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Identification of Rat Urinary and Fecal Metabolites of a New Herbicide, Pyribenzoxim

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Rats were treated with pyribenzoxim (*O*-[2,6-bis[(4,6-dimethoxy-2-pyrimidinyl)oxy]benzoyl]oxime), a new herbicide, to investigate the related metabolites in urine and feces. Metabolites were identified using LC/MS (electrospray ionization) and GC/MS (electron impact ionization) following the relatively simple and rapid extraction and purification procedures. Three metabolites were identified in urine either from oral gavage or intravenous (iv) injection. They were benzophenone oxime (BO), benzophenone oxime glucuronide (BOG), and 2-hydroxy-6-(4,6-dimethoxypyrimidin-2-yloxy)benzoic acid (HDB). Benzophenone oxime was present in larger quantity than BOG and HDB in urine from oral treatment, while the case was opposite in urine from iv treatment. Glucuronide conjugate was confirmed unambiguously by enzyme hydrolysis. 2,6-Bis(4,6-dimethoxypyrimidin-2-yloxy)benzoic acid (KIH-2023) and benzophenone were identified in feces. Benzophenone was confirmed by GC/MS and HPLC/DAD since LC/MS could not produce an ESI spectrum. On the basis of the results obtained, a metabolic map of pyribenzoxim is proposed.

KEYWORDS: Pyribenzoxim; herbicide; LC/MS; GC/MS; metabolites; rat

INTRODUCTION

Pyribenzoxim is a new postemergence herbicide providing broad-spectrum weed control in rice fields (*1*, *2*). Similar to sulfonylurea herbicides, this compound was known to inhibit acetolactate synthase (ALS), the enzyme involved in the biosynthesis of the branched-chain amino acids in plants (*3*, *4*). The oral acute LD_{50}(rat) was >5000 mg/kg and the dermal LD_{50} was >2000 mg/kg, indicating low mammalian toxicity (*5*); however, only limited information has been available on the environmental fate and metabolism of pyribenzoxim. A in vitro metabolism study using rat liver microsomes identified four metabolites, KIH-2023 (2,6-bis(4,6-dimethoxypyrimidin-2-yloxy)benzoic acid), benzophenone oxime, and monohydroxy- and dihydroxypyribenzoxim, using LC/MS/MS (*6*).

In addition to metabolism studies with isolated enzyme systems, in vivo studies with rats can provide specific and practical details of the chemical identity of metabolites, the pattern of their formation, and the metabolic pathway of pesticides. Furthermore, owing to the well-established techniques of LC/MS and MS/MS in the field of pesticide metabolism (*7*), problems of identifying the structure of metabolites, which are usually polar, have greatly been simplified (*6*, *8*−*11*). LC/MS can now be considered as a routine technique in pharmaceutical and agrochemical research with numerous applications in the identification of drug and xenobiotic metabolites from in vivo or in vitro studies (*12*).

In the present study, pyribenzoxim was administrated to rats so that sufficient quantities of metabolites could be formed for characterization purposes. The metabolites were identified by electrospray ionization liquid chromatography/mass spectrometry (ESI-LC/MS) and gas chromatography/mass spectrometry (GC/MS) with authentic compounds available.

MATERIALS AND METHODS

Chemicals. Pyribenzoxim (purity 98.3%) and KIH-2023 (purity 98.0%) were kindly provided by LG Life Sciences Ltd. Polyoxyethylene sorbitan monooleate (Tween-80), dimethylformamide, and benzophenone were purchased from Aldrich Chemical Co. (Milwaukee, WI). Benzophenone oxime (BO) was prepared according to the method reported previously (*6*). β-Glucuronidase (*Escherichia coli* type VIII-A) was obtained from Sigma Chemical Co. (St. Louis, MO). All solvents used for HPLC analysis were glass distilled (Duksan, Korea). All the other reagents and common chemicals were reagent grade or higher.

Animal Acclimatization and Chemical Administration. Five week old male Sprague−Dawley (SD) rats were purchased from SamTako Bio Korea (Osan, Korea). After arrival, the animals were acclimatized

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to the laboratory environment for at least one week. One day before the experiment started, the animals were separated and individually kept in a closed metabolism cage made entirely with glass and designed to collect urine and feces separately.

