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Short communication

Determination of the release of hydrolyzed demethylcantharidin from novel traditional Chinese medicine–platinum compounds with anticancer activity by gas chromatography

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Abstract

The present paper describes the development of a simple, accurate and reproducible gas chromatographic method for the determination of hydrolyzed demethylcantharidin release from a novel series of traditional Chinese medicine (TCM)–platinum compounds possessing potent anticancer and protein phosphatase 2A (PP2A)-inhibition properties. The salient features of the validated assay were a limit of detection (LOD) of 2 μg/mL, a limit of quantitation (LOQ) of 6 μg/mL, an intra- and inter-day precision of less than 11%, and an accuracy of more than 92%. The developed GC–flame ionization detection (FID) method was successfully utilized for the analysis of hydrolyzed demethylcantharidin, the TCM component that is slowly released from the novel compounds over 24 h, leading to PP2A inhibition. Further structural confirmation was achieved by GC–MS. The GC method is suitable for further mechanistic, pharmacokinetic and metabolic studies of the TCM–Pt compounds that might prove to be new anticancer agents with novel mechanisms of cytotoxic action. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Pharmaceutical analysis; Demethylcantharidin; Protein phosphatase 2A; Enzyme inhibitors

1. Introduction

A combination of demethylcantharidin (DMC) (7-oxabicyclo[2,1]heptane-2,3-dicarboxylic anhydride), a modified component of a traditional Chinese medicine (TCM), with a platinum moiety has produced a series of TCM-based platinum compounds $[Pt(C_8H_8O_5)(NH_2R)_2]$ (**1–5**) (Fig. 1) with anticancer activity [1]. The compounds showed selective cytotoxicity towards human liver cancer, circumvention of cisplatin cross-resistance, and are protein phosphatase 2A (PP2A) inhibitors, properties attributed to the inclusion of demethylcantharidin in the novel structures [2].

Demethylcantharidin and its diacid, endothall, are synthetic analogues of cantharidin and belong to a class of natural toxins and herbicides (Fig. 1). Cantharidin is also the active principle of *Epicanta gorhami* or *Mylabris* (“blister beetles”), which has long been used as a TCM for the treatment of liver, lung, intestinal and digestive tract tumors [3]. Cantharidin and its derivatives have been reported to have strong affinity and specificity for a “cantharidin-

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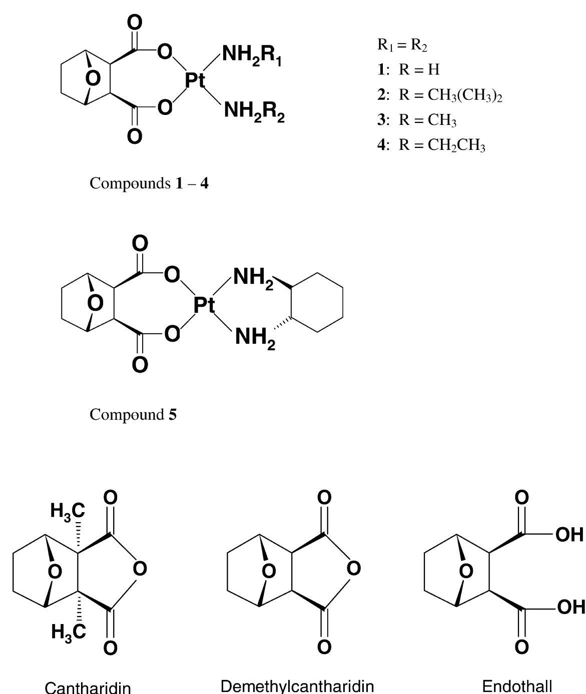

Fig. 1. Structures of the novel TCM–Pt compounds, cantharidin, demethylcantharidin, and endothall.

binding'' protein, identified as protein phosphatase 2A [4].

Previously, only a limited number of methods have been reported for the analysis of such compounds in environmental and biological samples, which include high-performance liquid chromatography (HPLC) with derivatization [5]. However, the HPLC method is time-consuming and requires more than 20 h alone for derivatization after extraction of cantharidin. Radio-labeled [^{14}C]endothall has been used in an investigation of its persistence in water and hydrosil [6].

