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Note

Removal of counter-ion reagent after semi-preparative reversed-phase ion-pair high-performance liquid chromatography

PHILIPPE GALLICE*, JEAN-PIERRE MONTI and AIMÉ CREVAT
Laboratoire de Biophysique, Faculté de Pharmacie, 27 Boulevard Jean Moulin, 13385 Marseille (France)
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For several years we have been investigating substances that accumulate in the plasma of uraemic patients[1,2]. These substances in molecular weight range 300–2000 are often called uraemic middle molecules (UMM). In order to isolate these compounds in a pure form, we developed a semi-preparative chromatographic procedure, the main steps of which are as follows. A gel permeation step (Sephadex G-15) yields seven fractions and fraction 2, corresponding to crude UMM, is separated into seven subfractions (2-1, ..., 2-7) using an anion exchanger (DEAE-Sephadex A-25). The last step is a reversed-phase (Spherisorb C_6, 5 μm) ion-pair high-performance liquid chromatographic (RP-IP-HPLC) separation of the 2-5 subfraction with 0.005 *M* tetrabutylammonium phosphate (TBAP) as a counter ion and methanol–water (20:80) as the mobile phase. This yields ten UMM fractions (2-5-1, ..., 2-5-10)[3]. However, the TBAP reagent interferes in the elucidation of the chemical structure and the study of the biological effect of the separated UMM.

In order to remove this counter ion from compounds of biological interest, we have developed a liquid chromatographic procedure that is an alternative to the previous technique[4], which turned out to be inappropriate.

EXPERIMENTAL

The compound eluted in the ion-pair form by RP-IP-HPLC is collected. The methanol in mobile phase[3] is removed by evaporation under reduced pressure at 37°C with a Rota-Vapor, then the volume is readjusted to the initial value with doubly distilled water. Subsequently, the solution is passed through a 30 × 1 cm I.D. glass column packed with DEAE-Sephadex A-25 anion exchanger (Pharmacia, Uppsala, Sweden) equilibrated with Tris–0.01 *M* HCl buffer (pH 8.6) at a flow-rate of 25 ml/h. After injection, the column is washed with the same buffer until complete elution of TBAP, which is not retained under these conditions.

Generally, three column volumes of Tris–HCl buffer are necessary for total removal of TBAP. Finally, the compound of interest sorbed by the anion exchanger is eluted as described previously using a sodium chloride gradient (0 to 0.15 *M*) in Tris–HCl buffer[3]. The eluate is monitored at 254 nm and collected in a Frac 100 fraction collector (Pharmacia). TBAP is determined both in the eluate washings and in the UMM fraction to Slonecker *et al.*'s method[5]: in phosphate buffer (pH 4.3) a

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picric acid solution with TBA^+ leads to TBA picrate. The absorbance of its chloroform solution is measured at 365 nm. As a supplementary control, we recorded proton nuclear magnetic resonance (^1H NMR) spectra of solutions in ^2H_2O before and after chromatography. ^1H NMR spectroscopy was performed at 200.13 MHz with a Bruker AM 200 spectrometer (Centre Universitaire de RMN, Faculté de Pharmacie, Marseille, France) in the pulsed Fourier transform mode. Solutes in ^2H_2O (2–3 mg of lyophilizate in 0.5 ml of ^2H_2O) were placed in a 5 mm O.D. tube and studied at 20.0 ± 0.5°C, with the solvent forming the internal lock. Proton chemical

TABLE I

DETERMINATION OF TBAP CONCENTRATION IN DIFFERENT SOLUTIONS OBTAINED DURING CHROMATOGRAPHIC STEPS

Solution	*Absorbance (365 nm)*	*TBAP concentration (M)*
HPLC mobile phase	0.456	0.005
HPLC eluate of UMM fraction	0.452	0.005
DEAE-Sephadex A-25 washing eluate	0.454	0.005
DEAE-Sephadex A-25 eluate of UMM fraction	0.000	0.000

