Original Article

A Novel Approach to the Quantitation of Coeluting Cantharidin and Deuterium Labelled Cantharidin in Blister Beetles (Coleoptera: Meloidae)

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Abstract

Blister beetles (Coleoptera: Meloidae) are the main natural source of cantharidin, but the compound titre is depended on several factors including, age, sex and mating status of the insects. In order to eliminate such uncertainty factors in physiological and chemical studies deuterium labelled cantharidin (D_2C) with no natural abundance is normally introduced into the beetles' body to use it as a model for studying the cantharidin behaviour in vivo. Experiments were achieved on *Mylabris quadripunctata* (Col.: Meloidae) from Southern France and the beetles were exposed to an artificial diet containing a defined amount of D_2C. On the other hand, because of the high similarity between the two compounds they cannot be well quantified by gas chromatography. In order to remove the burden, MRM technique was used for the first time which could successfully create well-defined cantharidin and D_2C peaks and hence a precise measurement. MRM technique was examined using a GC-MS Varian Saturn which collected MS/MS data of more than one compound in the same time window of the chromatogram. It is especially useful when coeluting compounds have different parent ions, i.e. m/z 84 for D_2C (coeluting isotopically-labelled compound) and m/z 82 for cantharidin (beetle-originated compound). Using the routine GC-MS runs, measurement accuracy may be significantly reduced because the D_2C peak is covered by the cantharidin huge peak while MRM could reveal the two coincided peaks of cantharidin and D_2C. Therefore MRM is hereby introduced as the method of choice to separate cantharidin from D_2C with high sensitivity and thus provide a precise base of quantitation.

Keywords: Cantharidin, Labelled cantharidin, Meloidae, GC-MS/MS, MRM

Introduction

Cantharidin ($C_{10}H_{12}O_4$), mainly found in blister beetles (Coleoptera: Meloidae), is among the most widely known insect natural products (McCormick and Carrel 1987, Dettner 1997). It is highly toxic to most animals (LD_{50} for human 10-60 mg/kg and intraperitoneal mouse LD_{50} 1.0 mg/kg) (Dettner 1997). Its reputation principally derives from descriptions of its physiological activities as an aphrodisiac and a blistering agent for humans and livestock. Cantharidin aphrodisiac property is initialized by inhibition of phosphodiesterase and protein phosphateses (PPs) activity and stimulation of ß- receptors (Sandroni 2001).

Current studies on the cantharidin are focused on the site of production in producing beetles, cellular detoxification of cantharidin, in vivo biosynthesis and the intermediate compounds as well as the PPs inhibitory properties (Knapp et al. 1998, McCluskey et al. 2001, McCluskey et al. 2002, Liu et al. 1995) which all require the precise measurement of cantharidin as a key ele-

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ment. Similarly, we had to precisely measure the titre of the compound in different organs of both sexes in order to study the cantharidin pharmacodynamics in male and female of *Mylabris quadripunctata*. Cantharidin titre in blister beetles is depended on several factors including, age, sex and mating status (McCormick and Carrel 1987, Carrel et al. 1993). Therefore to eliminate such uncertainty factors, deuterium labelled cantharidin (D_2C) in which a single hydrogen atom in each of the two angular methyl group has been replaced by deuterium atoms is introduced into the beetles' body. Because of the high structural similarity to cantharidin, D_2C is hence used as a model to predict the cantharidin behaviour in vivo. Since D_2C has no natural abundance, tracing and exact measurement of the compound is possible, however because of the very close structural similarity, they cannot be well separated by gas chromatography (Fig. 1 and 2). D_4C synthesizing is on the other hand difficult and at the same time purifying the very low yield of the product is another technical challenge. Since quantitation is based on the peak area of a single compound, the precise measurement of these two chemicals by GC-MS was faced with a technical limitation.

In order to create well-separated peaks, a kind of MS/MS technique called MRM (Multiple Reaction Monitoring) was used for the first time in this study to precisely separate cantharidin from D_2C and increases the quantitation accuracy.

Materials and Methods

Field collection of beetles and transport to laboratory

Specimens of *Mylabris quadripunctata* (Linné, 1767) were collected manually from St. Jéan du Gard, Department Gard, Southern France, while sitting on flowers or stems of different shrubs of the families of Astraceae, Compositeae and Leguminoseae. The material was directly transported to the University of Bayreuth, Germany, where the work was carried out. Voucher specimens are being kept at the TÖK II, University of Bayreuth, Germany.