After suspension in 0.5% Tween-80 solution, pyribenzoxim was given by oral gavage to the male rat at a dose of 1000 (mg/10 mL)/kg. For the intravenous (iv) administration, pyribenzoxim was dissolved in dimethylformamide and given to male rats via the tail vein at a dose of 100 (mg/mL)/kg. After administration of pyribenzoxim, the rats were individually housed in the cages. The urine and feces were collected separately for 24 h. Under the experimental conditions of this study, there were no observable toxicological effects in the pyribenzoxim-treated animals.

Instrumentation. *HPLC Systems.* HPLC was performed using an HP 1100 system (Hewlett-Packard, CA) with an HP Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm, Hewlett-Packard). For an analytical scale, the mobile phase consisted of water−acetonitrile containing 0.1% acetic acid. A one-step linear gradient [solvent A = water/acetic acid (999/1, v/v), solvent B = acetonitrile/acetic acid (999/1, v/v); for the analyses of feces samples, 50% A, 50% B at 0 min, 20% A, 80% B at 20 min; for the analyses of urine samples, 80% A, 20% B at 0 min, 20% A, 80% B at 20 min] was employed for >30 min with a flow rate of 1.0 mL/min. UV detection (244 nm) was performed with a variable-wavelength detector (HP 1100 series). The identity of the peaks was confirmed by cochromatography with reference compounds available.

LC/MS Systems. LC/MS was carried out by coupling an HP 1100 system to a Quattro LC triple-quadruple tandem mass spectrometer (Micromass, Manchester, U.K.) with electrospray ionization (ESI^+ mode). The source temperature, desolvation temperature, cone voltage, and capillary voltage were kept at 120 °C, 200 °C, 28 V, and 3.29 kV, respectively. An electron multiplier voltage of 640 V was used. The nebulizer gas and desolvation gas were ultrapure nitrogen set at 80 and 397 L/h, respectively. Mass spectrometry/mass spectrometry (MS/MS) was carried out using nitrogen as the collision gas. The collision energy was kept at 65.7 eV. The cone voltage and capillary voltage were adjusted to 25 V and 3.57 kV, respectively.

GC/MS Systems. The GC/MS analysis was carried out on a Shimadzu 17A GC instrument connected to a QP-5000 mass spectrometer (Shimadzu, Tokyo, Japan) with electron impact ionization (EI mode, 70 eV). The column was an Ultra 2 (30 m × 0.25 mm i.d., film thickness 0.25 μm, Hewlett-Packard, CA), and the oven temperature was raised from 150 to 230 °C at a rate of 10 °C/min with a 2 min hold at 150 °C and 5 min hold at 230 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The temperatures of the injection port, ion source, and interface were 240, 200, and 270 °C, respectively.

Identification of Urinary Metabolite M1 (BO). Urine samples were collected from rats dosed with pyribenzoxim via oral gavage. A Waters Sep-Pak Vac C18 cartridge (1 g, 6 cm^3, Millipore, Milford, MA) was conditioned with methanol (15 mL) followed by water (15 mL). Urine (5 mL) was loaded onto the conditioned cartridge and sequentially washed with 5 mL aliquots of varying percentages of methanol in water (10%, 20%, 40%, 60%, 80%, 100% methanol). Each fraction was analyzed by HPLC to check the presence of metabolites, and the fractions containing the appropriate metabolites were pooled and repurified on a C_{18} column (Waters μBondapak, 3.9 × 300 mm, 10 μm) using a mobile phase consisting of water−acetonitrile containing 0.1% acetic acid. A one-step linear gradient [solvent A = water/acetic acid (999/1, v/v), solvent B = acetonitrile/acetic acid (999/1, v/v); 100% A, at 0 min, 20% A, 80% B at 20 min] was employed for >30 min with a flow rate of 1.0 mL/min. The HPLC eluent was monitored at 244 nm. Major metabolite M1, eluting at 16.8 min, was collected from several injections, pooled, dried, and analyzed by LC/MS.

