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Gas chromatographic–mass spectrometric confirmation of atractyloside in a patient poisoned with *Callilepis laureola*

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Abstract

The South African traditional remedy *Impila* (*Callilepis laureola*) contains the mitochondrial toxin atractyloside. The plant is sold widely and continues to lead to fatalities in patients. We describe, for the first time, a simple GC–MS procedure for the identification of atractyloside, which we have applied to the gastric washing from a poisoned patient and to extracts of *Impila* tuber.

Keywords: *Callilepis laureola*; *Impila*; Atractyloside

1. Introduction

Plants containing atractyloside (ATR) are used worldwide, but especially in Africa and the Mediterranean regions. In South Africa, the Zulus use the tuber of *Callilepis laureola*, known as *Impila*, for the treatment of cough, as a vermicide and decongestant, to induce fertility, to correct impotence and to ward off ''evil spirits''. *Callilepis laureola* is a woody tuber with a bulbous shape and pungent odour. The tuber, either fresh or dried, is crushed, boiled in water and used as a tea [1]. The major human clinical studies have been carried out in South Africa, where severe hepatic pathology following the ingestion of herbal medicines is not uncommon. In most cases the identity of the plant is not confirmed [2]. ATR was first documented as leading to severe poisoning in humans in 1909 [3] and there have been regular reports since [4–8]. In a survey by Wainwright and Schonland in 1977 it was found that 30% of Zulus in KwaZulu Natal had used *Impila* at some time in their life [9]. The plant is widely available in the traditional remedy (muti) markets in South Africa [10]. In the largest two series, Wainwright et al. [11] found that 40% of cases were in children under the age of 10 and Watson et al. [5] reported 50 cases, all of which were children. The most recent series is that of Grobler et al. [8] who reported 10 cases. Our own experience consists of six cases, three of whom were adults. Presentation follows a history of convulsions and gastrointestinal symptoms. In 80%, there is confusion, stupor or coma. In fatal cases, death occurs either within a few hours due to hypoglycaemic coma [5,12], or within a few days (<4)

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from hepatic failure [6,8,9]. Survival figures are extremely low, with only 14% of patients surviving beyond 48 h in one series [5] and 10% in another [8].

ATR competitively inhibits ADP transport. This inhibition prevents the synthesis of ATP leading to cell death due to energy starvation [13,14]. Interest in the toxic effect of ATR in man was rediscovered in Europe after poisonings in Italy in 1955 [15], Morocco in 1969 [16] and Algeria in 1975 [17]. In South Africa, where severe hepatocellular necrosis following the ingestion of herbal medicines is not uncommon, the identity of the causative agent is seldom confirmed [2].

ATR was first extracted from the Mediterranean (glue) thistle, *Atractylis gummifera*, in 1868 and characterised by Lefranc [18,19]. Further studies by Piozzi et al. confirmed that ATR has a molecular mass of 803 a.m.u. [20]. Potassium atractylate is a hydrophilic glycoside, whose aglycone, atractyligenin, is an acidic hydrophobic, non-volatile diterpene with a pentahydrophenanthrene like structure [21]. The aglycone is joined to the glucose moeity via a β-glycosidic bond [22]. The carboxyl group on C_4 of the diterpene ring is most crucial to the toxicity and reduction of this group to the alcohol (atractylitriol) renders the molecule non-toxic [23].

Nuclear magnetic resonance [24] and mass spectrometry (MS) [25] have been applied to the analysis of plant material. A detailed study, using X-ray analysis has been made of the constituents of *Atractylodes lancea* [26]. There have also been a few studies using chromatographic techniques [27–29]. Thin-layer chromatography has been used on one occasion to screen for ATR in the gastric washing and urine of a poisoned patient [30] but there are no reports of confirmation of ATR in the clinical situation. Since we are regularly confronted with the need to confirm *Impila* ingestion in fatal cases, we embarked upon the development of such an assay using gas chromatography (GC)–MS.

2. Experimental

2.1. Materials

Ethyl acetate and methanol (HPLC grade) were obtained from Merck (Darmstadt, Gemany). Pyridine (silylation grade) and trimethyl silyl imidazole (TMSI) from Pierce (Rockford, IL, USA), were used for derivatisation. The internal standard, 5-α-androstane-3β,17β-diol, and the potassium salt of atractyloside were obtained from Sigma (Steinheim, Germany).

