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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC METHOD FOR THE QUANTITATION OF CANTHARIDIN IN HUMAN SERUM

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SUMMARY

A gas chromatographic–mass spectrometric (GC–MS) method utilising single-ion monitoring at a resolution of 3000 to quantitate cantharidin in post-mortem serum is described. Serum was acidified, extracted with toluene, and 1 μl of the toluene extract subjected to GC–MS analysis. Clofibrate was used as internal standard. The method displayed a high degree of specificity and was accurate and precise, with a linear response in the concentration range 15–150 ng/ml. A cantharidin concentration of 72.3 ng/ml was found in the post-mortem serum.

INTRODUCTION

Cantharidin (hexahydro-3a,7a-dimethyl-4,7-epoxyisobenzofuran-1,3-dione) (I, Fig. 1) is a terpenoid produced by blister beetles (*Coleoptera Meloidae*) as a defence against predators. This compound is a potent vesicant and poison and is also believed to be useful as an aphrodisiac.

We were recently requested, for forensic purposes, to determine the concentration of cantharidin in the serum of a corpse, death believed to have been caused by ingestion of this compound. Although several methods have been published for the determination of cantharidin [1–6], they were considered to be either not selective enough, too time-consuming or applicable to the analysis of this compound in media containing fairly high concentrations of cantharidin.

Initial gas chromatographic (GC) investigation of the sample at our disposal making use of a mass-selective detector in the selected-ion monitoring mode (Hewlett-Packard MSD Model 5970) and a Kratos MS80RF double-focussing mass spectrometer at low resolution indicated a concentration of cantharidin in the low ng/ml range. As can be seen from the chromatogram (Fig. 2), the nature

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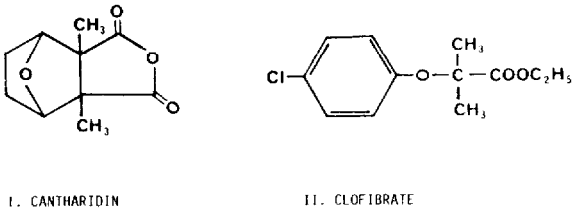

Fig. 1. Chemical structures of cantharidin (I) and clofibrate (II).

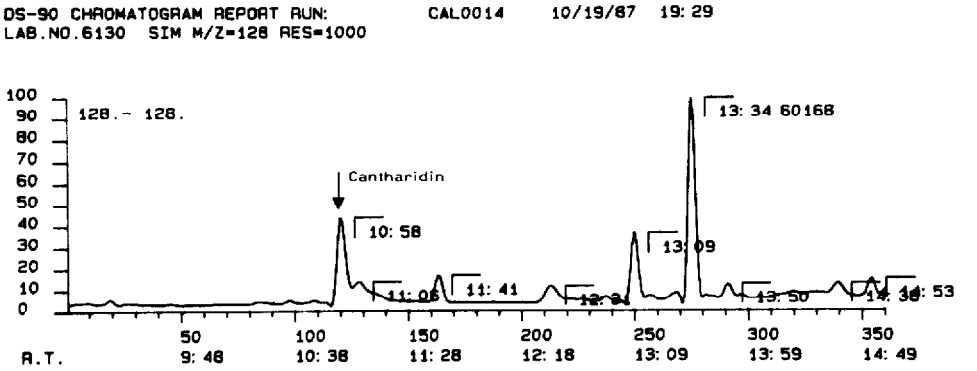

Fig. 2. Gas chromatogram of a serum extract for cantharidin monitoring *m/z* 128 at low resolution.

of the sample was such that substances that may interfere in the quantitation of cantharidin eluted in the same retention time region as this compound and the internal standard. It was therefore deemed appropriate to develop a method possessing a high degree of specificity and with the required sensitivity to quantitate this compound at the expected concentration. As a result of these analytical restrictions a gas chromatographic-mass spectrometric (GC–MS) method utilising single-ion monitoring (SIM) at medium resolution was developed for the quantitation of this compound.

EXPERIMENTAL

Reagents and chemicals

Cantharidin was supplied by the Health Chemical Laboratory (Johannesburg, South Africa) who obtained it from BDH Chemicals (Poole, U.K.). Clofibrate was supplied by I.C.I. South Africa Pharmaceuticals (Johannesburg, South Africa). Hydrochloric acid, methanol, acetone and sodium fluoride were of analytical grade and supplied by Merck Chemicals (South Africa). Toluene was of HPLC grade and was supplied by Rathburn Chemicals (Walkerburn, U.K.).

