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Note

Quantitative determination of cantharidin in biological materials using capillary gas chromatography with flame ionization detection

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Cantharidin (Fig. 1), a terpenoid produced by blister beetles (*Coleoptera, Meloidae*) for defense against predators [1], is a potent vesicant and poison. Painful skin blisters develop after people accidentally coat their skin with droplets of cantharidin-laden blood discharged reflexively by disturbed blister beetles [2]. This problem is common in warm regions where many blister beetles aggregate on crops and flock to lights at night [3]. Fatalities in humans resulting from the ingestion of cantharidin are rare now that various formulations, most notably the aphrodisiac "Spanish fly" made from powdered blister beetles, have become outmoded [4]. By contrast, cantharidin-poisoning in

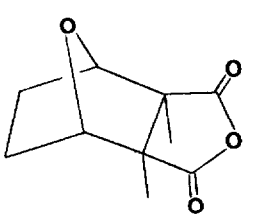

Fig. 1. Chemical structure of cantharidin.

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livestock, particularly horses, has grown recently because farm animals are increasingly being fed quickly baled leguminous hay contaminated with blister beetles that were trapped inadvertently while feeding on the foliage [5, 6].

Determination of cantharidin in biological materials is difficult. Recent high-performance liquid chromatographic (HPLC) techniques [7] can detect 1 ng of derivatized cantharidin, a sensitivity far better than blistering bioassays [8] and conventional gas chromatographic (GC) procedures [9]. But these HPLC methods are time-consuming; they require more than 20 h alone for preparative chromatography and derivatization after extraction of cantharidin. We here describe a quick capillary GC method that detects as little as picogram amounts of underivatized cantharidin in crude extracts of biological materials, including the tissues of blister beetles.

EXPERIMENTAL

Solvents, reagents and standards

Reagent-grade acetone was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Spectro-grade methylene chloride and chloroform and reagent-grade hydrochloric acid were from Fisher (Fair Lawn, NJ, U.S.A.). Reagent-grade sodium bicarbonate and sodium sulfate were from Baker (Phillipsburg, NJ, U.S.A.) and from Merck (Rahway, NJ, U.S.A.). Standard cantharidin was purchased from Inland Alkaloid (St. Louis, MO, U.S.A.). Standard benzophenone was from Aldrich (Milwaukee, WI, U.S.A.).

Apparatus

The gas chromatograph was a Varian Model 3700 equipped with a split/splitless injector and a flame ionization detector. Helium was used as the carrier gas at a flow-rate of approximately 1 ml/min, with a make-up helium gas flow-rate of 30 ml/min. Hydrogen and air flow-rates were 30 and 300 ml/min, respectively. The range and attenuator settings corresponded to $6 \cdot 10^{-12}$ A for full scale deflection. The column oven temperature was programmed from 100°C upon injection to 270°C at 20°/min. The injector and the detector were at 190°C and 320°C, respectively. The splitter (ratio 100:1) was turned on 0.5 min after injection to purge the inlet of solvent. A Hewlett-Packard Model 3390A integrator was used to quantitate the chromatograms. Samples (0.4 μl each) were injected using the splitless mode with a 1-μl syringe.

The fused-silica capillary column (27 m × 0.329 mm I.D.) was coated with 0.25-μm DB-5 (J. & W. Scientific, Rancho Cordova, CA, U.S.A.).

Extraction procedure

Cantharidin was extracted from animal tissues using a modified version of a published technique [1]. Wet biological samples ($\leqslant$ 1 g) were cut into small pieces or, in the case of liquids, were absorbed into cotton swabs or filter paper, then they were placed individually into microsoxhlet thimbles (Whatman Catalogue no. 2800-105) which had been pre-extracted for three days with acetone.

Each sample, contained in a Soxhlet thimble inside a glass test tube capped with a condenser, was hydrolyzed by treatment with three to four drops of

concentrated (12 *M*) hydrochloric acid and 1–2 ml of acetone for 4 h at 120°C. After hydrolysis, the thimble and the liquid remaining in the test tube were transferred to a microsoxhlet extractor (Corning Glass Works, Corning, NY, U.S.A.). The hyrolysis tube was rinsed four times with methylene chloride. The rinsings and additional methylene chloride were added to the extractor to bring the liquid to approximately 15 ml. The solvent was refluxed through the extraction apparatus for 12–15 h, and after cooling to room temperature, the solution in the extraction flask was concentrated to approximately 0.2 ml using a stream of nitrogen. The concentrate, neutralized and dried by passing it through a column packed with 0.8 g of anhydrous sodium bicarbonate layered on top of 0.8 g of anhydrous sodium sulfate, was collected in a 1-dram screw-capped vial. The extraction flask was rinsed four times with methylene chloride; each rinse was passed in sequence through the packed pipette into the vial. The pooled organic solution in the vial was concentrated to approximately 0.2 ml using a stream of nitrogen. Then the vial was capped with aluminum foil and stored at −20°C until just before analysis of its contents.

