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Note

Analysis of eumelanin-related indolic compounds in urine by high-performance liquid chromatography with fluorimetric detection

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Eumelanin-related indolic compounds are specific metabolites of pigment tissue. They are formed in melanocytes during melanogenesis, and a small part of them is then excreted from the cells into extracellular space after being partly methylated [1] The compounds are then transported in blood and eliminated from the body, some of them as conjugates with glucuronic [2] or sulphuric [3, 4] acid

Increased amounts of these substances are frequently found in the urine of patients suffering from malignant melanoma Seven indolic eumelanin-related compounds have been identified in melanotic urine [5–7] Two of them, namely 5,6-dihydroxyindole (5,6DHI) and 5,6-dihydroxyindolyl-2-carboxylic acid (5,6DHI2C), are direct eumelanin precursors, the others are their O-methylated derivatives· 5-hydroxy-6-methoxyindole (5H6MI), 6-hydroxy-5-methoxyindole (6H5MI), 5-hydroxy-6-methoxyindolyl-2-carboxylic acid (5H6MI2C), 6-hydroxy-5-methoxyindolyl-2-carboxylic acid (6H5MI2C) and 5,6-dimethoxyindolyl-2-carboxylic acid (5,6DMI2C).

The determination of the indolic substances may be of interest in studies dealing with melanin pigmentation and malignant melanoma In 1983, we described a gas chromatographic–mass spectrometric method employing deuterium-labelled analogues as internal standards [8]. To our knowledge, that method was the only one available for the profiling of eumelanin-related compounds. In order to set up a generally more accessible method for the analysis of eumelanin-related metabolites, we turned our attention to high-performance liquid chromatography (HPLC)

The present paper describes a procedure for separation and detection of

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eumelanin-related indolic substances in urine using reversed-phase HPLC with fluorimetric detection.

EXPERIMENTAL

Chemicals

Eumelanin-related indolic compounds were synthesized from 3,4-dihydroxyphenylalanine and 3,4-dihydroxyphenylethylamine as described elsewhere [9]. 5-Methoxyindolyl-2-carboxylic acid (5MI2C) was purchased from Janssen Chimical (Beerse, Belgium). Helix pomatia juice was from l'Industrie Biologique Francaise (Genneviliers, France). 5-Hydroxyindolyl-3-acetic acid (5HIAA) and indolyl-3-acetic acid (IAA) from Sigma (St Louis, MO, U S A) and indican from Fluka (Buchs, Switzerland) were used to confirm their presence in urinary extracts. All other chemicals and solvents were obtained from Merck (Darmstadt, F R.G) and used without any further purification.

Chromatographic conditions

Apparatus The chromatographic system consisted of a Varian Model 5000 liquid chromatographic pump, a Waters Model 710B WISP automatic sample injector and an RP-8 Spherisorb ODS-5 analytical column (10 cm × 4.6 mm I.D) with a 3-cm guard column (Brownlee Labs., Santa Clara, CA, U S A) The column was operated at ambient temperature Detection was accomplished by a Perkin-Elmer Model 650-10S fluorescence spectrophotometer with excitation and emission wavelengths set at 305 and 360 nm, respectively, and with the slit bandwidth set at 15 nm

Mobile phase The mobile phase consisted of (A) methanol and (B) sodium acetate buffer solution (0.05 mol/l) containing 0.01 mol/l ascorbic acid and 0 001 mol/l Na_2EDTA (pH 4 30) Methanol gradient elution was used starting from 15% (v/v) methanol with an increase to 40% (v/v) from 5 to 20 min The flow-rate was kept constant at 1 5 ml/min.

Sample preparation

With hydrolysis To 4 ml of urine, 1 μg of 5MI2C (internal standard, I S.) was added followed by 500 μl of 1.25 mol/l sodium acetate buffer (pH 6 2) and 100 μl of Helix pomatia juice containing β-glucuronidase and sulphatase activity The sample was shortly bubbled with nitrogen and incubated in a shaking water-bath at 37°C for 90 min. After the incubation, the sample was saturated with sodium chloride, bubbled again with nitrogen and extracted with 2 × 4 ml of diethyl ether Pooled extracts were evaporated and redissolved in 500 μl of the HPLC elution mixture containing 15% (v/v) of methanol Aliquots of 20 μl were usually used for injection on the column

Without hydrolysis Urine (4 ml) was mixed with internal standard (I S.) and sodium acetate buffer as described above The sample was then saturated with sodium chloride, bubbled with nitrogen and extracted with 2 × 4 ml of diethyl ether Pooled extracts were processed as described above

RESULTS AND DISCUSSION

A chromatogram of a mixture of synthetically prepared indolic eumelanin-

related substances with the I.S. is shown in Fig. 1 As can be seen, the chromatographic conditions made it possible to separate all of the seven indolic compounds of very similar structure.

