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IDENTIFICATION OF TWO THORMÄHLEN-POSITIVE COMPOUNDS FROM MELANOTIC URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

The isolation of two Thormählen-positive compounds from the urine of a patient with malignant melanoma and the elucidation of their structure by gas chromatography—mass spectrometry is described. The compounds were isolated using a poly-N-vinylpyrrolidone column and separated by preparative thin-layer chromatography. After elution they were analyzed by gas chromatography and gas chromatography—mass spectrometry as their trimethylsilyl derivatives and after hydrolysis also as their *tert.*-butyldimethylsilyl derivatives. The results showed the main Thormählen-positive compound A to be the glucuronide of 5-hydroxy-6-methoxyindole, whereas the minor compound AX appeared to be the glucuronide of its isomer 6-hydroxy-5-methoxyindole.

INTRODUCTION

The suggested biochemical pathway towards the production of eumelanin involves the formation of compounds of an indolic nature [1—3]. Therefore, it is not unreasonable to assume that the overproduction of melanin is the cause of

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an increased excretion of indolic compounds in the urine of patients suffering from pigmented malignant melanoma [4—6].

The group of so-called Thormählen-positive indoles [7] consists of at least five indolic compounds [4] which contain unsubsituted pyrrole rings [8]. The use of paper chromatography or thin-layer chromatography (TLC) for the investigation of their structure has been described by several authors [9—14]. Recently, the structure of one Thormählen-positive compound has been studied by nuclear magnetic resonance spectroscopy [15].

This paper describes the use of gas chromatography—mass spectrometry for the elucidation of the structure of two Thormählen-positive compounds which were isolated from the urine of a patient with melanoma using a poly-N-vinylpyrrolidone (PVP) column and TLC.

EXPERIMENTAL

Chemicals

Ethyl acetate (gas chromatographic (GC)—spectroscopic quality) was purchased from Baker Chemicals (Deventer, The Netherlands), beta-glucuronidase from *Escherichia coli* (100 U/ml) was from Boehringer (Mannheim, G.F.R.), *tert.*-butyldimethylchlorosilane—imidazole reagent was from Applied Science Labs. (State College, PA, U.S.A.), PVP was from Polyclar (Gaf, Austria).

5-Hydroxy-6-methoxyindole (5H6MI) and 6-hydroxy-5-methoxyindole (6H5MI) were prepared by decarboxylation of the corresponding indole-2-carboxylic acids and kindly supplied by Dr. Budešinská from Prague. All other chemicals and solvents were purchased from Merck (Darmstadt, G.F.R.). Glass-distilled water was used throughout.

Apparatus

Analyses were performed on Varian 3700 gas chromatographs equipped with capillary columns and flame-ionisation detectors. A capillary column, 25 m × 0.26 mm I.D., coated with SE-54 (Franzen Analysen-Technik, Bremen, G.F.R.) was used for the analysis of *tert.*-butyldimethylsilyl (*t*-BDMS) derivatives. The column temperature was programmed as follows: initial temperature at 120°C was maintained for 5 min and then programmed to 260°C at 4°C/min and maintained at 260°C for 30 min. Helium flow-rate was 0.73 ml/min. Injector and detector temperatures were 280°C. For the analysis of trimethylsilyl (TMS) derivatives a column 25 m × 0.26 mm I.D. coated with SE-30 (Jaeggi, Labor für Chromatographie, Trogen, Switzerland) was used. The oven temperature was programmed from 100 to 250°C at 4°C/min; detector and injector temperatures were set at 250°C. For the analysis of per-TMS derivatives of glucuronide conjugates, the temperature was programmed at 20°C/min.

