Journal of Chromatography, 110 (1975) 182–186

CHROM. 8306

Note

Gas chromatography and characterization of tetraethyl derivatives of uric acid

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(Received February 27th, 1975)

We are interested in assaying uric acid in serum with high accuracy by a similar principle to that described for serum calcium[1] using stable isotope dilution–mass spectrometry[2]. To facilitate both purification and introduction of samples into the mass spectrometer, it was desirable to find a derivative of uric acid which would be easily prepared and stable; soluble in common organic solvents; volatile enough for gas chromatography (GC); and give a suitable fragmentation pattern in the mass spectrometer. One or other of the methylated derivatives[3–6] of uric acid might meet these requirements, but to achieve specificity, it seemed preferable to use an ethyl derivative since methylated purine compounds are commonly found in biological materials. The synthesis of tetraethyl derivatives of uric acid has been attempted[7]; the present paper describes a simple method for the direct ethylation of uric acid, and the GC and characterization of the principal products.

EXPERIMENTAL

Materials

Diethyl sulphate (BDH, Poole, Great Britain), was used as received. Organic solvents and reagents were all of the analytical grade quality. [^{14}C]-Uric acid (specific activity 56.7 mCi/mmole) was purchased from the Radiochemical Centre (Amersham, Great Britain).

Preparation of derivatives

Solutions of uric acid were prepared by dissolution in 1 *M* KOH, with subsequent dilution, or addition of alkali, as required.

A solution of uric acid (2.5 mg in 5 ml of 5 *M* KOH) was mechanically stirred with diethyl sulphate (5 ml) in a flask kept at 65°. The pH of the reaction mixture was monitored by a Pye Autotitrator and was kept above pH 11 by adding 10 *M* KOH (approx. 1 ml) during 30 min. More diethyl sulphate (5 ml) was then added, together with more 10 *M* KOH (5 ml), and the reaction was allowed to continue as before for a further 30 min. A clear solution was then obtained, indicating utilisation of all the diethyl sulphate, and it was then extracted three times with 2-ml portions of diethyl ether. The ether extracts were combined and aliquots were taken for the GC.

Gas chromatography

Pye 104 and Becker 409 gas chromatographs were used, both with flame ionization detectors. The former was fitted with a stream splitter (1:10) and a preparative column (15 ft.) coated with 1% OV-17 on Gas-Chrom Q, 80–100 mesh, and was run isothermally at 220°; the Becker chromatograph was equipped with a 20 m open-tubular capillary column coated with OV-101, and was run with temperature programming from 100 to 200°, the rate of change being 2°/min. Liquid injection was used for the preparative chromatography, with caffeine as internal standard; solid injection was carried out on the Becker chromatograph.

RESULTS AND DISCUSSION

Gas chromatography of uric acid derivatives

Ethylation of uric acid gave two major ether-soluble volatile compounds, which, when chromatographed on OV-17, showed relative retention times (RRT) of 1.25 and 1.79 respectively, using caffeine as the internal standard (Fig. 1A). Although there are 12 possible isomers of tetraethyl uric acid, only five minor —quantitatively insignificant— peaks were noted. Using the stream splitter, the two major fractions were trapped for further characterization.

Each fraction was a white crystalline substance, readily soluble in water and the common polar solvents. Re-chromatography of the two fractions using the capillary column, with much higher resolution, showed that each gave only a single peak (Fig. 1B), so that each fraction may be regarded as a single compound (I and II respectively).

Spectroscopic characterization of the principal products

The mass spectra of compounds I and II were identical (Fig. 1C) and showed a prominent molecular ion at *m/e* 280, with four successive losses of 28 mass units (Fig. 2) giving peaks at *m/e* 252, 224, 196 and 168. The additional ions at *m/e* 265, 237, 209 and 181 corresponded to losses of 15 mass units (methyl) and were considered to arise from the ethyl substituents.

Although the mass spectrum is consistent with each compound being a tetraethyl derivative of uric acid, the fragmentation pattern does not permit differentiation between N-ethyl or O-ethyl substitution, since in both cases McLafferty γ-hydrogen rearrangement[8] could occur, to give rise to the loss of 28 mass units as shown (Fig. 2).