When we analysed the diethyl ether extracts of normal unhydrolysed urine (Fig 2A) we could, besides the isomeric 5H6MI2C and 6H5MI2C, always detect two other indolic substances 5HIAA and IAA Their identity was confirmed by comparing their chromatographic behaviour and mass spectra (Figs 3 and 4) with those of the standards After hydrolysis with Helix pomatia juice, a few other indoles excreted as conjugates (e g 5H6MI, 6H5MI and a substance tentatively identified as 3-hydroxyindole) appeared on the chromatograms (Fig 2B) The concentration of the indolic eumelanin-related compounds in normal urine samples was usually in the range of hundreds pmol/ml

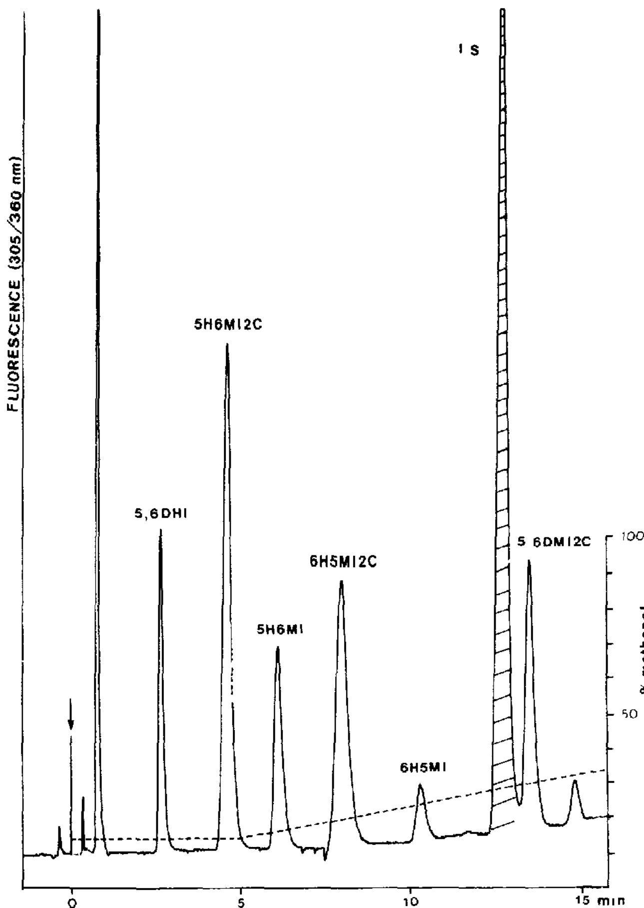

Fig 1 Separation of standard indoles using a methanol gradient elution with fluorescence detection The compounds were eluted from an RP-8 Spherisorb ODS-5 column (protected by a pre-column) with a mixture of methanol and acetate buffer containing ascorbic acid and Na_2EDTA, at a flow-rate of 1 5 ml/min Abbreviations 5,6DHI = 5,6-dihydroxyindole, 5H6MI2C = 5-hydroxy-6-methoxyindolyl-2-carboxylic acid, 5H6MI = 5-hydroxy-6-methoxyindole, 6H5MI2C = 6-hydroxy-5-methoxyindolyl-2-carboxylic acid, 6H5MI = 6-hydroxy-5-methoxyindole, I S = internal standard (5-methoxyindolyl-2-carboxylic acid), 5,6DMI2C = 5,6-dimethoxyindolyl-2-carboxylic acid

Fig 2 Typical chromatogram of an extract from a normal urine (A) without and (B) with enzymic hydrolysis of glucurono and sulpho conjugates. Abbreviations 5HIAA = 5-hydroxy-indolyl-3-acetic acid, IAA = indolyl-3-acetic acid, 3-OH indole = 3-hydroxyindole, for other abbreviations, see Fig 1