Apparatus

A Kratos MS80RF double-focussing mass spectrometer coupled to a Carlo Erba Mega-series 4000 gas chromatograph and a Data General (DG-30) data system utilising the Kratos DS90 software (Kratos Analytical, Manchester, U.K.) was used for the GC–MS analysis. Hard-copy print-outs of mass spectra and chro-

matograms were done on a Hewlett-Packard Model 7475A plotter [Hewlett-Packard (South Africa), Johannesburg, South Africa].

Chromatographic conditions

Chromatographic separation was achieved using a 25 m × 0.22 mm I.D. fused-silica column coated with chemically bonded 5% phenyl, 95% methyl silicone [(0.11 μm phase thickness (CPSIL-8-CB) supplied by Chrompack International, Middelburg, The Netherlands] operated under the following conditions: initial temperature 80°C for 3 min, followed by a temperature increase of 12°C/min to a final temperature of 280°C with a final time of 10 min. Injection port and transfer line temperatures were maintained at 280°C. Helium (linear gas velocity of 40 cm/s) was used as carrier gas. Under these conditions cantharidin elutes at 11 min ± 1 s and clofibrate at 11 min 5 s ± 1 s.

Injections were made using the splitless mode with toluene as solvent and a period of 3 min before opening the split valve.

Mass spectrometric conditions

Electron-impact (EI) acquisitions were done at an electron energy of 70 eV and an ion source temperature of 250°C. Quantitative analyses were done at a resolution of 3000. SIM acquisitions were done with a perfluorokerosene mass *m/z* 130.9920 as lock mass. Dwell time on the lock mass was 100 ms with a total cycle time of 700 ms, divided equally between the ions of the analyte (*m/z* 128.0473) and the internal standard (*m/z* 128.0029).

Internal standard and cantharidin stock solutions

A 1-μl volume of clofibrate (equivalent to 1.14 mg) was dissolved in 9 g of methanol (11.4 ml). This solution was further diluted to obtain a final solution containing 20 ng clofibrate in 10 μl of methanol (this volume being used in analysis). Cantharidin was weighed out accurately, dissolved in methanol and diluted with methanol to obtain a solution containing 75 ng per 100 μl. Serum standard solutions equivalent to 150, 105, 45 and 15 ng/ml were prepared by adding 100, 70, 30 and 10 μl of this stock solution to 0.5 ml drug-free human serum aliquots.

Extraction procedure

To 0.5 ml plasma (calibration standards, controls or unknowns) contained in a 2 ml disposable glass ampoule were added 10 μl of the internal standard solution, 250 μl of 6 *M* hydrochloric acid and 0.5 ml of toluene. The mixture was vigorously shaken on a vortex mixer for a period of 90 s followed by centrifugation for 10 min at 2000 *g*. A 1-μl volume of the supernatant toluene layer was used for GC–MS analysis.

RESULTS AND DISCUSSION

Choice of internal standard

The EI mass spectrum of cantharidin (Fig. 3A) shows an intense peak at *m/z* 128 (20% of the total ion current being resident in this peak) corresponding to