2.2. Plant specimen

A 1-g amount of *Callilepis* tuber was ground to a powder and extracted in water (10 ml) at 100°C for 15 min.

2.3. Standards

Individual solutions of ATR (potassium salt) and 5α-androstane-3β,17β-diol, were prepared in methanol. The concentrations of ATR and 5α-androstane-3β,17β-diol were 12.5 mmol/l and 34.2 mmol/l, respectively. The standard solutions were stored at 4°C.

2.4. Sample preparation

A 1-ml volume of standard solution was dried in a 10-ml glass tube. Internal standard solution (1 ml) was added to the dry ATR standard, human gastric wash (1 ml) and plant extract (1 ml) before the samples were acidified with hydrochloric acid (2 ml, 2 mol/l). The tubes were sealed with PTFE lined caps, vortexed and stored overnight at room temperature. The hydrolysates were extracted five times with ethyl acetate (2 ml). The mixtures were vortexed at each stage to enhance extraction efficiency. The combined organic extracts were dried under a stream of nitrogen at 40°C. Pyridine (200 μl) and TMSI (200 μl) were added to the dried extracts and derivatisation performed at 100°C for 2 h.

2.5. GC–MS conditions

A HP 6890 gas chromatograph equipped with a HP 7683 auto injector and a HP 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA, USA) was used for GC–MS analysis. Data collection and integration was performed with HP Chem Station

software. Chromatographic separations were performed with a J&W Scientific, DB-1 capillary column (30 m×250 μm I.D., film thickness: 0.1 μm) (Folsom, CA, USA).

Samples (2 μl) were injected in the pulsed splitless mode, the pulse time being 1.5 min and the pressure 200 kPa. The injector temperature was 250°C. Helium carrier gas flow-rate was 1.0 ml/min. The column temperature was programmed from 215 to 310°C at 1.6°C/min, with an initial isotherm of 3 min and a final isotherm of 10 min. Total run time was 72 min. All mass spectra were recorded at 70 eV. The mass range was 50–800 u.

2.6. Study of hydrolysis time

An 8-ml volume of standard solution was dried in a 50-ml flask. An equal volume of internal standard solution was added to the dry ATR standard. The sample was acidified with hydrochloric acid (16 ml, 2 mol/l). Samples (1 ml) of the hydrolysis mixture were taken every hour and subjected to the rest of the sample preparation procedure.

2.7. Identification

The mass spectra and chromatograms of the derivatised ATR standard were used to confirm the presence of ATR in the gastric wash and plant extract.

3. Results and discussion

A chromatogram obtained from the *Impila* tuber extract is shown in Fig. 1a and the mass spectrum of the penta-TMS derivative of atractyloside in Fig. 1b. The retention time for the trimethyl silyl ether derivatives of 5-α-androstane-3β,17-β-diol and ATR were 14.7 and 58.85 min, respectively.

The chromatogram of the extract of gastric lavage from the patient is shown in Fig. 2a and the mass spectrum of the ATR derivative at 58.31 min is shown in Fig. 2b.

The presence of ions with m/z 447 and 463 indicates that fragmentation of the molecule takes place on both sides of the glycosidic bond [25]. The position of fragmentation leading to the formation of these two ions is indicated in Fig. 3. Fragmentation on the glucose side of the glycosidic oxygen, coincidentally led to the formation of two ions with the same mass, i.e., m/z 463. Fragmentation on the diterpene side of the glycosidic oxygen led to the formation of ion m/z 447. The presence of both these ions confirms that the whole glycoside was indeed detected and that the glycosidic bond was not hydrolysed during acidic hydrolysis. Only the sulfate groups were hydrolysed to form the corresponding alcohol. The base peak m/z 289 is formed by loss of $[C_4H_9O]$ and $[OSiMe_3]$ fragments. The molecular ion at m/z 926, which is weak according to literature [25], could not be detected since the upper limit of the quadrupole was 800 a.m.u.

It was initially suspected that the glycosidic bond would undergo acidic hydrolysis to yield the aglycone of ATR, atractyligenin. It was for this reason that the non-endogenous steroid, 5α-androstane-3β,17β-diol, was selected as internal standard. The relative small amount of gastric lavage fluid that was available from the patient (1.5 ml in total) limited more extensive experimentation to find a more suitable internal standard. Typically, atractyligenin, parquine or carboxparquine would have been more suitable since they are structurally more related to ATR. The ATR concentrations in the aqueous extract and gastric washing were found to be 16.6 and 0.6 mmol/l, respectively.