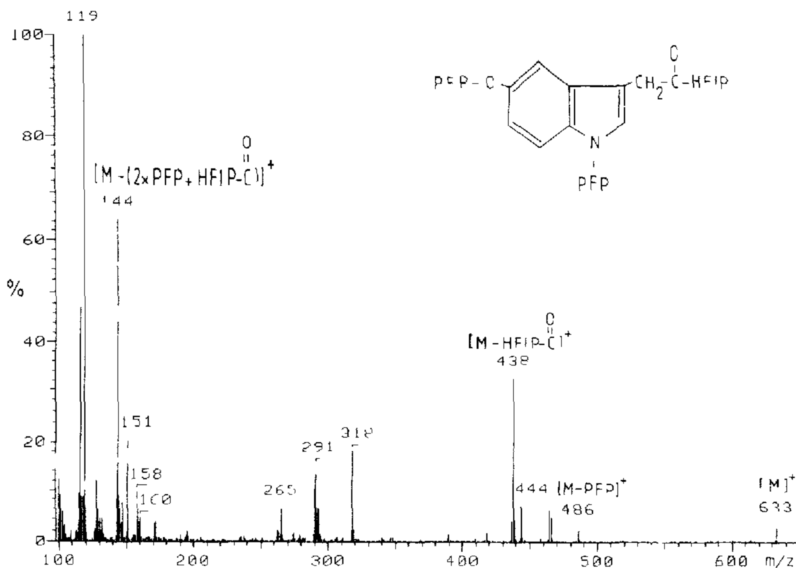

Fig 3 Mass spectrum of the pentafluoropropionyl (PFP) and hexafluoroisopropyl (HFIP) derivative of 5-hydroxyindolyl-3-acetic acid (5HIAA) Appropriate HPLC eluates from normal urines were pooled, extracted with ethyl acetate and the extracts were processed and analysed by gas chromatography–mass spectrometry as described elsewhere [8]

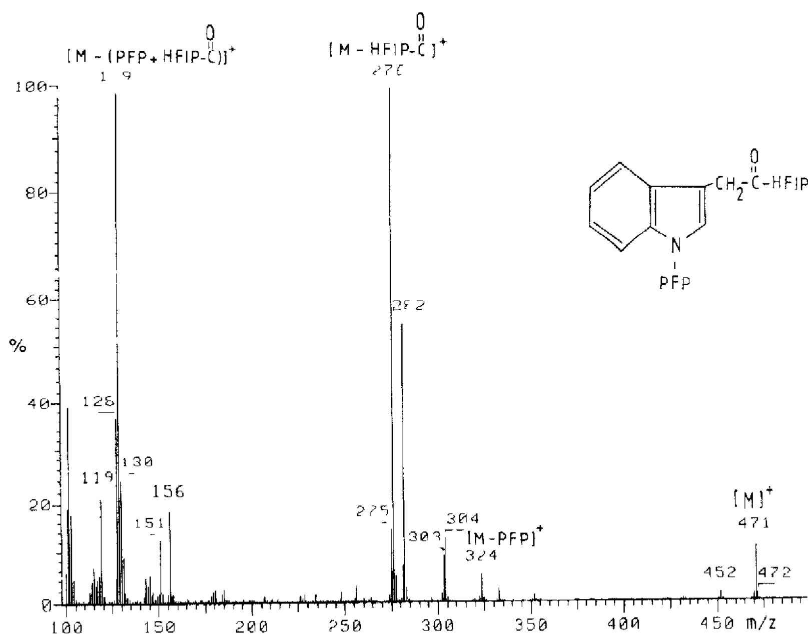

Fig 4 Mass spectrum of the pentafluoropropionyl (PFP) and hexafluoroisopropyl (HFIP) derivative of indolyl-3-acetic acid (IAA) Appropriate HPLC eluates from normal urines were pooled, extracted with ethyl acetate and the extracts were processed and analysed by gas chromatography—mass spectrometry as described elsewhere [8]

of urine. A detection limit of ca. 1 pmol injected on the column could be achieved

The HPLC analyses of melanotic urine provided quantitatively different pictures. Two dominant peaks, corresponding to 5H6MI2C and 6H5MI2C, were present on the chromatograms of unhydrolysed urine samples together with a small peak of 5,6DMI2C (Fig 5A) After enzymic hydrolysis, three other peaks, corresponding to 5,6DHI, 5H6MI and 6H5MI, appeared on the chromatograms (Fig. 5B). The differences between hydrolysed and unhydrolysed urine samples are in agreement with our previous findings, showing that the indoles without a carboxyl group are excreted as conjugates [2—4]

5,6DHI2C was not present in a detectable amount in our extracts. Although this eumelanin precursor was shown to be present even in normal urine [10, 11], its instability (i e strong inclination to oxidation and polymerization) makes its quantification unreliable.

