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Note

High-performance liquid chromatographic determination of urocanic acid isomers in biological samples

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It is known that urocanic acid is formed from L-histidine by an enzymatic reaction due to the enzyme histidine ammonia-lyase [1—5]. In the epidermis, where the enzymes of the catabolic pathway are absent, urocanic acid accumulates, forming as much as 0.5% of the dry weight of the epidermis [6—10]. The role of urocanic acid has still to be established, although it is believed that it acts as a sunscreen [11—14].

High-performance liquid chromatographic (HPLC) methods have been reported, but they suffer from the following drawbacks: a failure to resolve the two geometrical isomers of urocanic acid [15—18] and lack of sensitivity [19]. In our hands, the method of Morrison et al. [20] was unreliable since the elution conditions used resulted in a rapid deterioration of the stationary phases. Accordingly, we have developed and report here a simple, sensitive and reliable method for the quantitative determination of the two isomers of urocanic acid (Fig. 1) in biological samples.

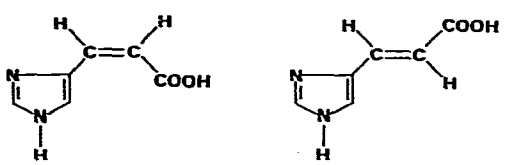

Fig. 1. Chemical structures of (*Z*)- and (*E*)-urocanic acid.

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EXPERIMENTAL

Instrumentation

HPLC was performed either on a Hewlett-Packard 1084 B chromatograph fitted with a variable-wavelength detector, or a system consisting of a Waters 6000 A pump, a Valco 7000 p.s.i. valve and a Waters M440 UV—visible dual-wavelength detector fixed at 254 and 280 nm.

The column used, LiChrosorb-NH_2 Hibar 250 RT-4 (25 cm × 4.6 mm I.D., 10 μm particle size) was purchased from Merck (Darmstadt, G.F.R.) and fitted with a 3 cm long precolumn filled with LiChrosorb-RP-18 (10 μm particle size).

The UV—visible spectrophotometer was a UVIKON 820 (Kontron) and the gas chromatography—mass spectrometry (GC—MS) apparatus was a 1010 C from Nermag.

Acetonitrile was chromatographic grade from Merck; KH_2PO_4, KOH and $HClO_4$ were analytical grade from Merck. Urocanic acid was supplied by Sigma (St. Louis, MO, U.S.A.). Water was purified, after deionizing, on a Milli Q system from Millipore.

The chromatographic eluent was filtered under vacuum on a Millipore filter (FHULP 0.45 μm).

To obtain (*Z*)-urocanic acid, we irradiated a solution of 1 mg/ml (*E*)-urocanic acid in the chromatographic eluent, with a 100 W Hanovia Xenon lamp filtered at 280 nm for 1 h. Under these conditions, we obtained a mixture containing 60% of (*Z*)-urocanic acid which was identified by its UV—visible spectrum and its mass spectrum, which were identical to the literature data [21].

We have confirmed in the same way the identity of the urocanic isomers in a healthy plantar callus extract, obtained by semi-preparative chromatography (same packing and eluent). In the routine procedure, the urocanic acid isomers are identified by the ratio of the absorbances at 254 and 280 nm.

Extraction procedure

After homogenization of the biological sample (50 mg) in 2 ml of 0.4 *M* perchloric acid with a Polytron homogenizer at 4°C (speed 8), the homogenate was centrifuged for 5 min at 6000 *g*, 4°C. A 1.5-ml portion of the supernatant was neutralized with KOH, diluted with 2 ml of acetonitrile, mixed and centrifuged for 5 min at 4500 *g* to remove the precipitated $KClO_4$ and other acetonitrile-insoluble material. An aliquot of the supernatant (50 μl) was then subjected to HPLC analysis.