Administration of the Labelled Compound

The beetles in the lab were fed for three days with an artificial diet (Selander 1986) to which 5 µl of a chloroform based D_2C (conc. 2000 ng/µl) had been added. After complete evaporation of the solvent, the beetles were exposed to the diet containing a defined amount of D_2C (Nikbakhtzadeh et al. 2007).

Sample Preparation and Extraction

The measurement of the total titre of cantharidin and D_2C, hydrolysis and extraction of the dissected tissues were carried out according to a well defined protocol (Holz et al. 1994).

Quantitative GC-MS

Normal GC-MS runs were performed by a GC 6000, Vega Series 2, Carlo Erba gas chromatograph equipped with a HT8 (8% phenyl polysiloxane-carborane, non-polar) bounded phase fused silica capillary column (SGE: FT 0.25 µm, ID 0.32 mm, OD 0.43 mm, Length: 25 m) which in turn connected to a Finnigan MAT Ion Trap Detector (ITD). Constant elium pressure was 53 mbar and injector temperature defined as 230 °C. Electron Impact Ionization (EI 70 eV) provides mass spectra with a characteristic fragmentation of cantharidin: the base peak with m/z 96 and two other fragments of m/z 128 and 67 (M^+: 197). D_2C provides mass spectrum with m/z 98, 130, 69 (M^+: 199). These provide an absolute proof if the beetle extracts truly bear the two compound of cantharidin and its isotopically labelled standard, D_2C (Fig. 3).

To analyse the data, the software IT Data System, version 4.00 and GCQ data software package 1997, Version 2.2 (Build 173), Finnigan Corporation were used.

MRM technique was examined using a GC-MS Varian Saturn 2000.40 equipped with a ZB-5 capillary column coated with 5% phenyl polysiloxane (Phenomenex: FT 0.25 µm, ID 0.25 mm, Length: 60 m). A 1079 injector was used and samples (1 µl) were injected splitlessly.

Trap and transfer line were kept at 175 °C and 260 °C, respectively. Mass spectra were taken at 70 eV (EI mode) at 1 scan sec^{-1}. Data were processed by a Saturn® GC/MS Workstation package, Saturn view ™ version 5.2.1, 1989-1998, Varian Associates, Inc. Helium at constant pressure served as the carrier gas (1.8 ml min^{-1}). The elution of compounds was programmed from 40 °C (2 min) to 130 °C at 100 °C min^{-1}, then to 195 °C at 3 °C min^{-1}, followed by a rapid heating to 250 °C at 100 °C min^{-1} kept for 2 min prior to cooling. Authentic cantahridin (purity 98%, SIGMA-ALDRICH, UK) served as standards for identification and calibration.

Results

MRM collects MS/MS data on more than one compound in the same time window of the chromatogram. It is especially useful when coeluting compounds have different parent ions, i.e. m/z 84 for D_2C (coeluting isotopically-labelled compound) and m/z 82 for cantharidin (beetle-originated compound). After MRM, several compounds could be directed towards different channels based on the defined parent ion which makes quantitation not only easier but more reliable (Fig. 4). Cantharidin and D_2C viewed and independently integrated by designating the appropriate channels in a file selection dialog (Fig. 5). It was even possible to send up to 10 different parent ions in each chromatographic segment. Our experiments indicated that the beetle feeding was inhibited if the D_2C titre of the artificial diet was higher than 2000 ng/µl, so only a very low amount of D_2C could be accumulated in each internal organ. Using the routine GC-MS runs, measurement accuracy may be significantly reduced because the D_2C peak -less than 50 Kcounts- is covered by the cantharidin huge peak with over 600 Kcounts (Fig. 6) while MRM could reveal the two coincided peaks of cantharidin and D_2C (Fig. 6). Therefore MRM is hereby introduced as the method of choice to separate cantharidin from the deuterium labelled cantharidin (D_2C) with high sensitivity.