Identification of Urinary Metabolites M2 (Benzophenone Oxime Glucuronide, BOG) and M3 (2-Hydroxy-6-(4,6-dimethoxypyrimidin-2-yloxy)benzoic acid, HDB). Urine (10 mL) from iv treatment of pyribenzoxim was applied to an XAD-2 column (2.5 × 5.5 cm) and washed with water (100 mL) and then methanol (8 mL). Urinary metabolites were eluted with methanol (20 mL). The methanol eluate was concentrated to near dryness, brought up to 5 mL with water, and purified with SPE as described above, and each fraction was analyzed by HPLC for the presence of metabolites. The fractions containing

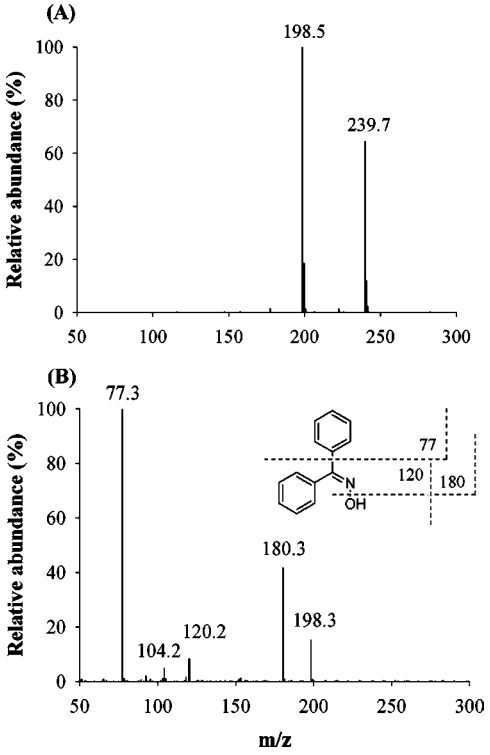

Figure 1. LC/MS (**A**) and MS/MS (**B**) spectra of BO (M1) from rat urine.

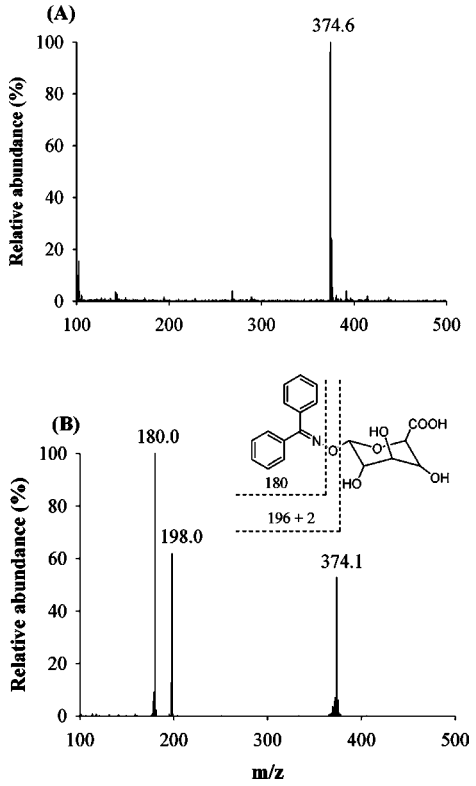

Figure 2. LC/MS (**A**) and MS/MS (**B**) spectra of BOG (M2) from rat urine.

metabolites M2 (7.9 min) and M3 (10.2 min) were pooled, dried, and analyzed by LC/MS.

Identification of Fecal Metabolites M4 (KIH-2023) and M5 (Benzophenone). Feces samples (5 g) from the rats treated with pyribenzoxim via oral gavage were homogenized in distilled water (5 mL), and aliquots (1 g) of fecal homogenate were extracted twice with methanol (5 mL) by sonication for 1 h. The methanol extract was centrifuged (4000*g*, 30 min), and the supernatant was evaporated at 50 °C under a nitrogen stream. The residue was dissolved in methanol (1

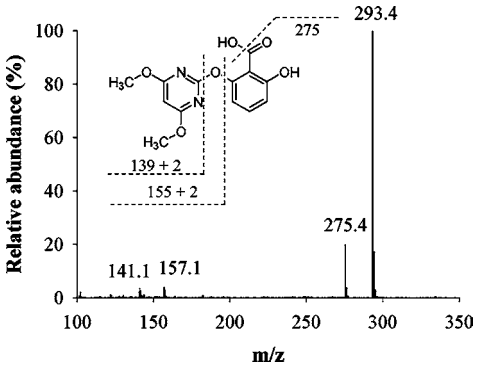

Figure 3. LC/MS spectrum of HDB (M3, $C_{13}H_{12}N_2O_6$) from rat urine.