Gas chromatographic—mass spectrometric (GC—MS) analyses were performed with a Varian Aerograph 1400 gas chromatograph coupled to a Varian MAT 112 mass spectrometer, equipped with a 17.5 m × 0.25 mm I.D. capillary column coated with SE-54 (Franzen Analysen-Technik). Helium flow-rate was 4 ml/min, ionisation energy 70 eV, injector temperature 250°C, source temperature 250°C, interface (all glass) temperature 275°C. Column temperature was programmed from 200 to 250°C at 10°C/min (glucuronide conjugates), or from 150 to 250°C at 4°C/min (unconjugated indoles).

Samples

Urine was obtained from a patient with generalized malignant melanoma. The generalisation was characterized in particular by an enlargement of the liver caused by melanoma metastases. The concentration of Thormählen-positive compounds was extraordinarily high — approx. 400 μg equivalents of indole per millilitre.

Procedure

The isolation of Thormählen-positive compounds from melanotic urine was performed using a 10 × 1 cm column filled with PVP (160—200 mesh). Two millilitres of melanotic urine were applied to the column, after which the compounds were eluted with water. The volume of each fraction was 1 ml. The content of Thormählen-positive compounds was measured using the Thormählen reaction as described previously [16]. The procedure was repeated four times, after which fractions of the second positive peak were pooled and lyophylized. The residue, originating from 8 ml of melanotic urine, was dissolved in 4 ml of methanol. The solution was preparatively applied to TLC plates (Cellulose F, precoated, 20 cm × 20 cm × 0.1 mm; Merck). Purification on TLC was carried out in an *n*-butanol—pyridine—water (1 : 1 : 1) solvent system. The bands were visualized on both edges by performing a Thormählen reaction [11]. Unsprayed bands were scratched off under UV light. The elution of two Thormählen-positive bands designated A and AX was performed overnight using the TLC solvent system. After elution the samples were divided into two parts and freeze-dried.

One part of each Thormählen-positive compound was hydrolyzed by adding 2 ml of sodium acetate buffer (0.5 mol/l, pH 6.5) and 100 μl of beta-glucuronidase solution, and incubating for 1 h at 37°C in a shaking water-bath. After incubation, the hydrolysate was extracted twice with 2 ml of ethyl acetate. Pooled extracts were dried over anhydrous sodium sulphate and evaporated under a stream of nitrogen at 40°C.

t-BDMS derivatives were prepared by treating part of the hydrolyzed samples with 100 μl of *t*-BDMS reagent mixture for 2 h at 80°C. After derivatisation, 0.5 ml of *n*-hexane was added, thoroughly mixed and centrifuged. The hexane layer was transferred to a clean tube and used for GC and GC—MS examination.

Per-TMS derivatives of Thormählen-positive compounds eluted from the TLC plates were prepared by treating the sample with 100 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 80°C for 30 min. TMS derivatives of extracted hydrolysates were prepared in a similar way; 1-μl aliquots were examined by GC and GC—MS.

RESULTS

A quantitative profile of Thormählen-positive compounds eluted from the PVP column is shown in Fig. 1. The control TLC carried out simultaneously indicated that the first peak was caused by a large amount of impurities forming a turbid solution. The second peak, however, was consistent with the compounds of interest. Fraction No. 5 contained a relatively pure compound that

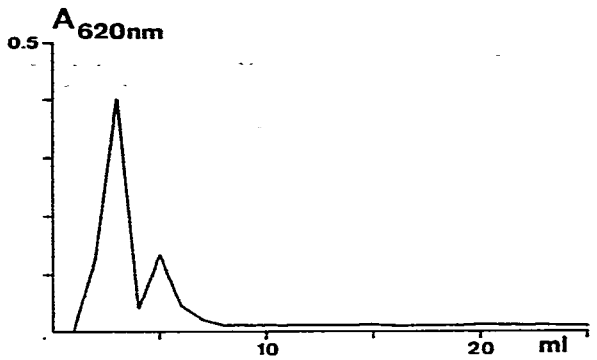

Fig. 1. A profile of Thormählen-positive compounds eluted from PVP. The second peak was found to consist of Thormählen-positive indolic compounds.