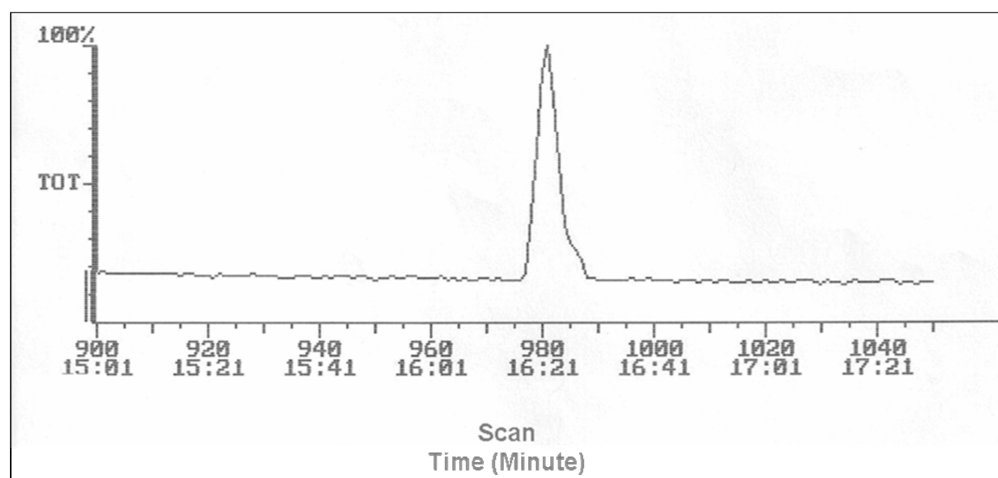
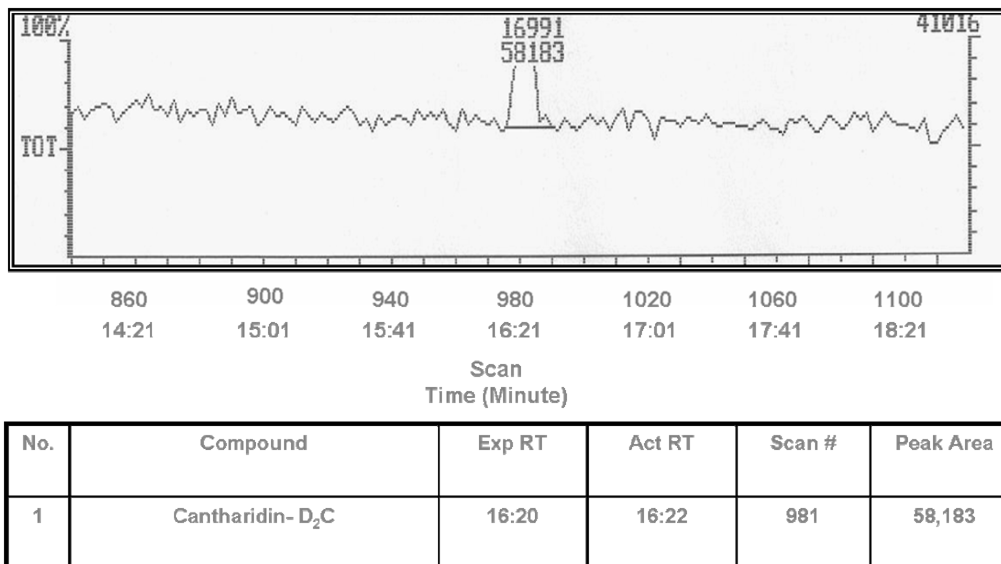


Fig. 1. Cantharidin-D_2C total ion chromatogram based on a GC 6000, Vega Series 2 gas chromatograph, injection vol.: 1 µl, Scan range: 900-1050, Int: 5156, 100%: 16991. Chromatogram appeared at scan 981 (RT 16:22)



No.	Compound	Exp RT	Act RT	Scan #	Peak Area
1	Cantharidin- D_2C	16:20	16:22	981	58,183

Fig. 2. Auto quantitative peak integration in GC 6000, Vega Series 2, Carlo Erba gas chromatograph by the IT Data System (version 4.00). The two compounds of cantharidin and D_2C are integrated in one peak and the peak area which is the basis of the qualitative measurement indicates the total amount of these two compounds together. Abbreviations: Exp RT= expected retention time, Act RT= Actual retention time.

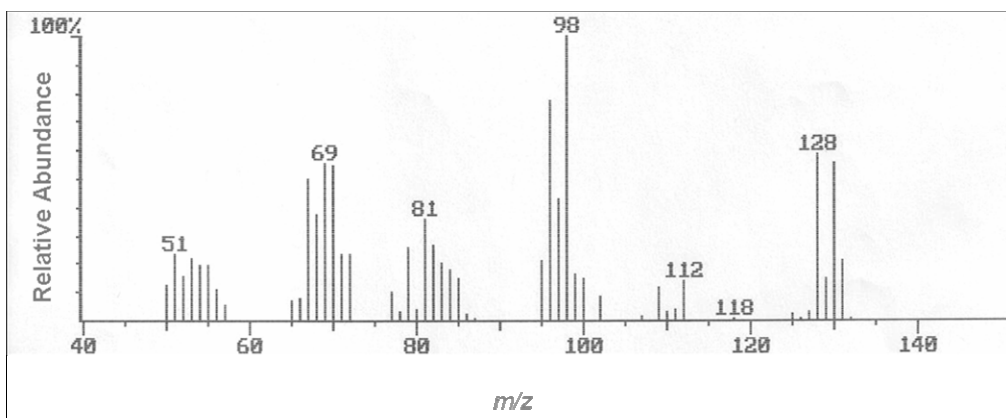


Fig. 3. EI mass spectra of cantharidin-D_2C mixture with base peaks at m/z 128, 96 and 67 for cantharidin and 130, 98 and 70 for D_2C, according to a GC 6000, Vega Series 2 gas chromatograph, 100%= 1805