In general, indolic compounds are known to exhibit natural fluorescent properties When excited at ca 300 nm, they emit fluorescent light usually in the range 330—430 nm, the precise wavelength depending on their structure The fluorescent properties of the eumelanin-related indolic compounds seem to be mainly influenced by the presence or the absence of the carboxyl group Under experimental conditions, the three indolic acids (5H6MI2C, 6H5MI2C and 5,6DMI2C) were characterized by excitation and emission maxima around 315 and 390 nm, respectively, whereas the indoles without the carboxyl group exhibited spectral maxima around 300 and 340 nm for the excitation and emission, respectively The approximate averages of the excitation and fluorescent maxima of both groups have therefore been used in our study

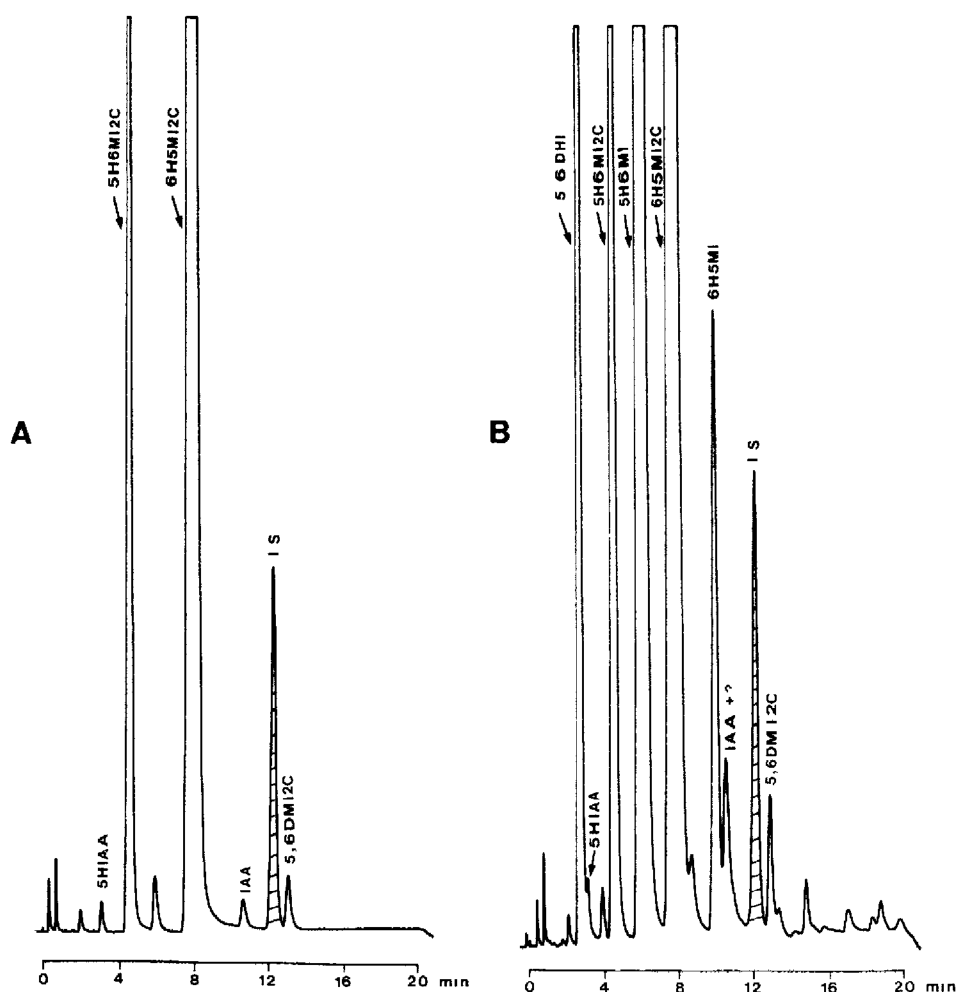

Fig 5 Chromatogram of an extract from a 10× diluted urine of a melanoma patient with hepatic metastases (A) without and (B) with enzymic hydrolysis of glucurono and sulpho conjugates For abbreviations, see Figs 1 and 2

In summary, the reversed-phase HPLC procedure makes it possible to separate six indolic eumelanin-related substances (specific metabolites of pigment cells). Spectrofluorimetry provides a selective and sensitive way to analyse these metabolites, enabling the detection of the compounds in normal urine samples The HPLC method may find its application in studies concerning melanin pigmentation and malignant melanoma

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