Novel TCM–Pt compounds **1–5**, incubated in normal saline over 24 h, have been shown to exhibit PP2A-inhibitory action, most likely due to the progressive release of demethylcantharidin (Fig. 2), and resulting in enhanced cytotoxicity and circumvention of cisplatin cross-resistance [2]. In cisplatin and carboplatin, the two Pt-based anticancer drugs commonly used worldwide, this PP2A-inhibitory effect is conspicuously absent.

The purpose of this study was to develop an appropriate analytical method to evaluate the release of demethylcantharidin from the TCM–Pt compounds in aqueous solutions. The main difficulty of analysis is the lack of ultraviolet absorption by demethylcantharidin, and the use of radioactive labels would be ineffective due to the probable successive dissociation of the bidentate demethylcantharidin from the novel TCM–Pt compounds (Fig. 2).

Compounds **1–5** are potentially a new class of anticancer agents with a novel dual mechanism of cytotoxic action: inhibition of PP2A and platination of DNA, the latter similar to the well established alkylating action of cisplatin (Fig. 2). A suitable analytical method is essential for further mechanistic, metabolic and pharmacokinetic studies of these compounds.

2. Experimental

2.1. Chemicals and materials

Cisplatin and carboplatin were obtained from Strem (Newburyport, USA). Demethylcantharidin and the novel TCM–Pt compounds were synthesized by the School of Pharmacy and Department of Chemistry at the Chinese University of Hong Kong [1,7]. Hydrolyzed demethylcantharidin was prepared by dissolving one part of demethylcantharidin in two parts of 1 *M* sodium hydroxide, and the resulting solution dried in vacuo to produce a white solid, after which the identity was confirmed by FT-IR and NMR. All chemical reagents used in the assay were reagent grade or better. High-purity nitrogen (>99.99%), compressed air and hydrogen [for flame ionization detection (FID) ignition] were supplied from Chun Wan Industrial Gas Company (Hong Kong, China). High-purity helium (99.999%) was obtained from Hong Kong Special Gas Company (Hong Kong, China).

2.2. Incubation conditions and extraction

Compounds **1–5** and the control, hydrolyzed demethylcantharidin (2 m*M*), were incubated in normal saline at 37 °C in a temperature-controlled water bath shaker (Reichert Scientific Instruments)

