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Biomedical Applications

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Note

Identification of an interfering compound in the gas—liquid chromatographic determination of N^2,N^2-dimethylguanosine*

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In the search for biological markers for cancer, the minor methylated nucleosides and pseudouridine, components of transfer RNA, have been suggested as indicators of cancerous growth. Since most of the minor nucleosides are excreted from the body in the urine, a gas chromatography method [1] has been developed to monitor the levels of pseudouridine (ψ), N^2,N^2-dimethylguanosine (m_2^2G) and N^1-methylinosine (m^1I) in urine. Reports by Waalkes et al. [2, 3] have indeed shown elevated levels for these markers in the urine of cancer patients with Burkitt's lymphoma, lung, colon, breast and other cancerous tumours.

In approximately 5—10% of the urine samples from cancer patients, an abnormally high m_2^2G value has been observed. Examination of these samples by gas chromatography—mass spectrometry (GC—MS) has confirmed the presence of a major interfering compound co-eluting with m_2^2G, which could not be readily resolved by the use of other GC columns. This paper reports on the isolation and identification of this interference.

EXPERIMENTAL

Reagents and equipment

A 402 F&M gas chromatograph with a two column oven bath, two hydrogen flame detectors, two differential electrometers, a linear temperature programmer, and a Honeywell Electronik 18 recorder was used for all gas chromatographic analysis. All gas chromatographic separations were done with a column of 3% SE-30 on 100—120 mesh Supelcoport.

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A CEC 21-110 mass spectrometer interfaced by means of a "Llewellyn type" silicone membrane separator with a Varian 1500 gas chromatograph was used for all GC—MS work. The mass marker was provided by a Jeolco JEC-6 spectrum computer. All high-resolution measurements were made either by peak matching or using a Jeolco JMA-1C-0 automatic data analyzer micro densitometer to read exact masses from the photographic plates.

A Waters Assoc. M-6000 high-pressure solvent delivery system was used as the pump for the liquid chromatographic isolation. A 30 cm × 0.9 cm column filled with Bio-Rad Aminex A-5 cation-exchange resin was used for the sample purification. The detector was a Laboratory Data Control Duo Monitor set to monitor at 254 and 280 nm.

A Cary Model 10 ultraviolet—visible spectrophotometer was used for all UV spectra.

A Bruker HX-90 Fourier Transform 90-MHz nuclear magnetic resonance (NMR) spectrometer equipped with a Nicolet 1080 signal average computer was used for ^{13}C and ^{1}H NMR spectra.

All other reagents and supplies were the same as previously reported [1].

Isolation of unknown compound

The unknown compound was isolated from urine by use of a modification of the charcoal adsorption method published by Chang et al. [1] followed by cation-exchange chromatography. The urine sample was filtered through a 3-μm millipore filter and then applied to a charcoal column. The ratio of urine to charcoal was normally 5:2 (v/v). The quantity of urine usually processed was 25—50 ml. After application of the urine sample to the column, it was washed with about 25 ml of water per milliliter of charcoal. The column was also washed with 1% pyridine in water with a volume of 25 ml being used per milliliter of charcoal. The unknown was then eluted with 95% ethanol. The volume of ethanol used was twice the column bed volume. The eluate from the charcoal column was taken to dryness at 60—70° under nitrogen gas and the residue dissolved in 1 *N* acetic acid. Approximately 1 ml of acetic acid was used for each 15 ml of urine. This solution was then filtered through a 1-μm millipore filter.

The elution characteristics of the unknown was then determined in the following manner. The equivalent of about 2 ml of urine in 1 *N* acetic acid was then placed on a cation-exchange resin column and eluted with deionized water using a UV detector to monitor the elution. The elution position of the unknown was determined by collecting all UV-absorbing peaks. Each fraction was evaporated to dryness, then derivatized as the trimethylsilyl derivative by the procedure of Chang et al. [1]. The derivatized fractions were checked by GC retention time and GC—MS. The unknown was eluted after three column dead volumes.

The unknown, in the eluate from the charcoal column, was then isolated on a preparative scale by loading the equivalent of 15—20 ml of urine on the cation-exchange column and collecting the appropriate fraction. The purified material was rechromatographed on the same column with approximately 60% of the peak being collected. These purified fractions were dried at 60—70° under a stream of nitrogen. The homogenity of the fraction was checked by gas—liquid and thin-layer chromatography. This residue was used for these structural investigations:

The trimethylsilyl and d_9-trimethylsilyl derivatives were subjected to both low- and high-resolution mass spectrometry.

The IR spectrum was obtained as a pellet in potassium bromide.

The ^1H and broad band ^1H decoupled ^{13}C NMR spectra of the unknown were obtained using ^2H_2O as the solvent with tetramethylsilane and hexafluorobenzene as the respective reference or lock compounds (capillary insert).