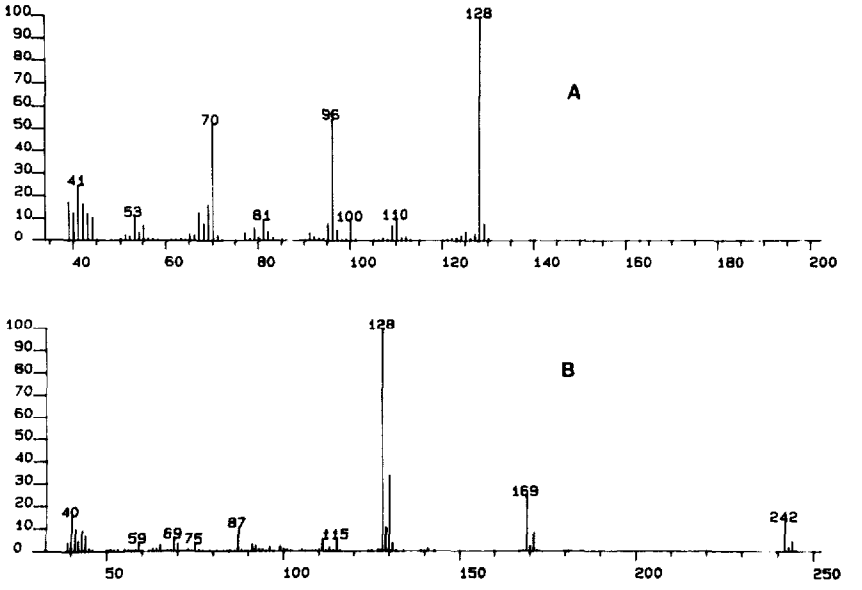

Fig. 3. EI mass spectra of cantharidin (A) and clofibrate (B).

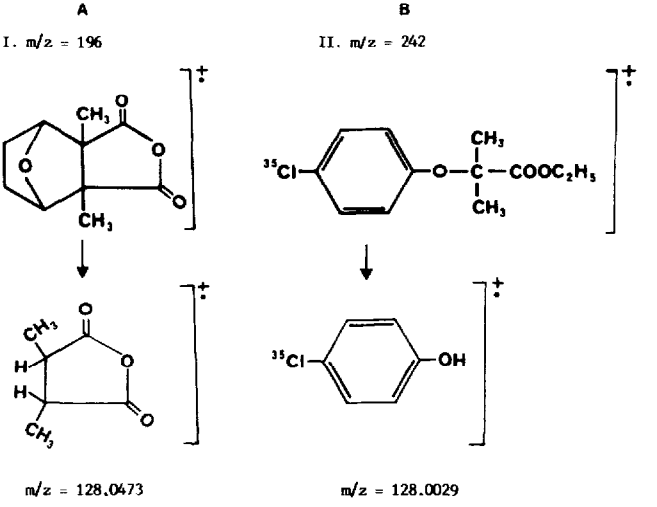

Fig. 4. Proposed MS fragmentation pathways for cantharidin (A) and clofibrate (B).

$C_6H_8O_3$ (Fig. 4A). We found that the drug clofibrate (II, Fig. 1) had GC properties similar to that of cantharidin and also had an intense peak at *m/z* 128 (30% of the total ion current being resident in this peak) (Fig. 3B) corresponding to $C_6H_5O^{35}Cl$ (Fig. 4B). The correctness of the empirical formulae for fragmentation assignations were confirmed by accurate mass determination at a resolution of 7000. For cantharidin the accurate mass of *m/z* 128 was determined as 128.0480 (expected: 128.0473) while for clofibrate the accurate mass of *m/z* 128 was determined as *m/z* 128.0022 (expected 128.0029).

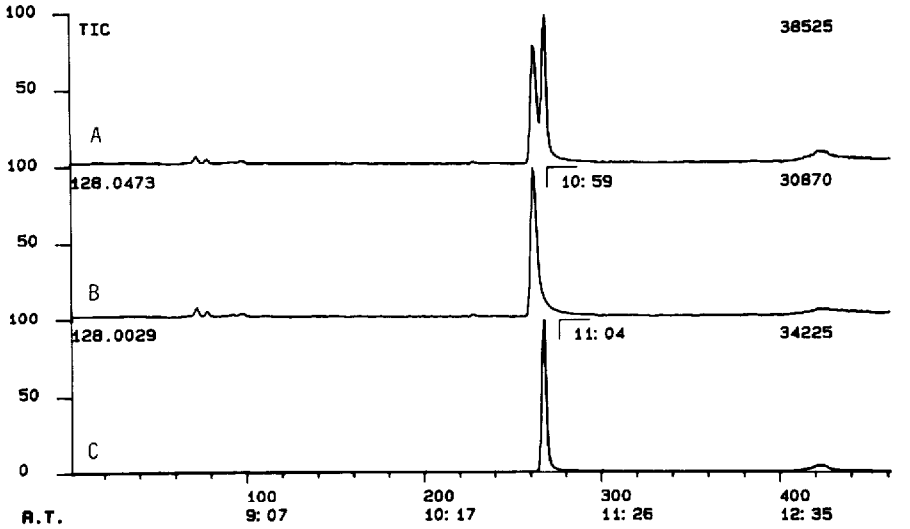

Fig. 5. MS separation of chromatographically unresolved cantharidin (retention time 10 min 59 s) and clofibrate (retention time 11 min 4 s). (A) Total ion current chromatogram. (B) Mass trace for m/z 128.0473 (cantharidin). (C) Mass trace for m/z 128.0029 (clofibrate).