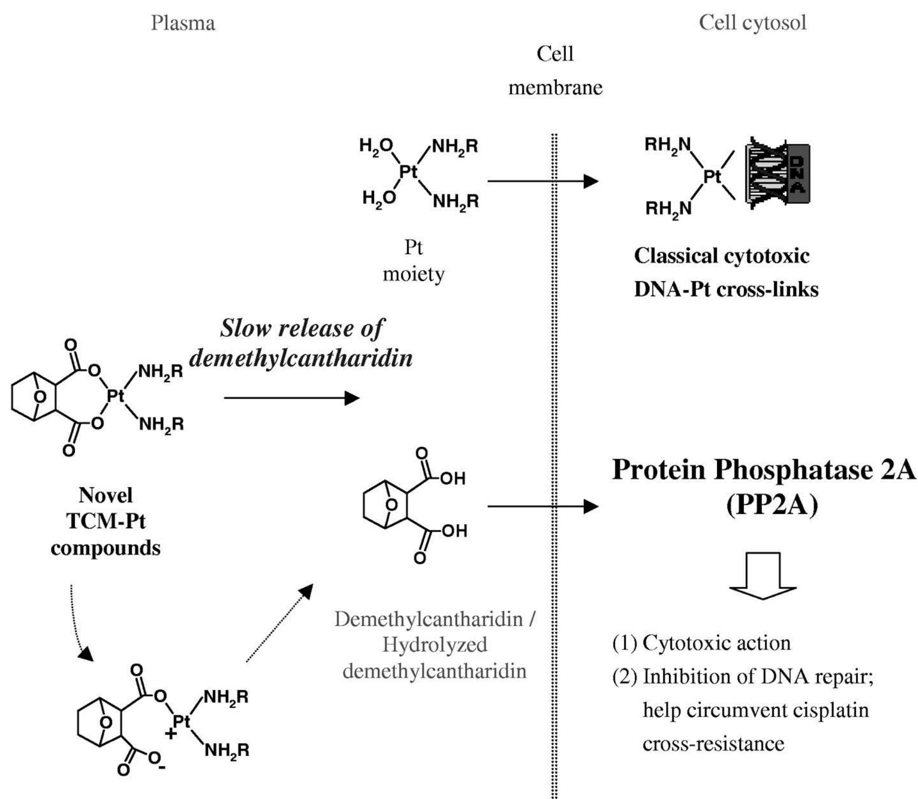

Fig. 2. Proposed dual mechanism of cytotoxicity of the novel TCM–Pt compounds.

set at 60 oscillations per minute. Aliquots were collected at designated times and lyophilized. The lyophilized solid was acidified with 3 *M* H_2SO_4, from which the released hydrolyzed demethylcantharidin was extracted by acetonitrile (three times). Undissolved solid was centrifuged and precaution was taken to avoid evaporation. The acetonitrile extracts were concentrated to a final volume of 0.5 mL and a 1 μL aliquot was injected into the GC.

2.3. Apparatus and chromatographic conditions

GC analysis was performed using a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, USA) equipped with a FID system, and interfaced to a Hewlett-Packard 3396 Series II integrator for integration and sample analysis. A capillary bonded fused-silica column, DB-23 column (30 m×0.248 mm, 0.25 μm) (J&W Scientific, Rancho Cordova, CA, USA), was used. Nitrogen was used as the carrier gas at a flow-rate of 1 mL/min. The initial oven temperature was set at 130 °C with the injector and detector temperatures at 230 °C. The temperature program consisted of a 20 °C/min temperature ramp to 220 °C. An aliquot (1 μL) of sample was injected with splitless injection and benzophenone (2 mg/mL) was used as the internal standard (I.S.).

Peak area ratios were calculated by dividing the area of each hydrolyzed demethylcantharidin 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 hydrolyzed demethylcantharidin-to-benzophenone ratio in standard solutions containing pure hydrolyzed demethylcantharidin. This calibration curve was used subsequently to quantify the amount of hydrolyzed demethylcantharidin released from the novel TCM–Pt compounds.

2.4. Extraction efficiency

Extraction efficiency was determined for hydrolyzed demethylcantharidin by evaluating the quantitative recovery of a single level spike. The spikes were prepared in normal saline as outlined previously and processed accordingly. Recovery was determined by comparison of direct injection data derived from hydrolyzed demethylcantharidin prepared in acetonitrile to data derived from samples of hydrolyzed demethylcantharidin in normal saline that were taken through the extraction process before GC analysis.

2.5. Accuracy and precision

Accuracy was measured by percent recovery of three different concentrations of hydrolyzed demethylcantharidin in normal saline that spanned the low, middle, and high range of the standard curve (10–1000 μg/mL). All samples were taken through the acetonitrile extraction process as described.

Intra-assay variation was determined by measuring the reproducibility of the detector response following repeated injections of a single standard containing hydrolyzed demethylcantharidin at three different concentrations. Inter-assay precision was determined by measuring the reproducibility of the assay result at three different concentrations on different occasions. In all cases, precision was expressed in terms of relative standard deviation (RSD).

2.6. Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by performing four different statistical analyses on data obtained from five different assays as described by Brittain [8]. For each method, LOD was determined by multiplying the noise by 3.3. LOQ was determined by multiplying the noise by 10.

2.7. Linearity and range

The linear dynamic range for the assay was determined by analysis of calibration curve data. Residuals determined from calibration curve data were evaluated for trending, non-randomness and deviation from the x-axis. Linearity was also evaluated by calculating the response/concentration ratio across the range of standards. The RSD was determined across this range. Range of the assay is defined as the inclusive intervals between the upper and lower levels of analyte that can be determined with precision, linearity, and accuracy [9].