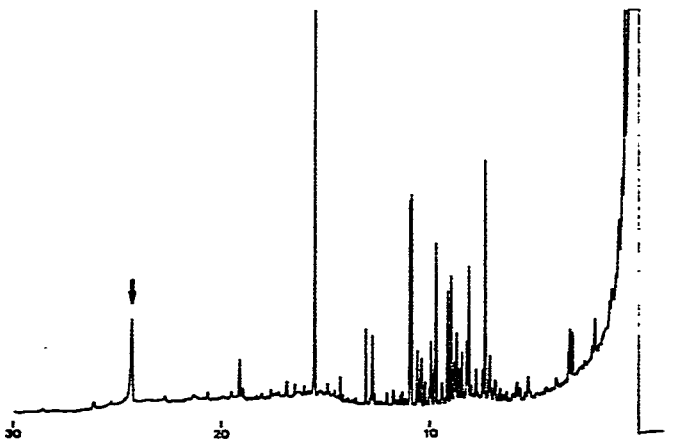

Fig. 2. Gas chromatogram of the per-TMS derivative of Thormählen-positive compound A. The arrow indicates its position in the chromatogram.

is believed to be the main Thormählen-positive indole in melanotic urine [4, 5, 9, 10, 12—14].

A more detailed study showed the presence of a small amount of a second Thormählen-positive compound. In accordance with earlier reports, the main compound was designated A and the minor compound AX. Hence, both compounds were separated on TLC having different R_F values in the solvent system mentioned above (A 0.55, AX 0.47). Derivatisation of these compounds after elution from TLC yielded per-TMS derivatives which formed a peak with a long retention time (Fig. 2). The Kovats retention index (isothermal, 250°C) was 30.67. It was not possible, however, to separate A and AX compounds, presumably because of the high similarity of their per-TMS derivatives. Mass spectra taken of these compounds showed a small molecular ion consistent with that calculated for methoxyhydroxyindole glucuronides (M^+ = 699). In accordance with other authors studying glucurono conjugates [17—19], we were able to assign fragment ions of a glucuronic acid moiety (m/e = 204, 217, and the very characteristic ion of m/e = 375) (Fig. 3).