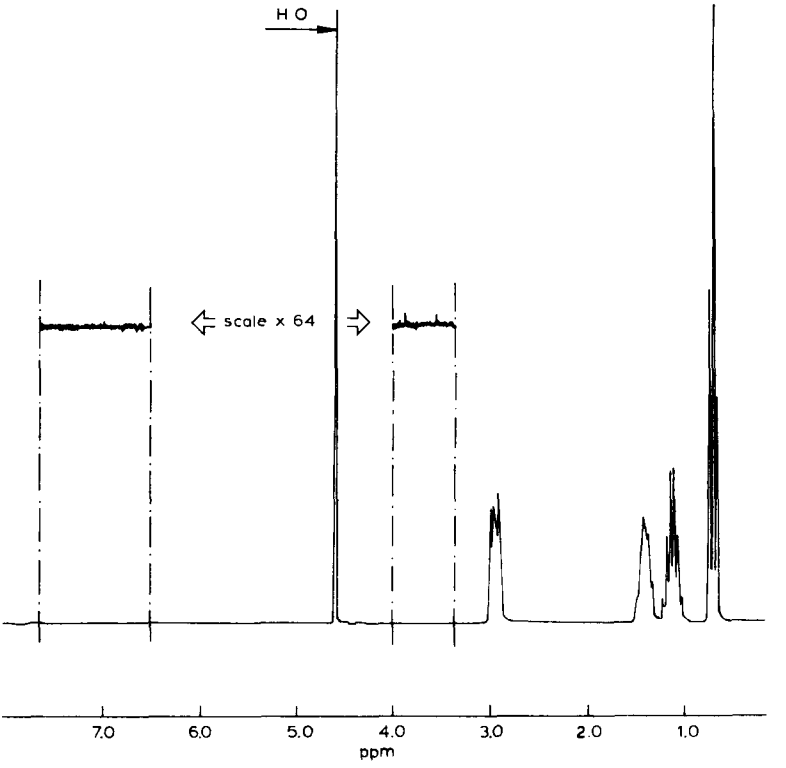

Fig. 1. ^1H NMR spectrum of HPLC eluate before TBAP removal (2–3 mg of lyophylizate in 0.5 ml of ^2H_2O). Only TBAP resonances are visible in NMR as the compound of interest represents less than 1% of the sample.

shifts were measured with reference to the resonance of 2,2-dimethyl-2-silapentane-5-sulphonate [3-(trimethylsilyl)-1-propane sulphonate] in ^2H_2O packed in a concentric capillary tube. The experimental ^1H NMR conditions were strictly identical for the two spectra (before and after chromatography): sweep width, 2400 Hz; pulse width, 4 μsec; acquisition time, 3.4 sec; number of scans, 75. An artificial line broadening of 0.3 Hz was used to improve the spectral signal-to-noise ratio.

RESULTS AND DISCUSSION

Table I shows the results of TBAP determinations in the washing solution and UMM fraction. Comparison of the TBAP concentrations in the HPLC mobile phase and in the HPLC eluate of UMM fractions shows that UMM do not interfere with the determination of TBAP concentration. TBAP is completely recovered in the DEAE-Sephadex A-25 washing eluate as the absorbance of the latter is identical with that of the TBAP–UMM mixture before chromatography.