RESULTS AND DISCUSSION

The effect of both the proportion of organic modifier and the ionic strength of the buffer used on the capacity factor k' of the *E*-isomer of urocanic acid is shown in Fig. 2. The results suggest that the separation on the amino group bonded phase is due to a mixture of partitioning and ion exchange. In fact, with up to 40% of acetonitrile there is practically no change in the capacity

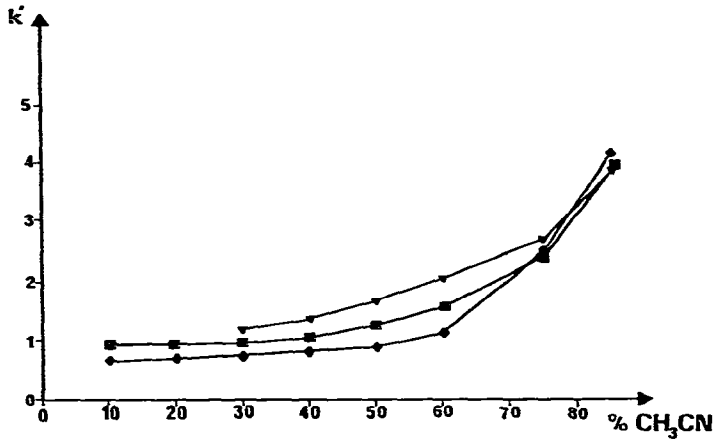

Fig. 2. Variation of the capacity factor, k', with the elution conditions: 0.1 *M* KH_2PO_4 (▼); 0.05 *M* KH_2PO_4 (■); 0.01 *M* KH_2PO_4 (◆).

factor on increasing the proportion of organic solvent, thus indicating that the column is working mainly in the ion-exchange mode, as shown by the variation of k' with ionic strength.

If we further increase the concentration of acetonitrile, the partition mode of separation becomes dominant, and the variation of the capacity factor with the proportion of organic solvent is important. The effect of the ionic strength thus becomes negligible for proportions of acetonitrile greater than 75%.

For the *Z*-isomer, the variation of the capacity factor is negligible up to 75% of acetonitrile. In this case, there is no effect at all of the ionic strength of the eluent on k', and the mechanism of separation seems to be only that of a partition effect.

In view of these results, we chose to perform the analysis under conditions giving the separation of a pure mixture of the isomers of urocanic acid shown in Fig. 3.

Under these conditions, the detection limit for the *E*-isomer of urocanic acid is 0.2 ng injected and 0.3 ng for the *Z*-isomer. This has to be compared to the respective limits of detection of 2 and 2.4 ng previously reported [19]. The response of the compounds is linear in the range of concentration studied (1—100 ng injected). By virtue of the symmetrical nature of the peaks, either peak area (HP 1084B) or peak height (Waters system) has been used for quantitation with the same accuracy. The reproducibility of five repeated injections of the same sample was better than 1%.

Our experiments on the extraction of urocanic acid from skin samples showed us the importance of the duration of the homogenization step. In fact, (*E*)-urocanic acid is stable at 4°C in 0.4 *M* perchloric acid, but when we perform the homogenization the recovery of urocanic acid is directly related to the time of treatment. At the homogenization speed used, as much as 40% of urocanic acid is decomposed in 8 min, mainly through a thermal process.