The limited amount of sample as well as the uncertainty of what other compounds would be present in the gastric lavage, led to the use of a low oven temperature ramp to obtain maximum separation. This resulted in excessive resolution and a prolonged separation time. The detection limit for ATR at signal-to-noise ratio of 3 in the gastric washing by the described method, was calculated as 0.0451 mmol/l. Focusing the analytes at a low temperature in the first few millimetres of the analytical column, followed by a more rapid increase in oven temperature, will not only decrease the separation time but will also result in less peak broadening and ultimately in lower detection limits.

In addition to decreased detection limits, the enhanced selectivity in the single ion monitoring (SIM) mode has the potential of decreasing the chromatographic separation time. The chromatogram of the gastric fluid, reconstructed with ion m/z 289 is

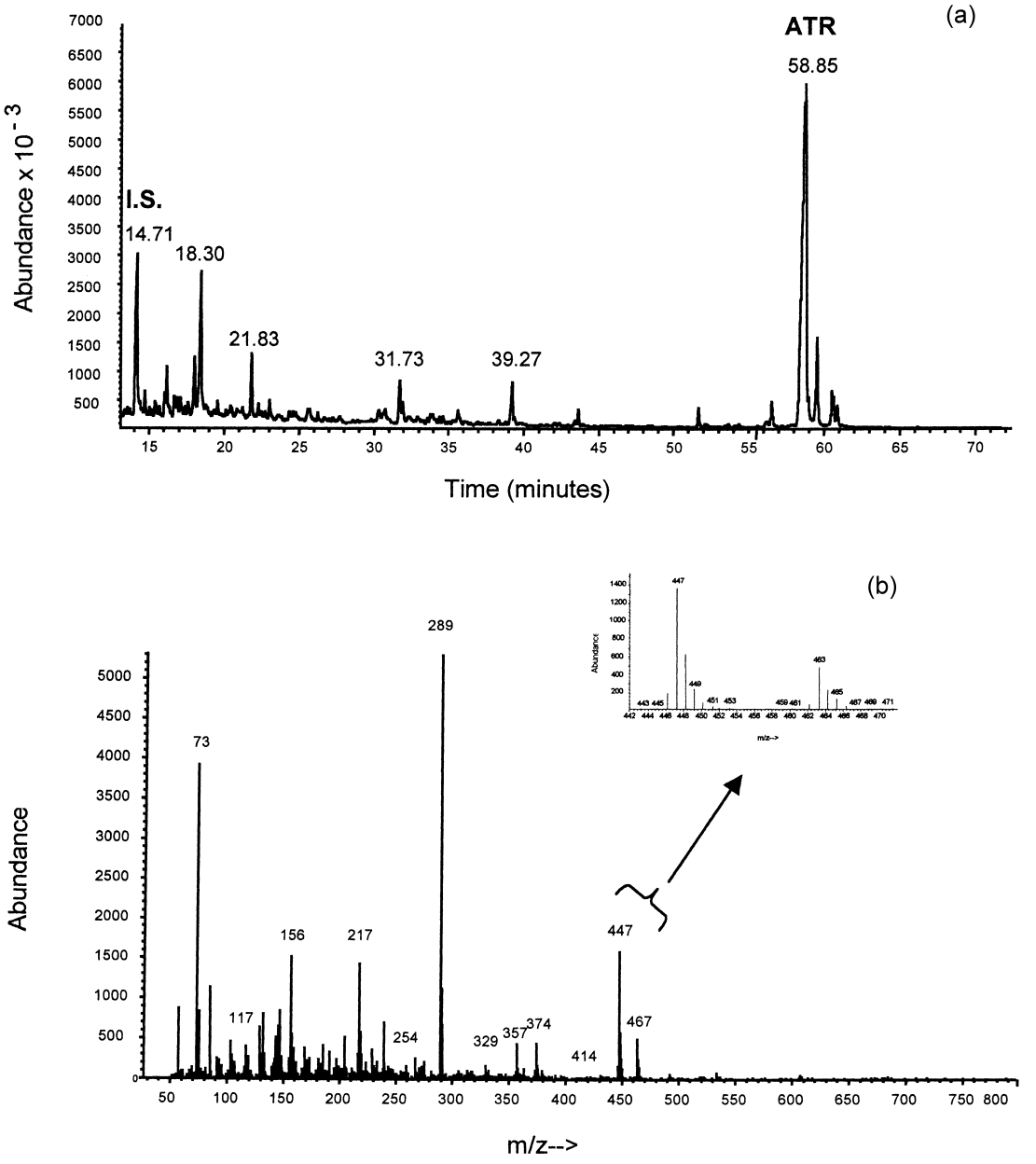

Fig. 1. (a) Chromatogram of the *Impila* extract. (b) Mass spectrum of the penta-TMS derivative of atractyloside in the tuber extract.

shown in Fig. 4. It shows that if the chromatogram was recorded in the SIM mode, the interference from other peaks would be low. Thus, by taking advantage of the high selectivity of SIM, the chromatographic analysis time could be reduced substantially by adjusting the oven temperature conditions for a faster separation. This procedure will be useful for screening but will give less structural information. Full scan mass spectrometry is preferable for confirmation of the presence of the toxin.

