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Note

Unexpected high-performance liquid chromatographic separation of Org GC 94 and [3,3,4,4-$^2H_4$]Org GC 94

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While developing an assay method for Org GC 94* (a new tetracyclic drug with anti-migraine activity[1]) in human plasma, some interesting phenomena were observed in the high-performance liquid chromatography (HPLC) of this compound. The assay method includes a hexane extraction of the plasma sample to which [3,3,4,4-$^2H_4$]Org GC 94 (Fig. 1) is added as internal standard, a HPLC clean-up step and a mass fragmentographic quantification[2]. For the clean-up step, analytical HPLC is used in the preparative mode: the eluate fraction containing Org GC 94 and its deuterated analogue is collected. For a proper purification, it is important to collect as small a fraction as possible. The start and end times of sample collection preferably coincide with the onset and end time of the Org GC 94 peak. While establishing the HPLC sample-collection time interval for the Org GC 94 assay, evidence was obtained for a remarkable separation between the compound and its deuterated analogue.

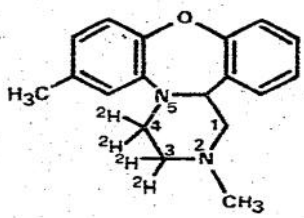

Fig. 1. The free base [3,3,4,4-$^2H_4$]Org GC 94.

EXPERIMENTAL

Deuterated Org GC 94

According to mass spectrometry, [3,3,4,4-$^2H_4$]Org GC 94 contained 1.5% of $^2H_3$ and 98.5% of $^2H_4$.

* 1,3,4,14b-Tetrahydro-2,7-dimethyl-2*H*-dibenzo[*b*,*f*]pyrazino[1,2-*d*][1,4]oxazepine maleate. In the text, Org GC 94 refers to the free base.

High-pressure liquid chromatography

HPLC was performed with a Waters Assoc. Model ALC 202 high-pressure liquid chromatograph equipped with a Type U6K injection system and a 280-nm ultraviolet detector. The separation was obtained on a column (30 cm × 4 mm I.D.) of μ-Porasil (Waters Assoc., Milford, Mass., U.S.A.) with *ca.* 2000 theoretical plates. The elution system used consisted of *n*-hexane–isopropanol (90:10, v/v) to which 4% of ethanol and 0.1% of concentrated (25%) ammonia were added. The flow-rate was 1 ml/min.

Mass spectrometry

Mass spectra were recorded with a Varian MAT CH7 mass spectrometer connected to a mass-spectrometer data system (Varian MAT SpectroSystem 100 MS). The instrumental conditions were: electron energy 70 eV; ion source temperature, *ca.* 150°; direct insertion probe temperature, *ca.* 90°; scan rate, 1 decade per second.

RESULTS AND DISCUSSION

The chromatograms of 3 μg of the individual compounds, Org GC 94 and [3,3,4,4-$^2H_4$]Org GC 94, and of the corresponding mixture are shown in Fig. 2. Both compounds are very well separated. Moreover, the non-deuterated compound is eluted first, followed by the deuterated compound. In order to confirm the identity of both peaks, the eluate was trapped in two fractions A and B (as indicated in Fig. 2)