2.8. Capillary gas chromatography with MS

Structural confirmation of the hydrolyzed demethylcantharidin released was performed with a GC–MS system from Finnigan MAT GCQ. High-purity helium was used as carrier gas at a constant flow of 1 mL/min. The gas chromatographic separation was performed with the same DB-23 capillary column and oven temperature program as described in Section 2.3. Ionization was in all cases carried out in the electron impact (EI) mode at 70 eV. Scan function with automatic gain control (AGC), using a scan rate of 1 scan/s, was applied.

3. Results and discussion

Under the chromatographic conditions, the retention times of hydrolyzed demethylcantharidin and the internal standard benzophenone were 13.92 and 6.51 min, respectively. Recoveries of hydrolyzed demethylcantharidin from spiked samples was maintained above 90% at all concentration levels examined (Table 1), demonstrating the excellent efficiency of the extraction process. Accuracy of the assay as tested by spiking three different concentrations of hydrolyzed demethylcantharidin into normal saline and measuring the respective recoveries is also summarized in Table 1, where recoveries ranged from 93 to 98%.

Intra-assay variation was evaluated by measuring hydrolyzed demethylcantharidin at three different concentrations in a single assay, and inter-day variation was determined in three separate assays on different occasions (Table 2). The precision levels for both are acceptable, as demonstrated by the relatively low RSD values (<11%). In addition, the developed assay demonstrated good sensitivity, and the LOD and LOQ for hydrolyzed demethylcantharidin, as determined by different statistical meth-

Table 1
Extraction efficiency of hydrolyzed demethylcantharidin (DMC) from normal saline[a] and assay accuracy

Hyd. DMC[a] (μg/mL)	Direct injection[b] (peak area)	Spiked[b] (peak area)	Recovery[b] (%)	Accuracy[c] (%)	Direct injection Hyd. DMC/I.S. ratio[d]	Spiked Hyd. DMC/ I.S. ratio[d]	Recovery[d] (%)	Accuracy[c] (%)
0	0	0	NA	NA	0	0	NA	NA
50	5210	4910	94.5	94.2	0.081	0.076	94.5	93.5
500	53 580	51 743	97.2	98.4	0.849	0.842	99.2	95.3
750	76 235	78 522	103.1	97.5	1.274	1.213	95.6	96.9

[a] Each data point represents the mean of triplicate injections.

[b] Peak areas are peak areas of hydrolyzed DMC. No normalization to internal standard was performed in order to demonstrate the absolute amount of analyte that was extracted (i.e. extraction efficiency).

[c] Accuracy (%) = [1 − (mean concentration measured − concentration spiked)/concentration spiked]·100.

[d] Ratios are calculated as Peak area hydrolyzed DMC/Peak area benzophenone (I.S.). Ratios are compared to peak areas to show that benzophenone extracts similarly to hydrolyzed DMC.

Table 2
Assay reproducibility (intra-assay[a] and inter-assay[b] response precision)

Hydrolyzed DMC in sample (μg/mL)	Intra-assay[a]		Inter-assay[b]	
	Mean response (Hyd. DMC/I.S.) (peak area)	RSD (%)	Mean response (Hyd. DMC/I.S.) (peak area)	RSD (%)
50	0.076	9.5	0.084	10.2
500	0.842	5.6	0.851	4.2
750	1.213	6.3	1.235	5.8

[a] Each sample was prepared in replicates of five. Each sample in turn was taken through the acetonitrile extraction process and analyzed in replicates of five by GC–FID in a single assay.

[b] Three independent assays were performed. Data represent samples analyzed by GC–FID in replicates of five. Response represents the ratio of hydrolyzed DMC/benzophenone (I.S.) peak areas.

Table 3
Release of hydrolyzed demethylcantharidin (DMC) from compounds **1**–**5**[a] as assessed by the GC–FID method

Hyd. DMC released (μ*M*)[b]	Incubation time in NS (h)							
	0	1	2	4	6	8	12	24
1	0	0.086	0.153	0.302	0.456	0.628	0.922	1.67
2	0	0.019	0.045	0.095	0.144	0.303	0.603	1.282
3	0	0.048	0.096	0.176	0.264	0.495	0.785	1.568
4	0	0.040	0.076	0.159	0.246	0.435	0.731	1.470
5	0	0.203	0.452	0.832	1.032	1.385	1.935	2.072
DMC (control)	2.043	1.985	2.063	1.995	2.012	2.001	1.998	2.053

[a] Aliquots of compounds **1**–**5** were collected from incubation mixture in NS at the designated time points and freeze-dried. Hydrolyzed demethylcantharidin released was extracted into acetonitrile after acidification. Concentration of all compounds at the beginning of incubation was 2 m*M*.