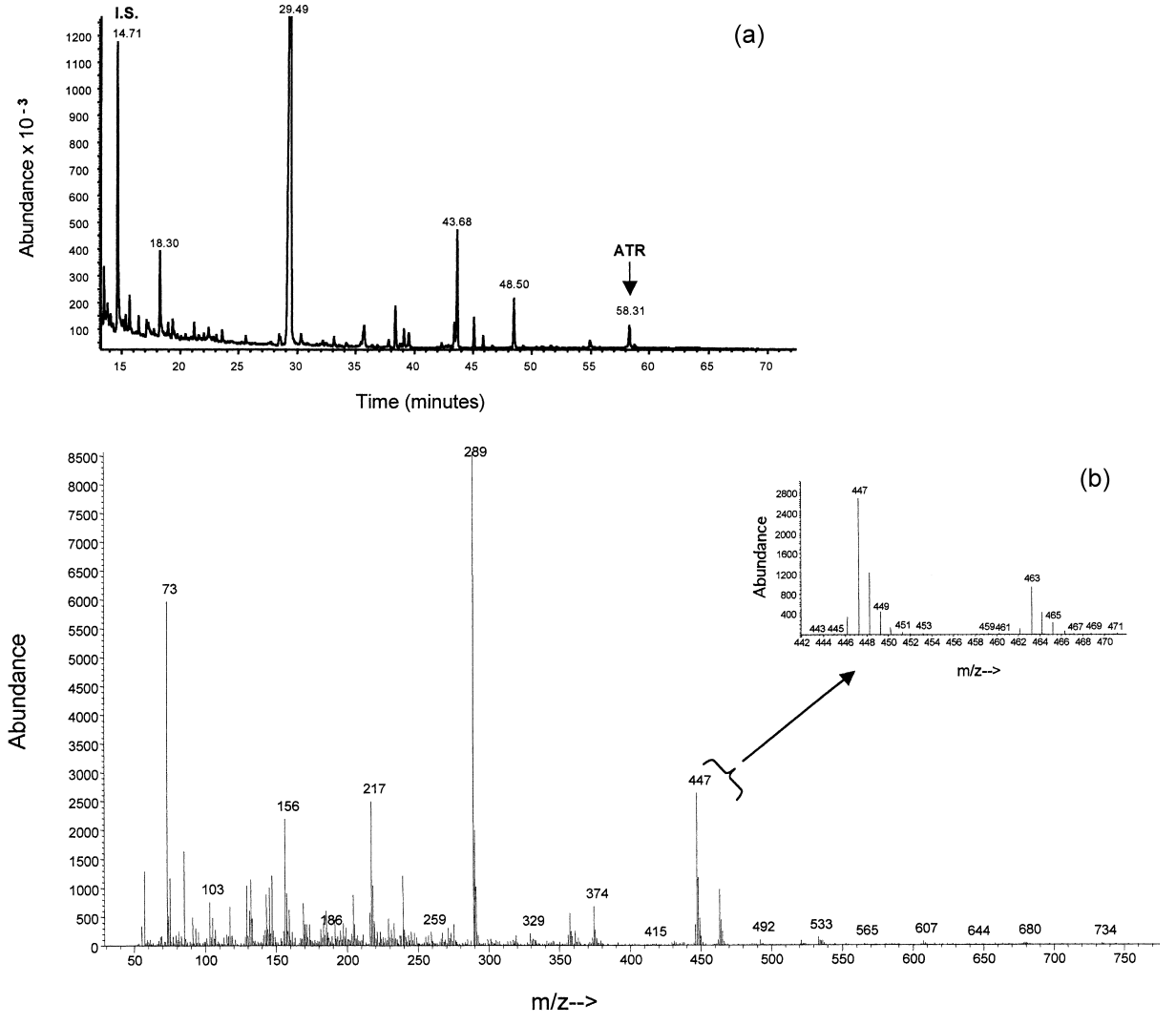

Fig. 2. (a) Chromatogram of the gastric wash extract of the poisoned patient. (b) Mass spectrum of the penta-TMS derivative of atractyloside in the gastric wash.

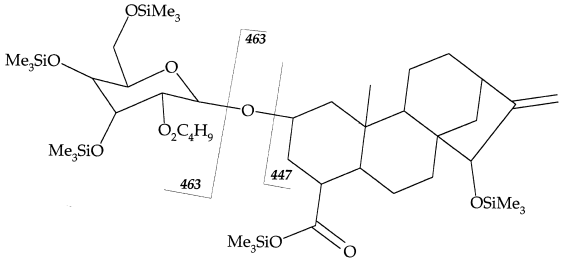

Fig. 3. Structure of the penta-TMS derivative of ATR and assignment of masses to the ion fragments.

Acidic hydrolysis during sample preparation was performed at room temperature. Attempts to hydrolyse standards at elevated temperatures resulted in poor derivatisation yields. Fig. 5 shows a plot of the response of ATR relative to that of the internal standard for acidic hydrolysis over a period of 14 h. The optimum hydrolysis time is approximately 10 h.

Moderate enzymatic hydrolysis with β-glycosidase will lead to the formation of the free diterpene. Screening for atractyligenin would reduce the total

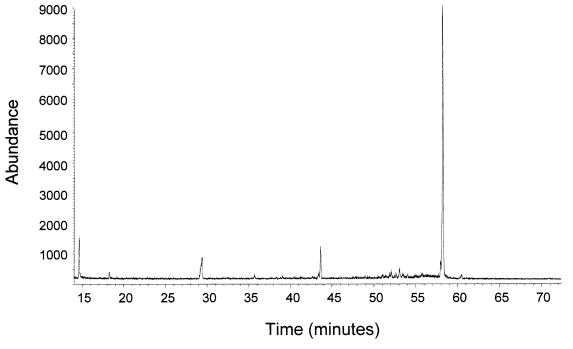

Fig. 4. A chromatogram reconstructed with ion m/z 289 from the full scan chromatogram of the gastric wash.