To differentiate the structure of the two isomers, infrared (IR) and nuclear magnetic resonance (NMR) analyses were carried out (Figs. 1D and E, respectively). The IR spectrum of compound II showed two strong bands in the carbonyl region at 1682 and 1712 cm^{-1} respectively. This indicates that only two of the three oxygen atoms are in the lactam form, and the third in a lactim associated with an ethyl; thus it is likely that compound I has three N-ethyl substituents and one O-ethyl substituent (Fig. 3).

By contrast, the IR spectrum of compound I showed three intense absorption bands in the carbonyl region at 1610, 1654 and 1700 cm^{-1}, corresponding unequivocally to a triketone structure, and showing that compound I is a tetra-N-ethyl derivative with the oxygen atoms at positions 2, 6, 8 in the lactam form.

The NMR spectrum confirmed that compound I corresponds to a structure

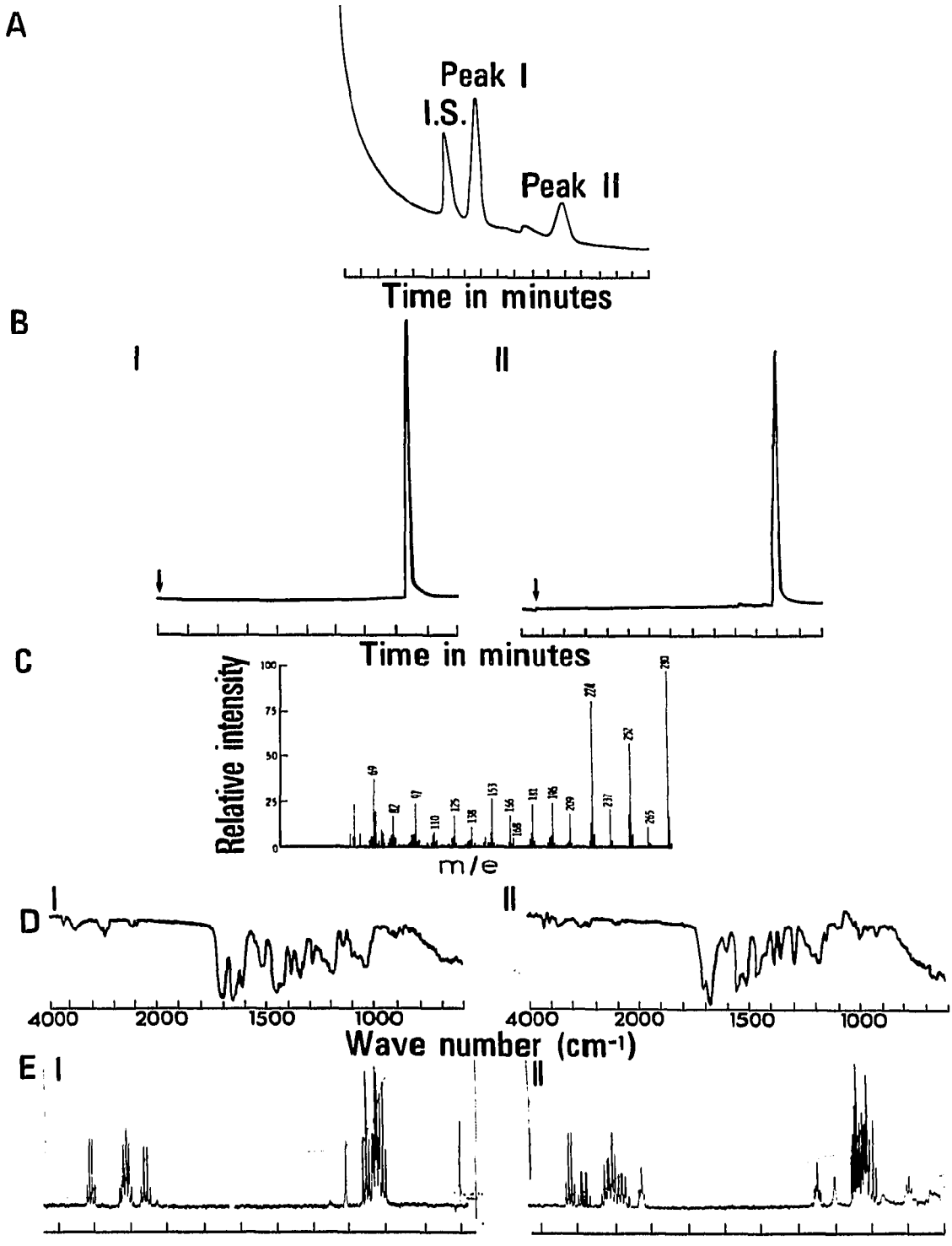


Fig. 1. (A), GC of ethylated derivatives of uric acid with caffeine as the internal standard (I.S.); (B), re-chromatography of trapped peaks I and II on the capillary columns; (C), mass spectrum of the two tetraethyl uric acid derivatives; peaks I and II gave identical spectra; (D), IR spectra of peak I and of peak II, both in chloroform solution; (E), NMR spectra of peak I and of peak II, both in chloroform solution.