Although the two compounds were not completely separated chromatographically under the conditions used, the masses of the two fragments of interest differed to such an extent that a resolution of 3000 was sufficient to separate them by MS (Fig. 5). Using this resolution therefore had the effect of ensuring the required selectivity not obtained with a flame ionisation detector or with a mass spectrometer operated at lower resolution.

Since clofibrate is an ester which is reported to be hydrolysed rapidly in vivo to clofibric acid it was important to investigate whether it would be necessary to add an enzyme inhibitor to the serum before addition of the internal standard. Inactivation of the enzymes by means of 100 μl of a 2 M sodium fluoride solution and comparison of the yield of clofibrate obtained from serum treated in this way with the yield obtained from untreated serum indicated that no hydrolysis occurred in the short period between addition of the internal standard and its extraction with toluene.

Influence of the acid concentration on the efficiency of the extraction

In a publication by Mari et al. [3] it is evident that an acidic environment is required for the efficient extraction of cantharidin from biological specimens. We determined the optimum amount of acid to be added to 0.5 ml serum as follows: Six aliquots of 0.5 ml of serum were each spiked with 150 ng of cantharidin contained in 10 μl of methanol. A 250-μl volume of one of the following hydrochloric acid solutions (1.0, 2.0, 3.0, 6.0 and 11.0 M) was added to one of the serum aliquots. To one serum aliquot 250 μl of water only were added. Each mixture was extracted with 1.0 ml toluene as described above. A 0.5-ml volume of the supernatant toluene was removed and 30 ng of clofibrate contained in 10 μl toluene were added to each aliquot. A 1.0 μl volume of each toluene extract was analysed,

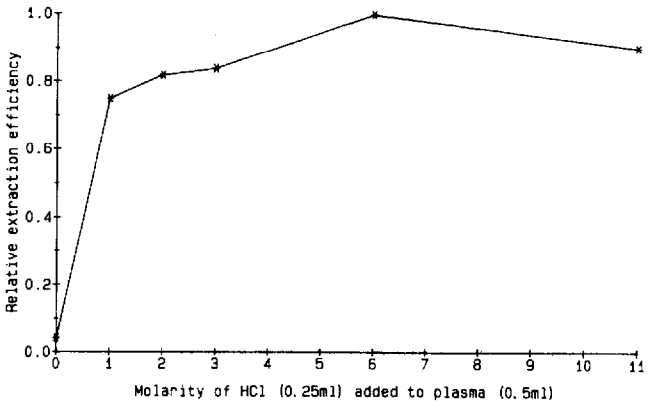

Fig. 6. Influence of acid concentration on the extraction efficiency of cantharidin from serum.

the peak areas of the cantharidin and the internal standard peaks determined and the peak-area ratio cantharidin clofibrate calculated. The results of this experiment indicated that the best yield was obtained when 6 *M* hydrochloric acid was used (Fig. 6).

Extraction efficiency

Cantharidin (150 ng) was added to 0.5 ml of serum and extracted with 0.5 ml of toluene in the way described above. To a 250-μl aliquot of the supernatant toluene were added 20 ng of clofibrate contained in 10 μl of toluene. A 1-μl volume of this solution was subjected to GC–MS analysis. The peak area of cantharidin so obtained was compared with that obtained by injecting 1 μl of a solution prepared to contain 75 ng of cantharidin and 20 ng of clofibrate in 250 μl of toluene after normalising the peak areas of clofibrate to the same value and adjusting the peak areas of cantharidin accordingly. The results indicated that an extraction efficiency of 88% was achieved.

Quantitation

Calibration and calculation. Four serum standards covering the expected concentration range were processed as described above. The calibration graph was established by plotting cantharidin concentrations (y) versus peak-height ratio I/II (x) and performing a least-squares straight line regression analysis on the data points. This regression equation was then used to calculate concentrations of I in unknown serum samples by calculating the peak-height ratio I/II and substituting this value in the equation for the calibration graph. The calibration graph was linear in the concentration range up to 150 ng/ml ($y=1.38+63.81x$; $r^2=0.997$).