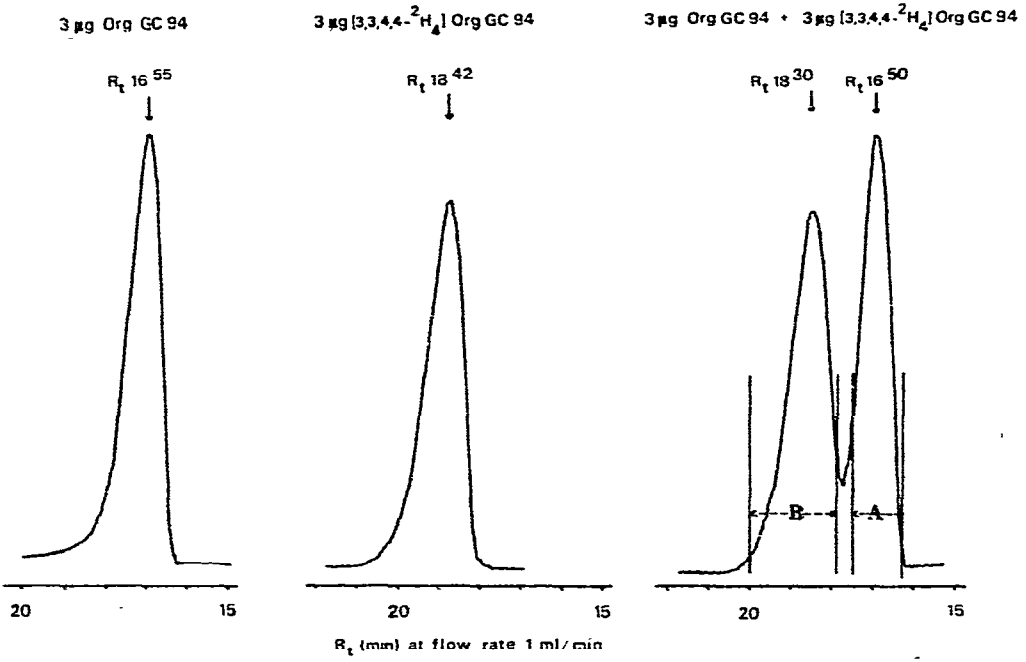

Fig. 2. High-pressure liquid chromatograms of Org GC 94, [3,3,4,4-$^2H_4$]Org GC 94, and of a mixture of both compounds. For A and B, see text.

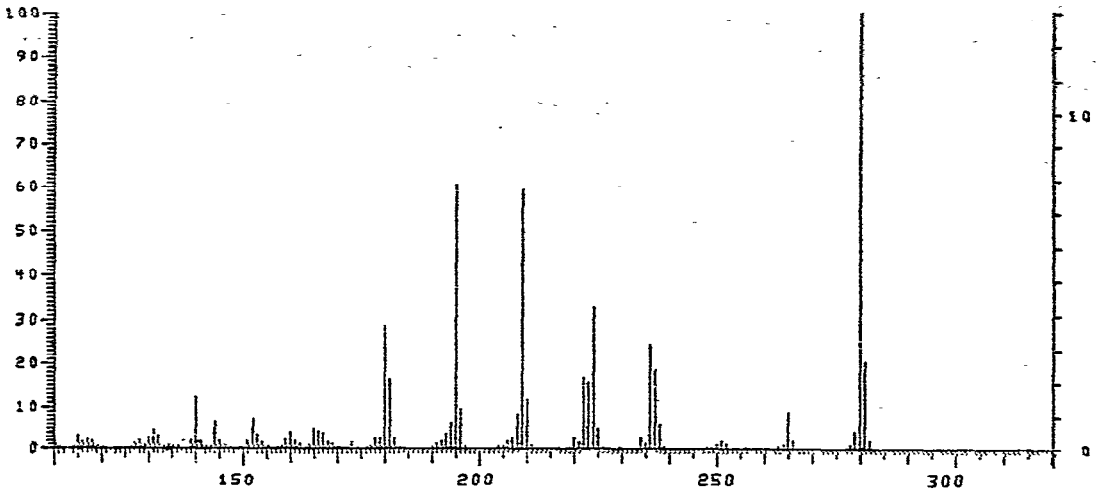

Fig. 3. Mass spectrum of HPLC eluate fraction A showing the molecular ion at *m/e* 280 for Org GC 94.

for mass spectrometric analyses. The mass spectra of both fractions are given in Figs. 3 and 4. They yield evidence for the authenticity of both compounds.