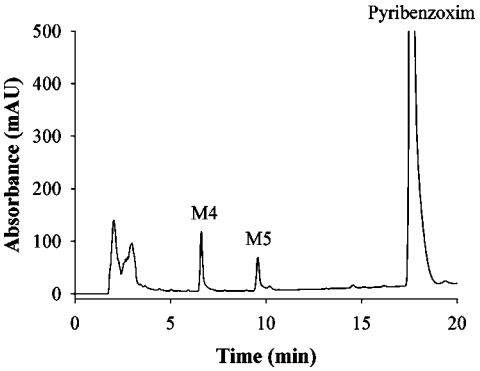

Figure 4. HPLC chromatograms of the methanol extract of rat feces.

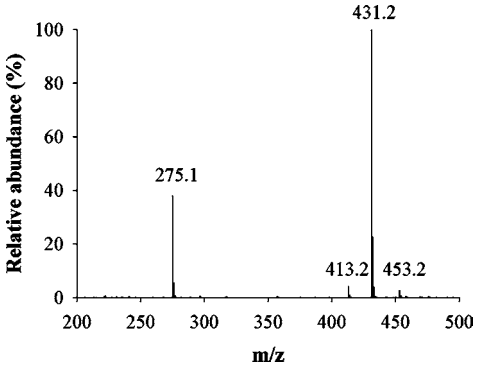

Figure 5. LC/MS spectrum of KIH-2023 (M4, $C_{19}H_{18}N_4O_8$) from rat feces.

mL) and purified by HPLC with an analytical C_{18} column (Waters μBondapak, 3.9 × 300 mm, 10 μm) using a mobile phase consisting of water−acetonitrile containing 0.1% acetic acid. A two-step linear gradient [solvent A = water/acetic acid (999/1, v/v), solvent B = acetonitrile/acetic acid (999/1, v/v); 70% A, 30% B at 0 min, 55% A, 45% B at 10 min, 20% A, 80% B at 20 min] was employed for >30 min with a flow rate of 1.0 mL/min. The HPLC eluent was monitored at 244 nm. Metabolites M4 and M5 and pyribenzoxim, eluting at 6.9, 7.9, and 16.5 min, respectively, were collected from several injections, pooled, dried, and analyzed by LC/MS and GC/MS.

Enzyme Hydrolysis. Aliquots (100 μL) of urine extract from the rats treated with pyribenzoxim via iv were adjusted to pH 6.8 using 1 M hydrochloric acid. After β-glucuronidase (100 unit) was added, the samples were incubated at 37 °C overnight (~18 h) in a shaking water bath. Control samples of pH-adjusted urine extract were also incubated. All samples were analyzed by HPLC with UV detection as described above.

RESULTS AND DISCUSSION

Purification of Metabolites in Urine and Feces. M1 (BO), M2 (BOG), and M3 (HDB) were present as a metabolite in urine from the rat treated via oral gavage. M1 was purified successfully from urine using a Sep-Pack C18 cartridge and HPLC; however, it was difficult to purify M2 and M3 from this sample due to their low quantity. In the case of the iv administration of pyribenzoxim, M2 and M3 in urine were purified using Amberlite XAD-2 and a Sep-Pak C18 cartridge. In feces samples via oral gavage, M4 (KIH-2023), M5 (benzophenone), and pyribenzoxim were purified using solvent extraction and HPLC.

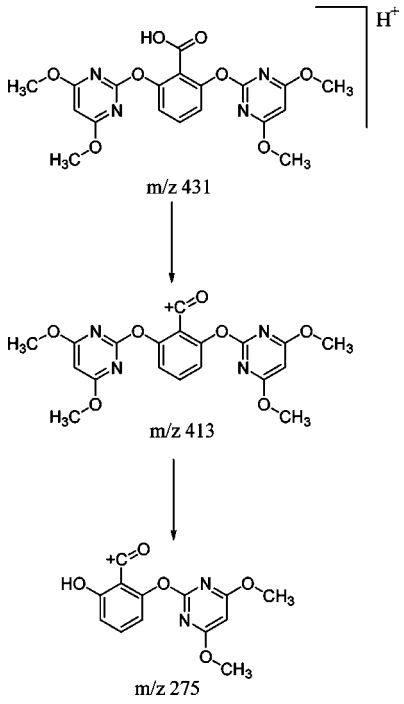

Figure 6. LC/MS fragmentation scheme for KIH-2023 (M4).

