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Note

High-performance liquid chromatography of metribuzin and non-polar metabolites extracted from leaf tissues

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Metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4- triazin-5(4H)-one] (Fig. 1) is an asymmetric triazinone herbicide which effectively controls certain grass and broadleaf weeds infesting soy bean, tomato and other crops. The rate of metabolism of metribuzin to less phytotoxic compounds is thought to determine the relative tolerance of different crop cultivars to metribuzin[1–4]. Frear *et al.*[5] have identified two polar metabolites, the β-D-(N-glucoside) conjugate and the malonyl β-D-(N-glucoside) conjugate, as the initial and major degradation products in tomato.

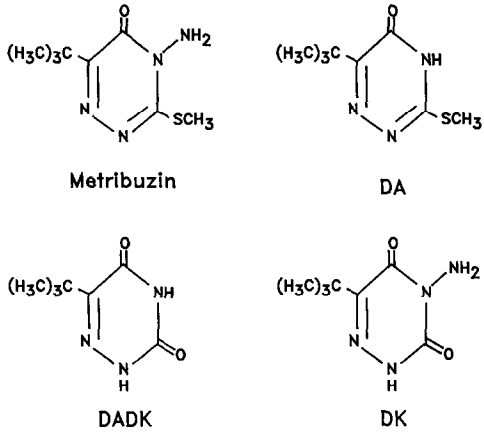

Fig. 1. Structure of metribuzin and non-polar metabolites.

In contrast, the major pathway of metabolism of metribuzin in soy bean involved an initial oxidation of the methylthio group to yield a reactive sulfoxide intermediate[6]. This sulfoxide could then undergo peptide conjugation with homoglutathione or possibly degrade to non-polar metabolites, specifically the diketo (DK) and the deaminated diketo (DADK) forms of metribuzin. Small amounts of the deaminated (DA) form of metribuzin, DK and DADK (Fig. 1) were reported as plant metabolites in sugar-cane[7], potato[8] and several weed species[9,10]. These non-polar

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products were also formed as degradation products in the soil[11] and by photochemical reaction on surfaces and in solution[12,13].

Metribuzin and DA, DADK and DK metabolites have previously been resolved and quantified by thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) procedures[1,3,4,14–16].Some GLC analyses utilizing electron-capture detectors[14,16] required extensive sample cleanup on liquid chromatography columns, and also that metribuzin be separated from non-polar metabolites and analyzed independently[16]. The use of flame photometric detection in GLC can eliminate interferences present in non-purified extracts[17]. Jarczyk[18,19] has described detailed GLC determinations of metribuzin and metabolite residues using a nitrogen-specific alkali flame ionization detector.

Parker *et al.*[20] developed an alternative technique based on an isocratic HPLC system for the resolution of metribuzin, DK, DADK and DA on a C_{18} column with detection by absorbance at 254 or 280 nm. Quantitation limits by direct peak integration ideally were 1–2 ng for standard mixtures of metribuzin and each of the non-polar metabolites. These limits of detection were not achievable in extracts from biological material because of interferences from naturally occurring components that could not be separated satisfactorily from the compounds of interest.

In this report, we describe an HPLC mobile phase providing increased resolution of metribuzin, DK, DADK and DA on a C_{18} column. We have also developed a new procedure for the rapid removal of interfering compounds extracted from leaf tissues together with the herbicide and non-polar metabolites. This routinely allowed quantitative detection limits as low as 0.2 ng. Radioactivity in ^{14}C-labelled non-polar metabolites and the parent compound can be determined simultaneously by coupling UV detection with an in-line radioactivity monitor.

EXPERIMENTAL

Standards and solvents

Standards of metribuzin and the metabolites DA, DADK and DK were provided by Mobay (Kansas City, MO, U.S.A.). Stated purities for these compounds were 97.3, 99.9, 92.9 and 99.1%, respectively. [5(ring)-^{14}C]Metribuzin (20.8 Bq/mmol) (96.3% purity) was also supplied by Mobay. All organic solvents used were HPLC-grade (Fisher Scientific, Fairlawn, NJ, U.S.A.). Water used in HPLC analyses was purified through NANOpure II and ORGANICpure units (Barnstead, Sybron Corp., Boston, MA, U.S.A.). All HPLC solvents utilized were filtered through 0.5-μm Millipore filters (Millipore, Bedford, MA, U.S.A.).