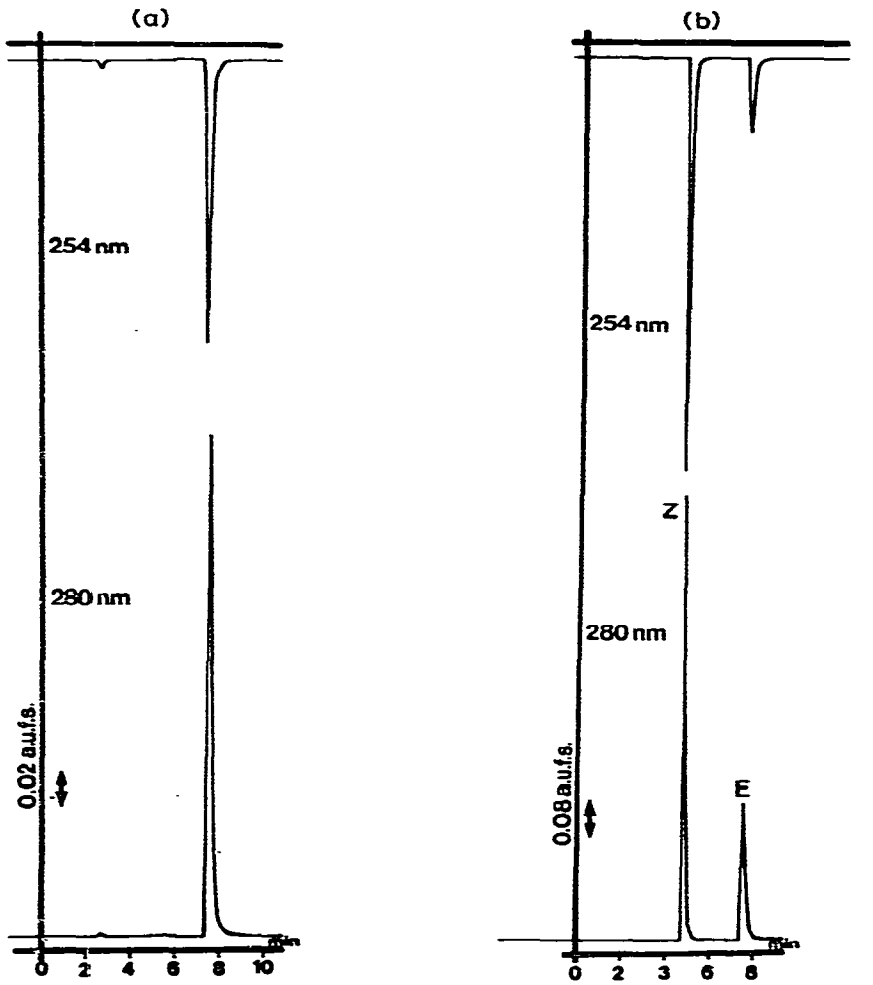

Fig. 3. Chromatograms of: (a) pure (*E*)-urocanic acid (0.5 μg); (b) a mixture of pure *Z*- and *E*-isomers (1.5 and 0.5 μg, respectively). Eluent: KH_2PO_4 (0.05 *M*)—acetonitrile (1:1, v/v), pH 7. Flow-rate: 1 ml/min. Pressure: 80—100 bars.

Addition of 0.1 *M* EDTA increased the total recovery of about 15%, but the decomposition is still significant.

The best conditions were found to be a 4-min homogenization in steps of 1 min, with a 1-min pause between each step to allow sufficient cooling of the Polytron shaft. Under these conditions, we obtained a 90% recovery of (*E*)-urocanic acid after addition of a known amount of urocanic acid to healthy plantar callus.

The reproducibility of the complete analytical procedure was found to be better than 5%.

Fig. 4 shows the chromatogram obtained with an extract of plantar callus of a healthy subject. We can see only minor interferences with (*E*)-urocanic acid. Dual-wavelength detection (254 and 280 nm) was usually used to identify the compounds by the ratio of the signals at these two wavelengths which, for a given compound, is constant.

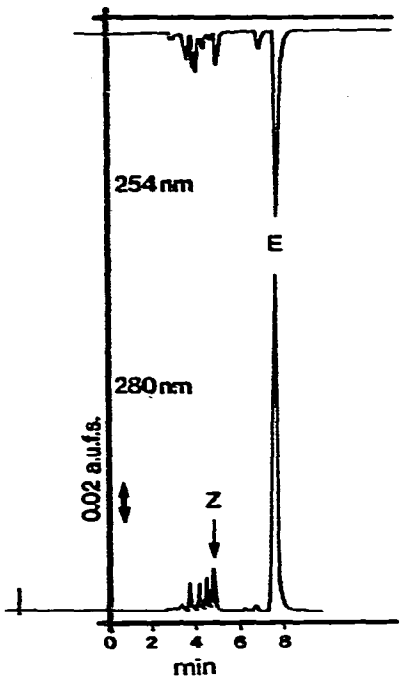

Fig. 4. Chromatogram of a healthy plantar callus extract. Conditions are the same as in Fig. 3.

The concentration of (*E*)-urocanic acid found in plantar callus is in good agreement with the literature values [14], ranging from 0.5 to 0.3% of dry weight, depending on the subject under study.

We are currently studying the urocanic acid content of the skin by this improved analytical procedure in patients with dermatological diseases.

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