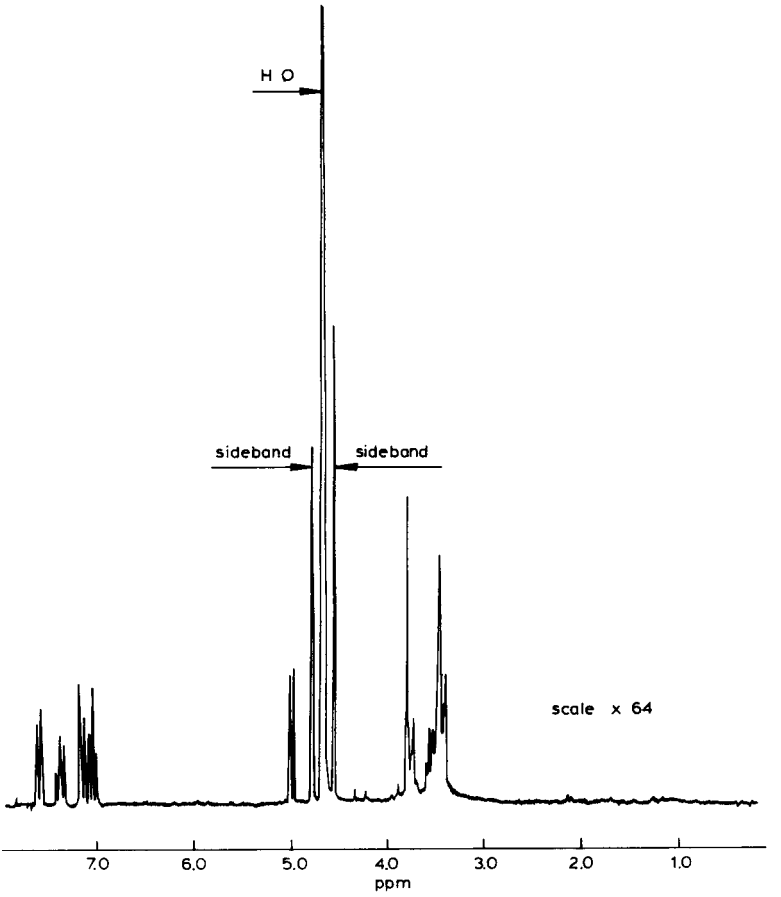

Fig. 2. ^1H NMR spectrum of HPLC eluate after TBAP removal (2–3 mg of lyophylizate in 0.5 ml of ^2H_2O). The observed resonances correspond to the compound of interest in a high state of purity (*ca.* 100%).

Figs. 1 and 2 show the ^1H NMR spectra before and after the TBAP chromatographic removal step, respectively. Before TBAP removal (Fig. 1), the compound of biological interest gives no NMR trace as its concentration is too low. In contrast, after chromatography (Fig. 2) it is clear that no trace of TBAP is visible, which confirms the chemical determination. Hence the removal of TBAP makes possible structural studies of unknown molecules by NMR spectroscopy.

In comparison with the previous technique[4], our method seems more suitable because it allows the use of the same chromatographic support (DEAE-Sephadex A-25) as in preceding steps, so it is possible to use the same elution volume calibration. Moreover, the proposed technique has the advantage of eliminating TBAP when the compound of interest is concentrated at the top of the column. Hence we can conclude that this efficient chromatographic procedure would be useful in semi-preparative or preparative reversed-phase ion-pair HPLC.

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REFERENCES

1 J. B. Rinaudo, P. Bernard, M. Crest, J. M. Moret, A. Murisasco, S. Saingra, R. Frayssinet, A. Crevat, P. Gallice, N. Fourier and F. Sicardi, *Int. J. Artif. Organs*, 4 (Suppl.) (1980) 90.
2 D. Braguer, A. M. Chauvet-Monges, J. C. Sari, A. Crevat, C. Durand, A. Murisasco and R. Elsen, *Clin. Nephrol.*, 20 (1983) 149.
3 P. Gallice, N. Fourier, A. Crevat, M. Briot, R. Frayssinet and A. Murisasco, *Kidney Int.*, 23 (1983) 764.
4 F. C. Baker and D. A. Schooley, *Anal. Biochem.*, 94 (1979) 417.
5 J. H. Slonecker, J. B. Mooberry, P. R. Schmidt, J. E. Pittsley, P. R. Watson and Allene Jeanes, *Anal. Chem.*, 37 (1965) 243.