Analytical procedure

For GC analysis each extract was diluted to approximately 0.3 ml with chloroform. As an internal standard, 19.53 μg of benzophenone dissolved in 0.1 ml of chloroform were added to the diluted extract. After mixing, 0.4 μl of the benzophenone-containing chloroform solution of the extract was injected into the gas chromatograph.

Peak area ratios were calculated by dividing the area of each cantharidin peak by the area of the corresponding internal standard peak. A calibration curve was constructed by plotting peak area ratio as a function of the known cantharidin-to-benzophenone ratio in standard solutions containing pure cantharidin. This calibration curve was used subsequently to calculate unknown amounts of cantharidin in each extract of biological materials.

RESULTS AND DISCUSSION

Use of benzophenone as an internal standard in the quantitation of cantharidin is largely responsible for the success of the analytical method. An internal standard in general obviates the need to make highly accurate sub-microliter injections into the GC. The choice of benzophenone was made after attempts to use three compounds having closer structural resemblance to cantharidin, namely benzocantharidin [10], 3,6-endoxo-1,2,3,6-tetrahydrophthalic anhydride, and 1,2,3,6-tetrahydrophthalic anhydride, were unsuccessful. The latter compounds proved to have unsatisfactory GC behavior. Benzophenone is ideal in its GC behavior relative to cantharidin: the two compounds display similar retention times (5.74 and 5.20 min, respectively) but are completely resolved.

Fig. 2 illustrates the separation of cantharidin from blood of the margined blister beetle, *Epicauta pestifera*, along with the internal standard. Typical gas chromatograms obtained with the method using other tissues from this insect species are also shown in Fig. 2.

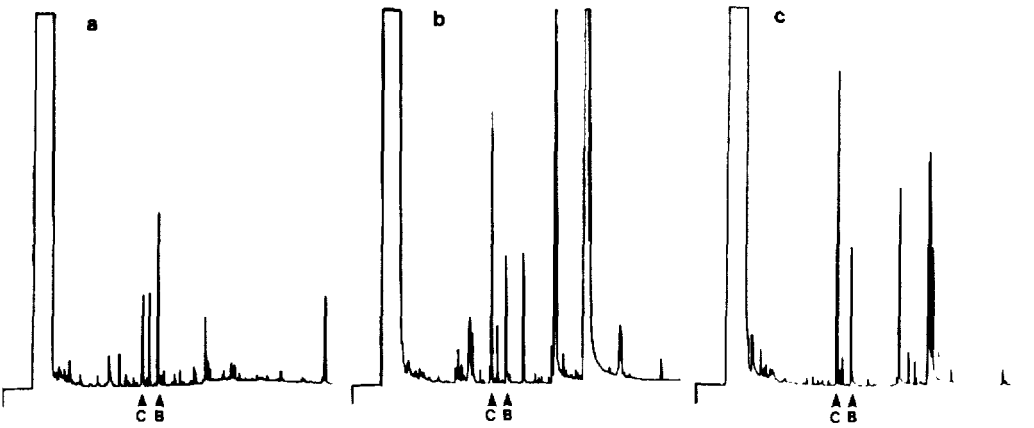

Fig. 2. Gas chromatograms showing the quantitation of cantharidin (C) in extracts of the margined blister beetle using benzophenone (B) as an internal standard. Peaks of interest are indicated by arrows. Retention times are 5.20 min for C and 5.74 min for B, using a 27-m 0.25-μm DB-5 column with an oven temperature increasing from 100°C to 270°C at 20°/min. Figures show injections containing 33.2 ng of C (a), 149 ng of C (b), and 140 ng of C (c), all with 40.7 ng of B added, in extracts of blood droplets discharged by a male beetle (a), somatic tissues of a female (b), and the testes of a male beetle (c).

cantharidin standard and a constant amount of the internal standard, was shown to be linear in the range 4 ng to 2 μg of cantharidin for a single injection. Under the best conditions, the maximal sensitivity of the analytical method for a single injection was found to be 30 pg of cantharidin. Under typical conditions in a biological matrix, we find it useful to determine as little as 500 pg of cantharidin.

Measurements made for standards and for various insect tissues, including mealworms (larval *Tenbrio molitor* that lack cantharidin) and female blister beetles [adult *Epicauta pestifera* which have at most a small amount (35 μg) of cantharidin], spiked with 1—300 μg of cantharidin showed that the accuracy of the technique is greater than 95% and that its precision is within 2—3%. Overall recovery of cantharidin from spiked and unspiked samples, which was determined by submitting them to hydrolysis and extraction a second and third time, is approximately 99%.

We have experienced little long-term variability using this method to analyze cantharidin in a variety of biological matrices. In other work to be reported elsewhere in detail, we have found the intra-assay error to range from 1 to 7% across all animal samples examined, including tissues and excrement from vertebrates poisoned by cantharidin.

The analytical method is relatively quick. Neither preparative chromatography nor derivatization is required before sub-nanogram amounts of cantharidin can be detected. An analysis of cantharidin in a sample can be completed in one day if the extraction is performed overnight.

This analytical method is being applied to a number of projects, including the biosynthesis of cantharidin in blister beetles and the uptake and systemic distribution of the substance in animals that have eaten blister beetles.

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