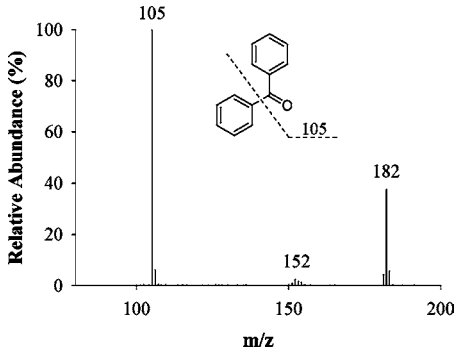

Figure 7. GC/MS spectrum of benzophenone (M5) from rat feces.

Metabolites in Urine. No pyribenzoxim was observed in urine either from oral gavage or from iv treatment. M1 was identified as BO from the LC/MS and MS/MS analysis with authentic compounds. The mass spectrum of BO has an intense $[M + CH_3CN + H]^+$ ion at *m*/*z* 239 and a base peak at *m*/*z* 198 which arises from the MH^+ ion (**Figure 1**). The MS/MS spectrum of BO (**Figure 1**) by fragmenting *m*/*z* 198 through collision gave the characteristic daughter ions of benzophenone oxime at *m*/*z* 180, 120, and 77.

M2 gave an MH^+ molecular ion at *m*/*z* 374. With low cone voltage (10 V), only the MH^+ ion was detected to confirm it was MH^+ (**Figure 2**). M2 was confirmed as BOG by the diagnostic loss of the glucuronyl moiety (176 amu) (*13*) to form the aglycon product ion at *m*/*z* 198, which is a benzophenone oxime (**Figure 2**). Another characteristic fragment included an ion at *m*/*z* 180. To confirm the glucuronide conjugate, the urine extracts containing BOG were treated with β-glucuronidase and

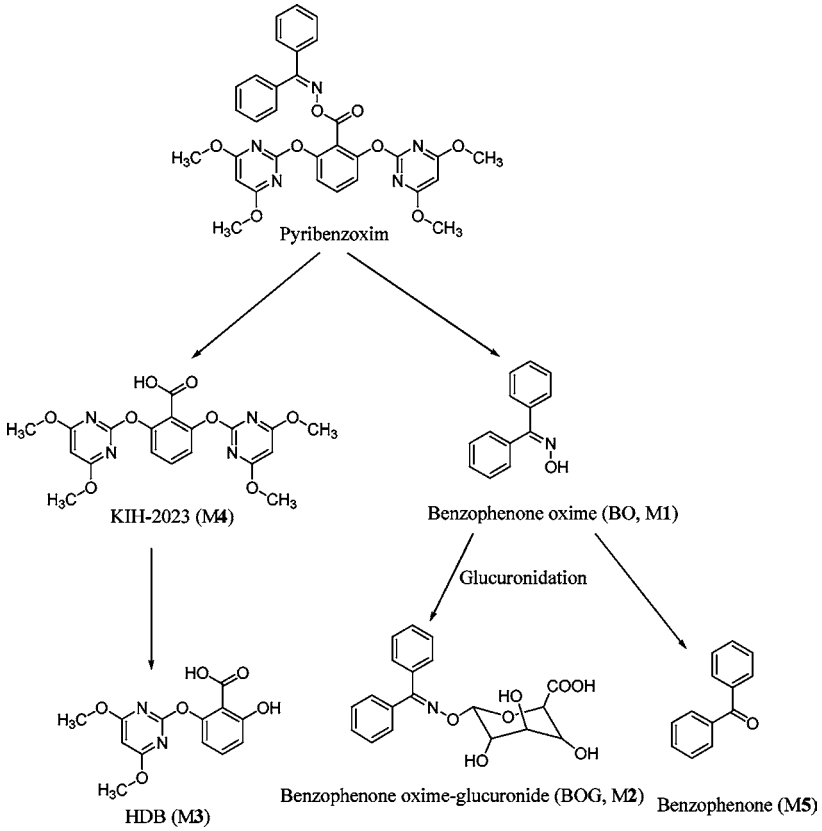

Figure 8. Proposed metabolic pathways for pyribenzoxim in rats.