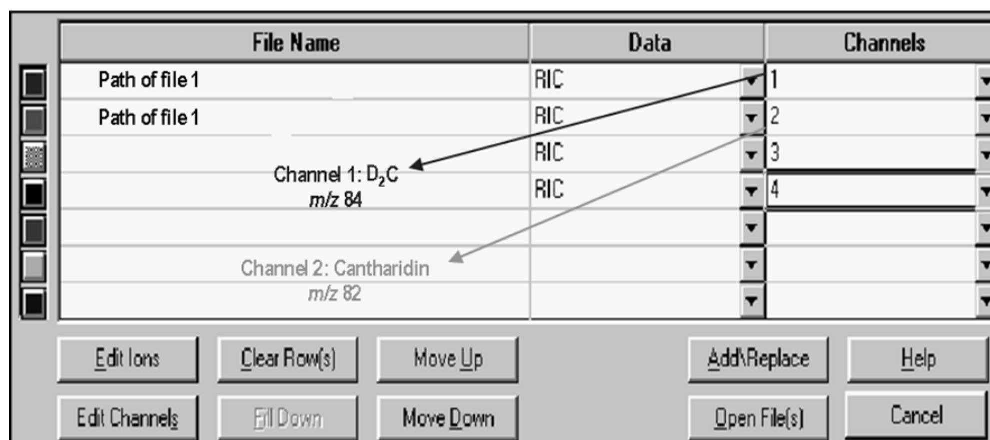


Fig. 4. MRM setting shows different channel options based on the defined parent ion of cantharidin (*m/z* 82) and D_2C (*m/z* 84)

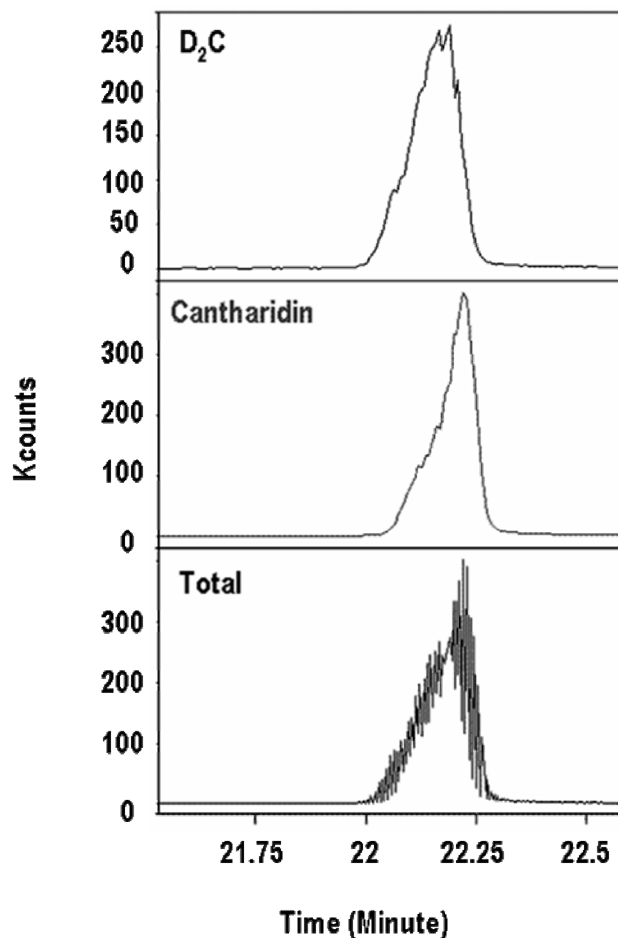


Fig. 5. Three windows of the same segment indicate channel one at 1st window (D_2C characteristic peak), channel two at the 2nd window (cantharidin peak) and both peaks have been finally shown together in the last window