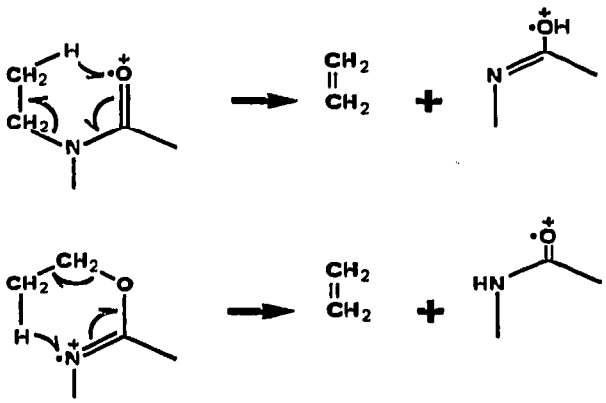
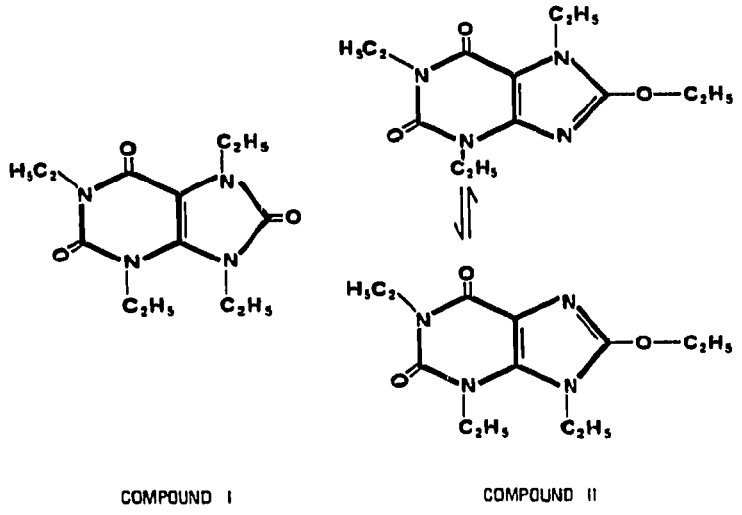


Fig. 2. Scheme of McLafferty γ-hydrogen rearrangement.

containing four distinct ethyl groups. With compound II however, the NMR spectrum suggested that one ethyl group is attached to an oxygen, (probably that of the pyrazine ring), two are attached to N-1 and N-3 of the pyrimidine ring, while the position of the fourth ethyl substituent is uncertain, being either at N-7 or N-9, or both, in tautomeric equilibrium (Fig. 3).



COMPOUND I COMPOUND II

Fig. 3. Structural formula of compounds I and II.

CONCLUSIONS

With the reaction conditions described, compound I was produced in approximately twice the yield of compound II, as judged by the relative peak areas on the gas chromatogram. The sum of the two isomers accounted for an overall yield of 50–70%, as estimated by using [^{14}C]-uric acid as an internal standard, and by comparison with caffeine as the internal chromatographic standard, and correcting for the molecular response.

The two tetraethyl derivatives of uric acid were found to be stable in diethyl ether or chloroform solution for up to 7 days at room temperature, and stable in a dry form for at least one month.

These derivatives of uric acid are simple to synthesise, stable, can be gas chromatographed readily, and afford a characteristic mass fragmentation pattern. These properties make them very suitable for use in the assay of uric acid in biological matrices, and for stable isotope-dilution studies.

ACKNOWLEDGEMENTS

The encouragement and advice of Dr. S. S. Brown and Mr. J. F. Pickup is gratefully acknowledged. Our thanks also go to Dr. C. H. L. Shackleton and Dr. A. M. Lawson for their help with the capillary GC and mass spectrometry.

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