[b] Data represent mean of three independent determinations.

ods, ranged from 1.27 to 1.97 and from 3.86 to 5.97 μg/mL, respectively. As noise measurements can vary from method to method, conservative estimates are a LOQ of 6 μg/mL and a LOD of 2 μg/mL.

The linearity of the GC–FID assay was determined from an average of five different assays of a typical standard curve with concentrations of hydrolyzed demethylcantharidin ranging from 0 to 1000 μg/mL. The least squares linear regression analysis showed the standard curve to be linear with a coefficient of determination (R^2) of 0.994±0.005. The data also demonstrate that the assay response is

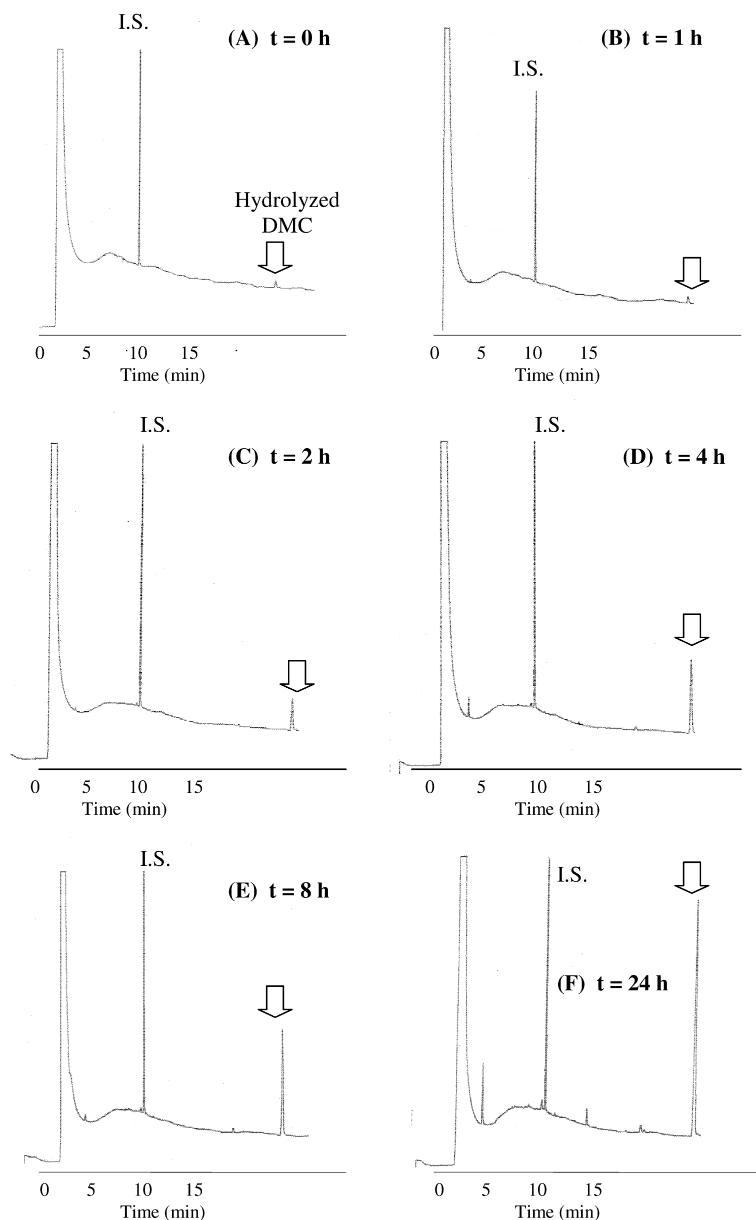

Fig. 3. Representative GC–FID chromatograms showing the progressive release of hydrolyzed demethylcantharidin from compound **1** incubated in NS (0 to 24 h) [I.S.=internal standard (benzophenone 2 mg/mL in acetonitrile)].

proportional to concentration across the range of standards. Additionally, the residuals do not show any trending or non-randomness, which is consistent with assay linearity. Thus, the linear range of the assay is 10 to 1000 μg/mL of hydrolyzed demethylcantharidin.