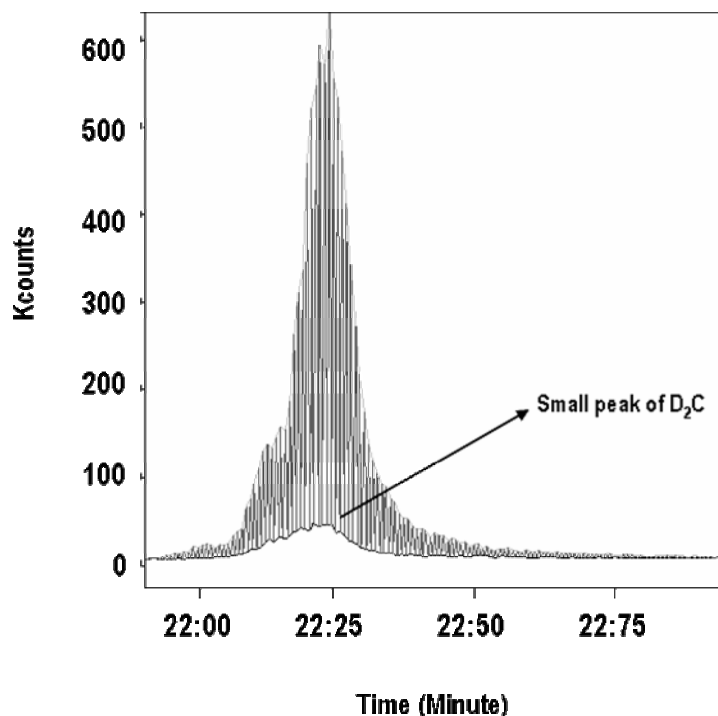


Fig. 6. MRM of the introduced D_2C into *Mylabris quadripunctata*. The bigger peak belongs to cantharidin. In normal GC-MS, these two peaks cannot be individually observed and the calculation shows the total amount of the both compounds.

Discussion

However HPLC has sometimes been used by the workers (McCormick and Carrel 1987) to find cantharidin, it was widely replaced by GC since 1980s and has proved to be a more reliable method in both detecting and quantifying cantharidin of the insect-derived materials (Capinera et al. 1985, Carrel et al. 1986). GC is in particular more attractive because of the quick sample extraction and derivatization. Low vapour pressure and the good thermal stability of cantharidin on the other hand have made gas chromatography the method of choice. Using authenticated cantharidin in GC analyses, former researchers had no applied restriction to confirm detection of cantharidin in biological samples. Their data were easily confirmed by a MS detector which provided the molecular fragment information. Much of the recent studies require not only the detection of cantharidin trace amounts, but a very precise quantitation. It is known that cantharidin is mainly produced by male blister beetles and transfer to females in large quantities along with sperm during copulation (Dettner 1997, Nikbakhtzadeh et al. 2007). Studying the cantharidin pharmacodynamics in the field collected materials is therefore challenging because most of the collected beetles have previously copulated and their original reservoir of the compound has been dramatically altered. One alternative could be working with the laboratory cultured beetles, although rearing of blister beetles and especially their feeding in captivity was difficult with an uncertain yield (Selander 1986). In the absence of such laboratory controlled beetles, the only robust tool to study the cantharidin movement and tissue accumulation and also researches on the compound's site of production is introducing the very similar mimic of cantharidin, D_2C, into the tissues of the living beetles of both sexes (Holz et al. 1994). Using the EI-mass spectra, Holz and their co-workers (Holz et al. 1994)

easily traced cantharidin and its isotopically-labelled compound by a GC-MS machine, however they had no intention to make a quantitative analysis. The exact quantitation of Cantharidin and D_2C which is based on the precise separation of their chromatogram peaks in gas chromatography is very important if the labelled compound is going to be applied in any biological studies. We used three GC-MS machines if there was a difference in the accuracy of their normal GC-MS runs, but a considerable miscalculation was observed in all these runs because cantharidin and D_2C coelute on the GC column and their chromatogram peaks have almost the same retention time. Modification of the technical parameters such as column type, oven programme, carrier gas and its velocity were also examined whether they had any sharpening effect on the coeluting cantharidin/D_2C, but no well-defined peak was obtained.

As an alternative to the normal MS, we hereby suggest MRM technique which proved to be of high precision in the quantitation of D_2C when the compound was present in a cantharidin rich biomaterial, e.g. tissues of a blister beetle. In this way, the peak area of a well defined chromatogram is independently integrated and the total amount of a compound calculated. It should be noted that although D_2C has two mass units more than cantharidin, it is eluted earlier because the deuterium-replaced hydrogens affect the spatial angles of the molecule which in turn increases the molecule interaction to the internal film of the compact capillary column.

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