Plant material and ^{14}C-incubation

Sweet potato (*Ipomoea batatas*) and soy bean (*Glycine max*) plants were grown in greenhouse conditions in a potting soil consisting of a 2:1 (v/v) mixture of composted potting soil and sand. Leaf tissue was harvested from the apical portion of the growing stem and used in the extraction and ^{14}C-incubation experiments.

Sweet potato stems with attached leaves were excised and transferred to 125-ml erlenmeyer flasks containing 100 ml of tap water for the ^{14}C-incubation experiments. Roots had developed on the cuttings after incubation for 3 days in an 18 h photoperiod (200 μEinsteins m^{-2} s^{-1}) at 26°C wih a 17°C dark period. The isolated cuttings were

then incubated for 48 h in flasks containing 100 ml of $5 \cdot 10^{-6}$ *M* metribuzin containing 0.5 μCi [^{14}C]metribuzin (20.8 Bq/mmol). At the end of the incubation period, cuttings were rinsed with running water and placed in 100 ml of $5 \cdot 10^{-6}$ *M* metribuzin for an additional 72 h.

Sample preparation

Leaf tissue (2.5 g fresh weight) was homogenized with 20 ml of methanol–water (8:2, v/v) using a Polytron. Spiked samples were prepared by adding 2.5 μg each of metribuzin, DA, DADK and DK, dissolved in methanol to the homogenates. An equivalent amount of methanol was added to blank samples. Homogenates were vaccum filtered, and then plant residue on the filter was rinsed with 20 ml of methanol–water (8:2, v/v) followed by 10 ml of methanol. Combined filtrates were evaporated *in vacuo*. The sample was dispersed in 25 ml of chloroform and partitioned with 20 ml of 0.1 *M* potassium chloride. The chloroform layer was recovered and the aqueous phase re-partitioned twice with chloroform. Combined chloroform fractions were dried *in vacuo* at 35°C and the residue re-dissolved in 200–500 μl of diethyl ether. This re-dissolved residue was then applied to a C_{18} Sep-Pak cartridge (Waters Assoc., Milford, MA, U.S.A.). Diethyl ether was evaporated by passing nitrogen gas through the Sep-Pak. Metribuzin and non-polar metabolites were then eluted with 20 ml of acetonitrile–water (50:50, v/v). The eluate was dried *in vacuo* and the residue next solubilized in chloroform and applied to a silica Sep-Pak cartridge (Waters Assoc.). Metribuzin and non-polar metabolites were eluted from the Sep-Pak cartridge with 20 ml of chloroform. The final extracts were dried and then re-dissolved in the mobile phase prior to HPLC analysis.

HPLC analyses

HPLC analyses were conducted utilizing an ACS Model 351 solvent delivery system (Applied Chromatography Systems, Luton, U.K.), a Rheodyne Model 7125 syringe-loading sample injector with a 50-μl loop and a 25 cm × 4.6 mm I.D. Beckman Ultrasphere (5 μm) reversed-phase C_{18} column. The mobile phase was 0.05 *M* acetic acid–acetonitrile–methanol (67:28:5, v/v/v) delivered at a flow-rate of 0.9 ml min^{-1}. Samples for injection (50 μl) were taken from final extracts dissolved in this mobile phase. Compounds were detected at 280 nm with a Linear UV1S 203 variable wavelength detector, and elution profiles were recorded and integrated by a HP 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). After injection of 20 samples, the column was cleaned with tetrahydrofuran at a flow-rate of 1 ml/min for 2 h to remove strongly retained contaminants. Radioactivity was determined after the peaks were first detected at 280 nm and then eluted through a Flo-One Beta (Model IC) HPLC radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.) with Flo-Scint III (Radiomatic Instruments and Chemical Co.) as scintillator mixed with the HPLC eluate by means of the Flo-One component pump operating at 4.5 ml/min. Under these conditions, the counting efficiency for ^{14}C was approximately 80%.