The indolic moiety was characterized by two fragments. The cleavage of the

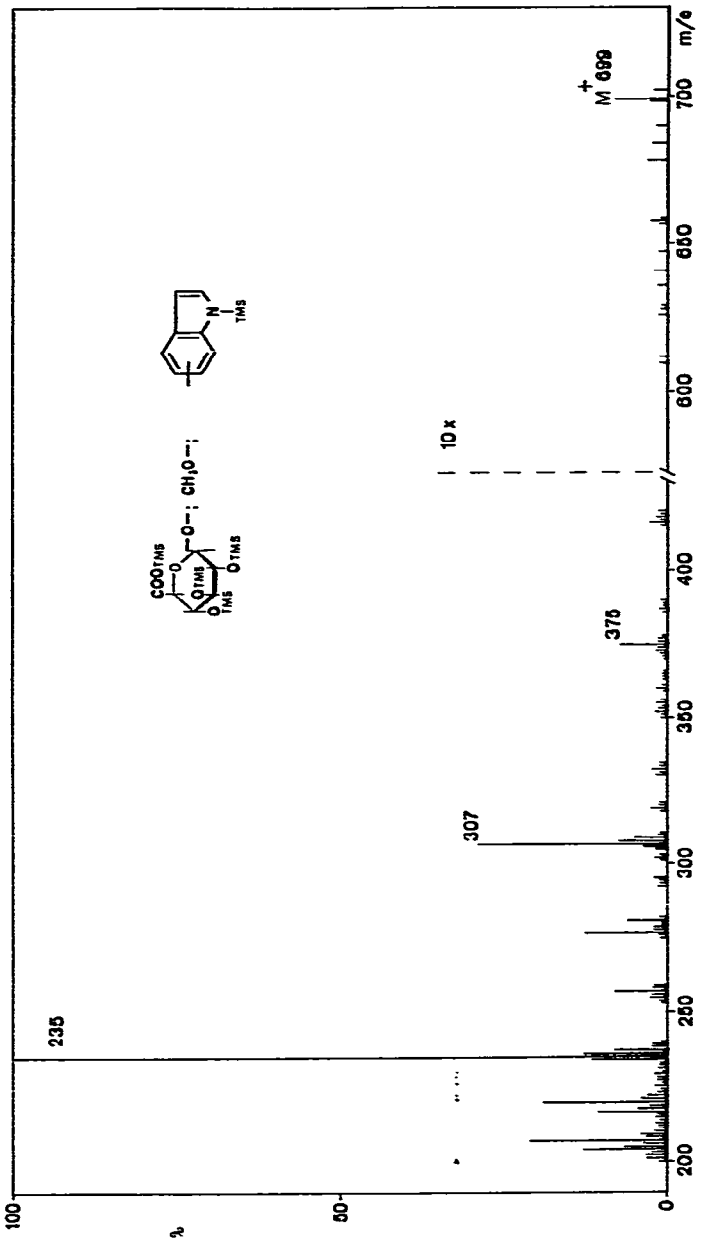

Fig. 3. Mass spectrum obtained from the per-TMS derivative of the glucuronide conjugate of hydroxymethoxyindole.

ether link led to the loss of the glucuronic moiety, resulting in the formation of a fragment ion with m/e = 235. In analogy to similar cases [17, 19], the fragment ion with m/e = 307 is considered to be formed by the elimination of the glucuronic acid moiety and rearrangement of a TMS group onto the hydroxymethoxyindole.

In order to elucidate the position of the substituent in the indolic ring, the hydrolyzed samples of both Thormählen-positive compounds were derivatized and analyzed by GC—MS. Mass spectra of TMS derivatives (Fig. 4) as well as of *t*-BDMS derivatives (Fig. 5) indicated the presence of a methoxyhydroxyindole

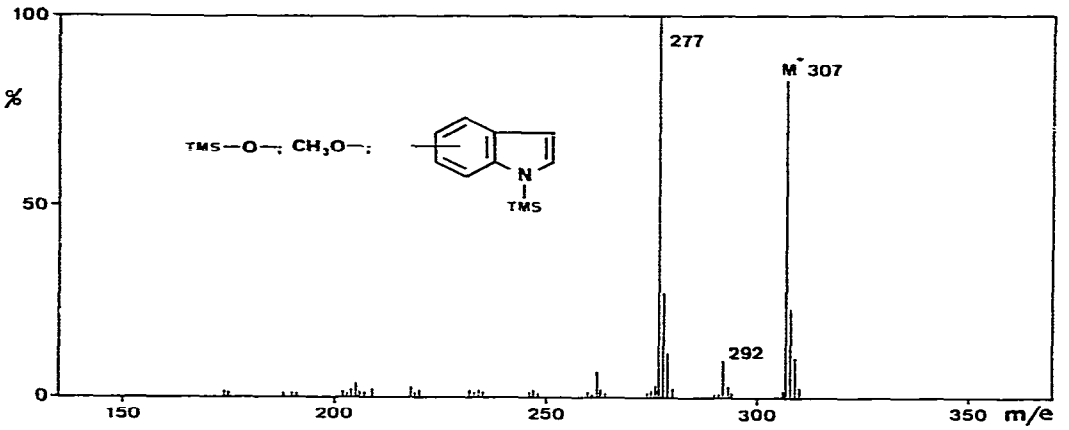

Fig. 4. Mass spectrum of the TMS derivative of hydroxymethoxyindole.