The validated GC–FID assay was applied to the determination of hydrolyzed demethylcantharidin released from the novel TCM–Pt compounds. Authentic hydrolyzed demethylcantharidin used as the control is readily detected and its concentration remained unchanged with incubation time in normal saline (NS). No hydrolyzed demethylcantharidin was detected from freshly prepared solutions of all novel TCM–Pt compounds. However, it became apparent that the release of hydrolyzed demethylcantharidin from compounds **1**–**5** occurred progressively over 24 h, which was readily followed by the GC–FID method, as illustrated in Table 3. The GC–FID chromatograms of compound **1**, being the representative of the TCM–Pt novel compounds, are shown in Fig. 3.

Evidence for the release of hydrolyzed demethylcantharidin from the novel TCM–Pt compounds is obtained by retention time indexing with authentic standard and further confirmed by mass spectrometry. The significant mass spectral fragments identified from the released DMC were m/z 68, 100, 169. The fragmentation pathway appears to follow a retro-Diels–Alder reaction (Fig. 4), which is the reverse of the classical Diels–Alder reaction between furan and maleic anhydride, the first step in the synthesis of demethylcantharidin. A retro-Diels–Alder reaction from the hydrolyzed demethylcantharidin yielded furan (m/z 68) as the diene fragment and elimination of 2,5-furandiol (m/z 100) as the ene entity. This behavior is supported by reports of similar thermal dissociation of bicyclic dienes via retro-Diels–Alder reactions [10,11].

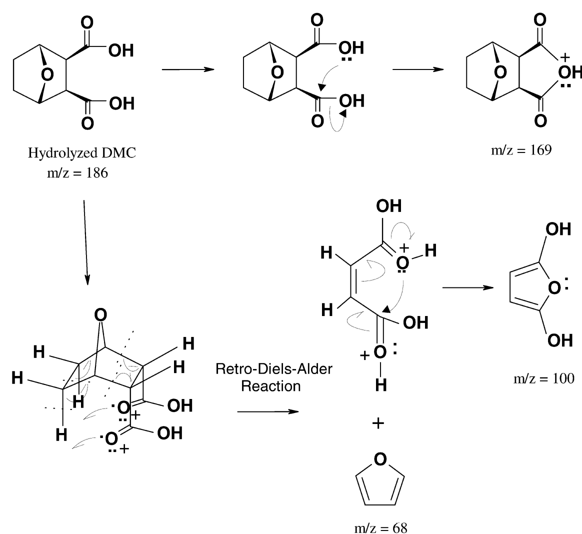

Fig. 4. Proposed fragmentation pathway for the EI mass spectra of hydrolyzed demethylcantharidin released from the novel TCM–Pt compounds.

4. Conclusion

The developed GC–FID/GC–MS method has been shown to be fast, simple and accurate for the determination of hydrolyzed demethylcantharidin that is released from novel TCM–Pt compounds **1**–**5**. This new GC–FID method has the added advantage that almost none of the clinically useful Pt-based drugs can be analyzed by conventional gas chromatographic methods because of their ionic nature and low vapor pressure [12]. This successful method development enabled us to clearly establish that compounds **1**–**5**, after incubation in normal saline, released the hydrolyzed demethylcantharidin progressively over a period of 24 h, leading to a gradual increase in protein phosphatase inhibitory effect. Based on this result, we were able to propose a potentially new mechanism of cytotoxic action for Pt-based anticancer agents.

The simplicity and reliability of the GC–FID method should prove to be a valuable tool for future pharmacokinetic and metabolic studies of the novel TCM–Pt compounds where the released hydrolyzed demethylcantharidin can be readily identified and quantified.

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