An explanation for the different behaviour of the compounds in this type of adsorption chromatography might be the shielding by the deuterium atoms of the polarity centres located at the N-2 and N-5 atoms. The polarity at N-2 can be affected by use of different concentrations of ammonia or acetic acid in the elution solvent. In strongly alkaline solution both compounds will be present as the free bases, while in an acidic medium they will be protonated. Deuteration of Org GC 94 at C-3 and

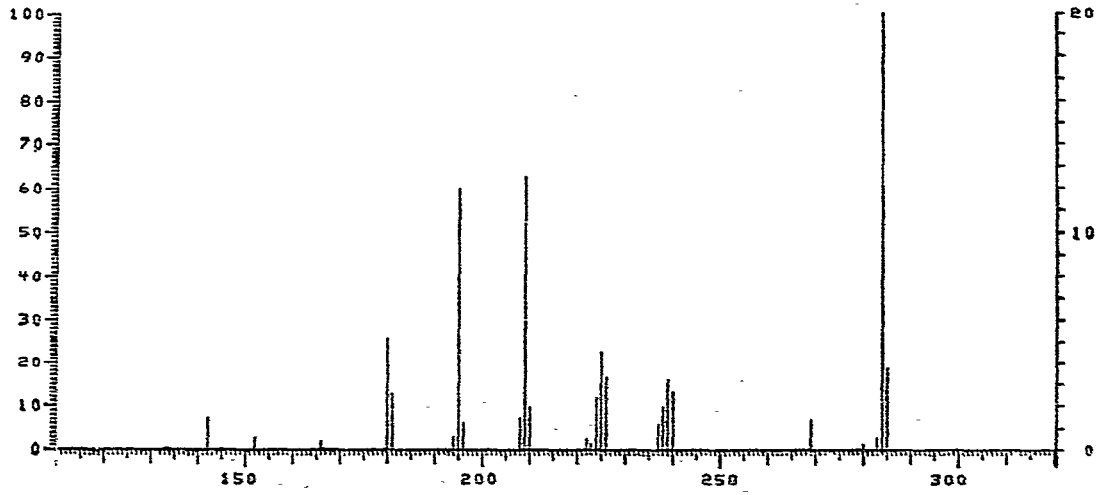

Fig. 4. Mass spectrum of HPLC eluate fraction B showing the molecular ion at *m/e* 284 for [3,3,4,4-$^2H_4$]Org GC 94.

C-4 might influence the pK_a to some extent, resulting in slightly different adsorption characteristics. However, it was not possible to determine the pK_a values in the elution solvent; in methanol–water (6:94, v/v), the pK_a of Org GC 94 is 6.28. Variation of the ammonia concentration from 0 to 1% caused a decrease in the resolution of the compounds, whereas by use of 0.3% acetic acid the separation was improved (while disturbing the peak shape at the same time). This effect is illustrated by the data in Table I in which the resolution is expressed as the difference in retention times of the compounds relative to the mean retention time ($\Delta t_R/\bar{t}_R$).

TABLE I

EFFECT OF CONCENTRATED AMMONIA (25%, w/w) AND GLACIAL ACETIC ACID WHEN ADDED TO THE ELUTION SYSTEM *n*-HEXANE–ISOPROPANOL–ETHANOL (86.5: 9.6: 2.8, v/v) ON THE HPLC RESOLUTION OF Org GC 94 AND [3,3,4.4-$^2H_4$]Org GC 94

Concentration	$\Delta t_R/\bar{t}_R$
1.0% Ammonia	*ca.* 0
0.5% Ammonia	0.04
0.1% Ammonia	0.10
0	0.14
0.3% Glacial acetic acid	0.2

A detailed study to explain the unexpected and remarkable separation of these compounds goes beyond the scope of our present investigations for assay development. However, since it is expected that other basic drugs might also elicit analogous phenomena, one should be aware of the possible consequences for assay procedures of this type. For assay methods using deuterated internal standards and a HPLC clean-up step, the retention times of both compounds must be measured accurately. In the case of separation or of peak broadening, relatively large eluate fractions should be collected or other elution systems or columns should be selected in order to prevent loss of one of the compounds.

On the other hand, the reported phenomenon yields possibilities for the preparative enrichment of analogous compounds from a mixture in which deuteration in the vicinity of a centre of polarity occurs only to a limited extent.

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