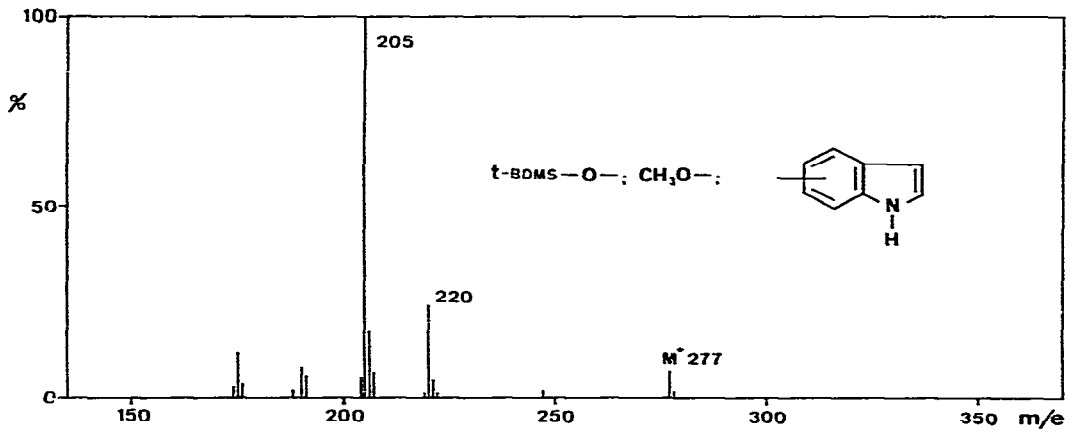

Fig. 5. Mass spectrum of the *t*-BDMS derivative of hydroxymethoxyindole.

TABLE I

METHYLENE UNIT VALUES OF ISOMERS OF METHOXYHYDROXYINDOLE

	TMS	*t*-BDMS
5H6MI	18.72	21.26
6H5MI	18.59	21.13

in the hydrolyzed samples. The comparison of retention times (Table I) with the synthetic 5H6MI and 6H5MI showed that Thormählen-positive compound A contains 5H6MI and minor AX contains isomeric 6H5MI.

DISCUSSION

Attempts to elucidate the structure of Thormählen-positive compounds are numerous. However, few reports have been conclusive until now. Almost half a century ago, Linnel and Raper [20] considered these compounds to be glucuronides or sulphates of 5,6-dihydroxyindole. Leonhardi [9] discovered three Thormählen-positive compounds using paper chromatography and named them A, B and C. He proposed that the structure of compound A was consistent with 5,6-dihydroxyindole bound to a dipeptide from pyrrolidonecarboxylic acid and glutamine [21]. In 1962 Anderson [10] reported the identification of esteric sulphates and the remarkable quantity of glucuronic acid in two different fractions of Thormählen-positive compounds. A reliable proof for the presence of glucuronic acid in Thormählen-positive compound A was provided by Pechan [5, 13]. However, most authors were unable to provide the correct structure of the indolic part of the molecule. Atkinson [12], who also reported glucuronic acid to be part of the molecule of Thormählen-positive compound A, showed the indolic component to be a mixture of 5H6MI and 6H5MI in the approximate ratio of 5 : 1. In the light of our findings, it is most likely that his compound A was a mixture of A and AX. Recently, the structure of compound A was also studied by nuclear magnetic resonance spectroscopy [15]. On the basis of the results obtained, the structure of the glucurono conjugates of 5H6MI has been proposed.

The existence of compound AX has only been described a few times [4, 5, 13, 14]. This compound could be overlapped by the main component A in most cases. In our samples the concentration of A was estimated to be approximately ten times higher than that of AX. Although methylation on position 6 is preferred [22] the ratio between the isomers may be rather variable.

In conclusion, this study shows that GC and/or GC—MS can be very useful for the elucidation of the structures of melanin precursors and their conjugates. In the past, the high instability of unconjugated indolic compounds could have been the main cause of unsuccessful studies. GC and/or GC—MS analysis, besides providing high sensitivity and specificity, offers the possibility of studying volatile derivatives of these compounds that are much less sensitive to oxidation. Quantitative measurements of these compounds using GC and GC—MS are in progress.

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