained conjugates of other cannabinoid metabolites, as significant immunoreactivity was still present after alkaline hydrolysis. Similar HPLC-EMIT profiles were reported by Law et al [*22*]. It might be expected that both the EMIT and Roche antisera would cross-react with all 9-substituted metabolites, some of which may not be hydrolyzed by methanolic sodium hydroxide. On the other hand, this early eluting peak of immunoreactivity was reduced after hydrolysis in the HPLC-RIA profile obtained using the RTI-RIA procedure for COOH-THC.

Surprisingly, use of the RTI-RIA procedure for Δ^9-THC resulted in immunoreactivity at elution volumes corresponding to COOH-THC and metabolite conjugates. COOH-THC has been reported [*18*] to cross-react to a very small extent ($<0.1\%$) to this antiserum. The early eluting peak of immunoreactivity is presumably due to conjugates of Δ^9-THC metabolites in which the antibody recognition site has not been altered. A number of mono and dicarboxylic acids have recently been identified in human urine following oral administration of marihuana [*26,27*]. These, both unconjugated and conjugated, could account for the unexplained immunoreactivity seen in this study.

Summary

This study has demonstrated that the HPLC-IA profiles of cannabinoid metabolites pre- and post-alkaline hydrolysis depend upon the specificity of the antisera used. These distinct profiles could be used to confirm presumptive positive IA results. Certainly fractionation, collection of the eluent corresponding to the retention volume of COOH-THC, and reanalysis by IA is a possible procedure for confirmation of presumptive positive urine screening results.

Acknowledgments

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