An ultraviolet spectrum of the unknown was obtained from its solution in water at pH's 1, 7, and 10.

RESULTS AND DISCUSSION

The mass spectral fragments from the trimethylsilyl and d_9-trimethylsilyl derivatives of the unknown are presented in Table I. Table II presents the ^{13}C and ^1H NMR spectral data.

TABLE I

MASS SPECTRAL DATA OF UNKNOWN

TMS-derivative *m/e* (intensity)	d_9-TMS-derivative *m/e* (intensity)	No. of silicon atoms
73 (100)	82 (100)	1
147 (84)	162 (78)	2
217 (69)	232 (74)	2
223 (51)	235 (27)	2
295 (96)	313 (92)	2
375 (71)	402 (54)	3
464 (7)	499 (8)	4
672 (2.5)	714 (3.0)	5
687 (0.3)	732 (0.3)	5

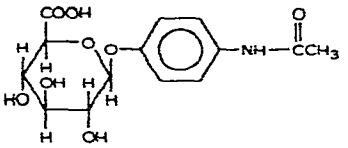
TABLE II

NMR DATA ON UNKNOWN

^{13}C NMR		^1H NMR	
δ	intensity	δ	intensity
23.28	1	2.71	3
70.83	1	4.26	4
71.83	1	7.88	4
73.23	1		
75.82	1		
101.89	1		
118.01	2		
124.81	2		
137.86	1		
154.69	1		
173.79	1		
178.00	1		

The IR spectrum showed a broad band centered at 3400 cm^{-1} and multiple bands between 1650 and 1800 cm^{-1}. These absorption bands are indicative of hydroxyl groups and multiple carbonyls. The ultraviolet spectrum showed a maximum at 241 nm, a shoulder at 280 nm, and typical end absorption and did not vary upon changing the pH. These are typical characteristics of a substituted aromatic ring, lacking ionizable functionality in conjugation with the aromatic system. The absence of phosphorus and sulfur was verified by chromatographing the derivatized unknown on a gas chromatograph equipped with a flame photometric detector in both the P and S modes.

The above data taken in conjunction with the following NMR and MS spectral designations are consistent with the assignment of the following structure of the glucuronide of 4-hydroxyacetanilide (I) to the unknown.



(I)

The acetyl methyl group appears as a singlet at δ 2.71 in the ^{1}H NMR spectrum and at δ 23.78 in the ^{13}C NMR spectrum. The methide protons of the glucuronic acid group appear as a multiplet centered at δ 4.26 in the ^{1}H NMR and the corresponding carbons are located at δ 70.83, 71.83, 73.23, 75.82, and 101.89 (anomeric carbon) in the ^{13}C NMR spectrum. The *para* disubstituted benzene ring is observed as a characteristic AB quartet at δ 7.88 in the ^{1}H NMR spectrum and at δ 118.01, 124.81, 137.86 (N-bound C), and 154.69 (O-bound C) in the ^{13}C NMR spectrum. The exact mass measurement of 687.2999 for the molecular ion is in good agreement with this elemental composition. This compound would be expected to be silylated at five sites as shown by the mass spectrum of the d_9-trimethylsilyl derivative. Additional proof is provided by the fact that the ultraviolet spectrum of 4-hydroxyacetanilide in water is identical with that for this unknown.

The origin of the glucuronide of 4-hydroxyacetanilide from the human body would be through the administration of 4-hydroxyacetanilide (paracetamol), which is a common acetylsalicylic acid substitute.

Final confirmation of the origin for the unknown was obtained by the administration of paracetamol to two people. The unknown was not present in the urine before, but was confirmed to be present by GC—MS after administration of paracetamol. This further substantiates the structural assignment of the unknown to the glucuronide of 4-hydroxyacetanilide.

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REFERENCES

1 S.Y.Chang, D.B. Lakings, R.W. Zumwalt, C.W. Gehrke and T.P. Waalkes, *J. Lab. Clin. Med.*, 83 (1974) 816.
2 T.P. Waalkes, C.W. Gehrke, R.W.Zumwalt, S.Y.Chang, D.B.Lakings, D.C. Tormey, D.L. Ahmann and C.G. Moertel, *Cancer*, 36 (1975) 390.
3 T.P. Waalkes, C.W. Gehrke, W.A. Bleyer, R.W. Zumwalt, C.L.M. Olweny, K.C. Kuo, D.B. Lakings and S.A. Jacobs, *Cancer Chemother. Rep.*, 59 (1975) 721.
4 G.C. Levy and G.L. Nelson, *Carbon-13 Nuclear Magnetic Resonance for Organic Chemist*, Wiley-Interscience, New York, 1972.