BOG was converted almost entirely to a benzophenone oxime, implying M2 was the benzophenone oxime glucuronide conjugate.

M3 was identified as HDB, giving MH^+ at *m*/*z* 293 (**Figure 3**). This compound apparently formed by loss of a pyrimidine moiety of KIH-2023 as reported in the study of the metabolism of KIH-2023 in rats (*14*).

Metabolites in Feces. The HPLC chromatograms of the feces extracts show two metabolites (M4, M5) in addition to nonmetabolized pyribenzoxim, which is present in a major portion (**Figure 4**). From the LC/MS analysis with authentic compounds, M4 was identified as KIH-2023 on the basis of the MH^+ at *m*/*z* 431 (**Figure 5**). A weak $[M + Na]^+$ ion at *m*/*z* 453 confirmed the MH^+ ion, and other characteristic fragments ions at *m*/*z* 413 and 275 also supported the correct structure (**Figure 6**). This metabolite was also observed earlier from in vitro metabolism of pyribenzoxim using rat liver microsomes (*6*).

For M5, although the peak was well separated in the HPLC chromatogram (**Figure 4**), a proper spectrum was not obtained in the LC/MS analysis, suggesting that ESI was not a proper technique for the compound to form a positive ion. This phenomenon was confirmed in a separate experiment in which even 5 μg of the benzophenone standard hardly produced a good spectrum compared to the other compounds in this study.

By HPLC/DAD analysis, instead, it was identified as benzophenone by comparing the retention time and UV spectrum with those of authentic compounds. The result was confirmed again by GC/MS analysis (**Figure 7**), obtaining an intense M^+ ion at *m*/*z* 182 and a base peak at *m*/*z* 105, which arises from the loss of phenyl from it.

Metabolism of Pyribenzoxim by Rats. As expected, the in vivo metabolism of pyribenzoxim was somewhat different from the in vitro metabolism by rat liver microsomes. KIH-2023 and benzophenone oxime were also observed in in vitro studies (*6*); however, glucuronide, HDB, and benzophenone were detected only in in vivo metabolism, suggesting that the main metabolic reactions for pyribenzoxim absorbed in the body may be enzymatic hydrolysis and conjugation.

In general, foreign compounds having a high molecular weight ($\geq 325 \pm 50$) were excreted in bile (*15*−*18*), rationalizing that pyribenzoxim (MW = 609) and KIH-2023 (MW = 430) were excreted in feces. Especially pyribenzoxim was expected to exhibit the preferential biliary excretion, when compared with renal excretion into the urine, due to the molecular weight cutoffs in the proximal tubule of the kidney (*17*). It was reported that 69.0% of orally treated [^{14}C]pyribenzoxim was excreted in feces 24 h after treatment (*19*). From the result of the present study (**Figure 4**), it could be concluded that most of the radioactivity of feces was due to unchanged pyribenzoxim. Benzophenone oxime glucuronide was excreted not in feces but in urine. This result was supported by a number of investigations which reported that glucuronides were hydrolyzed by intestinal microorganisms of mammals and caused enterohepatic circulation (*15*, *20*−*22*) to be excreted in urine.

Conclusion. Pyribenzoxim, in rats, was metabolized via hydrolysis to give BO (M1) and KIH-2023 (M4), which in turn was hydrolyzed to HDB (M3). BO was conjugated to form glucuronide BOG (M2) or further oxidized to benzophenone (M5). BO, BOG, and HDB were observed in urine, and KIH-2023 and benzophenone were observed in feces. Possible metabolic pathways of pyribenzoxim in the rat are proposed in **Figure 8** on the basis of the urinary and fecal metabolites found in this study.

LC/MS and GC/MS were successfully employed for the identification of rat urinary and fecal metabolites, connected with careful adaptation or modification of extraction and purification methods. The MS/MS technique proved to be an excellent and powerful supplementary tool for confirming the

identity of metabolites present in biological samples such as urine and feces.

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