TLC separation of metribuzin and metabolites

Silica gel HF plates (Analabs, Norwalk, CT, U.S.A.) were used for TLC of [^{14}C]metribuzin, DA, DADK and DK. Tissue extracts for TLC were first purified

through C_{18} and silica Sep-Paks, then re-dissolved in ethanol and applied to plates, developed in benzene–chloroform–*p*-dioxane (4:3:3, v/v/v)[1]. Metribuzin, DA, DADK and DK standards were used for co-chromatography and detection by UV light. The resolved compounds were scraped from the TLC plate and extracted from the silica with 50% ethanol and counted for radioactivity using a Beckman LS 1801 liquid scintillation counter.

RESULTS AND DISCUSSION

Standards of metribuzin and the non-polar metabolites, DK, DADK and DA, were optimally resolved by reversed-phase HPLC separation utilizing 0.05 *M* acetic acid–acetonitrile–methanol (67:28:5, v/v/v) as the mobile phase (Fig. 2). Reproducible quantitation by peak integration at 280 nm was recorded between 0.2 ng and 100 μg of the standards. Parker *et al.*[20] used reversed-phase separation with a mobile phase consisting of methanol–0.05 *M* acetic acid (62:38, v/v), delivered at 1 ml/min, to separate these same compounds with detection limits of 1–2 ng at 280 nm.

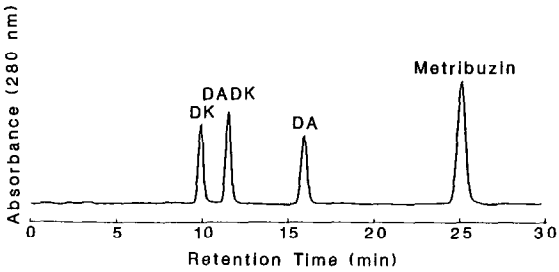

Fig. 2. Reversed-phase HPLC separation of metribuzin and non-polar metabolite standards. Column: Beckman Ultrasphere (5 μm, 250 mm × 4.6 mm). Mobile phase: 0.05 *M* acetic acid–acetonitrile–methanol (67:28:5, v/v/v), 0.9 ml/min; detection by UV absorbance (280 nm).

Chromatograms of samples from sweet potato leaf extracts which were spiked with a mixture of metribuzin and non-polar metabolite standards (2.5 μg per 2.5 g tissue) before or after filtration through C_{18} and silica Sep-Paks are compared in Fig. 3A and B, respectively. The peaks defined correspond to approximately 25 ng each of metribuzin, DK, DADK and DA. Without Sep-Pak purification (Fig. 3A), unidentified components eluted immediately before or concurrently with the compounds of interest and background signals were elevated. Successive elution of the extracts through C_{18} and silica Sep-Pak with the solvents described, removed most of the interfering contaminants (Fig. 3B). The unidentified peaks remaining in samples filtered through Sep-Paks were also found in blank tissue extracts. They were sufficiently resolved from metribuzin, DK, DADK and DA to allow reproducible quantitation of metribuzin and the metabolites to a limit of 0.2 ng. An equivalent chromatogram of samples from soy bean leaf extracts purified by Sep-Pak filtration is shown in Fig. 4. The recoveries of metribuzin and each of the non-polar metabolites added to the tissue extracts of both sweet potato and soy bean were 92–94%.