Accuracy and precision of the analytical procedure. Four control serum samples, containing different concentrations of cantharidin spanning the expected concentration range, were analysed in quadruplicate. Each set of determinations was done utilising a separate calibration graph. The results presented in Table I indicate that the method performs satisfactorily in the range between 15 and 150 ng/ml.

TABLE I

ACCURACY AND PRECISION OF THE ANALYTICAL PROCEDURE ($n=4$)

Control No.	Concentration expected (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Q1	125.4	122.2 ± 6.1	4.9
Q2	62.5	62.6 ± 4.41	7.0
Q3	31.3	33.2 ± 1.76	5.3
Q4	15.8	17.6 ± 1.0	5.7

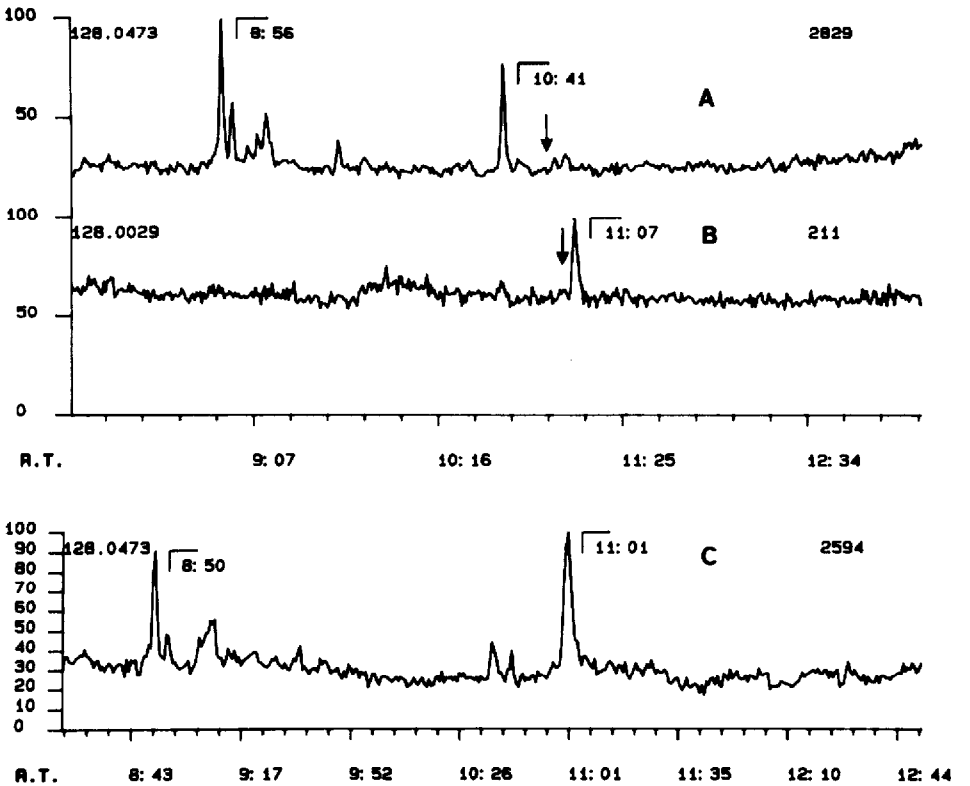

Fig. 7. (A) Blank serum extract monitored for m/z 128.0473 (cantharidin). (B) Blank serum extract monitored for m/z 128.0029 (internal standard). (C) Serum extract containing 5 ng/ml cantharidin monitored for m/z 128.0473.

Selectivity and sensitivity. Chromatograms representing a blank serum extract being monitored for cantharidin (m/z 128.0473) (Fig. 7A) show that the trace for cantharidin is devoid of any interfering peaks at the retention time of cantharidin (11 min ± 1 s).

The trace for the internal standard (m/z 128.0029) (Fig. 7B) shows a small peak at a retention time of 11 min 7 s (internal standard retention time = 11 min 5 s ± 1 s. The contribution of this peak is, however, of negligible proportions.

The sensitivity of the method is illustrated in Fig. 7C, showing a signal-to-noise ratio of 5:1 for cantharidin (retention time 11 min ± 1 s) at a concentration of 5 ng/ml serum.

Analysis of post-mortem serum. Using the method as described above, the cantharidin concentration in the post-mortem serum was determined as 72.3 ng/ml.

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