analysis time since the aglycone will elute earlier than the glucoside. This procedure could be used as an additional confirmatory procedure for the presence of atractyloside.

The method described was carried out on the only tissue available to us, namely gastric washings. At the time of the analysis ethical clearance had not been obtained for collection of the extra blood and urine specimens, which would have been necessary for studies in these fluids. The method will be used in future cases to quantitate ATR and possibly also to study the pharmacokinetics.

4. Conclusions

A novel confirmation procedure for the presence of ATR in a biological fluid from a poisoned patient is reported which has the potential to be employed as an emergency screening procedure. GC–MS with its superior analytical sensitivity and selectivity, can also be used to confirm the presence of ATR in traditional remedies and plant material.

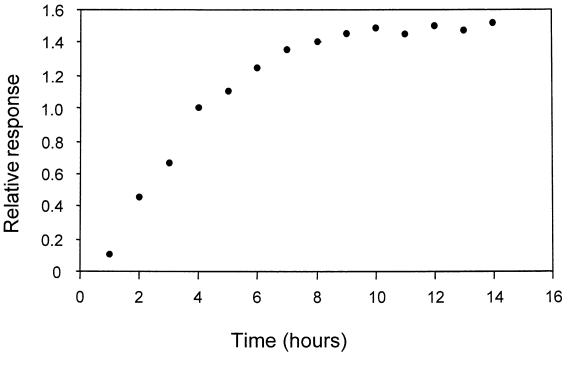

Fig. 5. Relative response of the penta-TMS derivative of ATR vs. time during acid hydrolysis.

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