The excellent HPLC resolution allowed the direct determination of [^{14}C]metri-

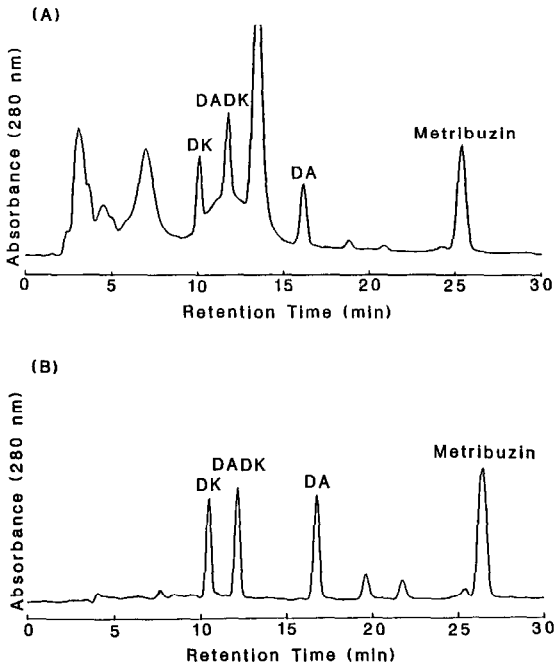

Fig. 3. Chromatograms of sweet potato leaf extracts spiked with metribuzin and non-polar metabolites and then injected (A) before or (B) after filteration through Sep-Paks. HPLC conditons. see Fig. 2.

buzin, DK, DADK and DA by liquid scintillation counting after they first eluted through the UV detector and then eluted through an in-line radioactivity detector. Flow-rates of HPLC mobile phase and scintillation fluid of 0.9 and 4.5 ml/min, respectively, were optimal for peak resolution and counting efficiency. Fig. 5 records a radiogram of [^{14}C]metribuzin and ^{14}C-metabolites resolved by the HPLC system. The sample mixture was extracted from sweet potato leaf tissue. The overall recovery of radioactivity from the HPLC column in a 30-min run time was 93–95%. The distribution of counts between the different compounds resolved was in close

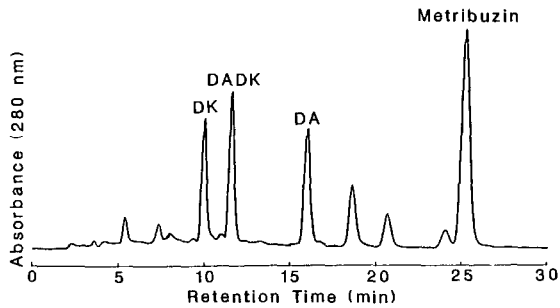

Fig. 4. Chromatograms of soy bean leaf extracts spiked with metribuzin and non-polar metabolites. HPLC conditions, see Fig. 2.

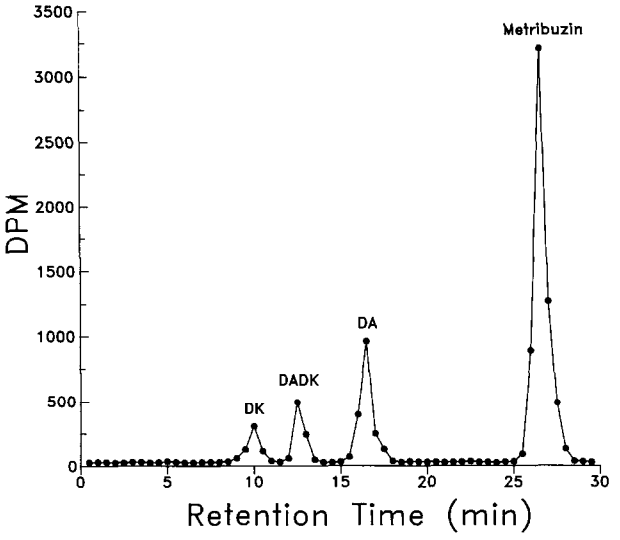

Fig. 5. Radiogram of ^{14}C-metribuzin and ^{14}C-metabolites extracted from sweet potato leaf tissue and resolved by HPLC. Conditions, see Fig. 2. Flow-rates: mobile phase, 0.9 ml/min, scintillation fluid, 4.5 ml/min.

agreement with values determined in aliquots of the same sample mixture separated by TLC. Utilization of HPLC for resolution avoids possible losses during collection of zones from TLC plates and allows simultaneous quantitation.

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