Journal of Chromatography, 168 (1979) 526–529

CHROM. 11,385

Note

Method for fluorescence detection in the high-performance liquid chromatography of Δ^4-3-ketosteroids

RIKIZO HORIKAWA*, TAKENORI TANIMURA* and ZENZO TAMURA
Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113 (Japan
(Received June 20th, 1978)

We have recently developed a fluorometric method for the determination of Δ^4-3-ketosteroids[1]; the steroids react with isonicotinylhydrazine (INH) in a methanolic aluminium(III) salt solution to form hydrazones, which fluoresce owing to complex formation with aluminium ions.

This paper reports the application of the reaction to fluorescence detection in high-performance liquid chromatography of Δ^4-3-ketosteroids, where the UV detection method has previously been exclusively used.

EXPERIMENTAL

Reagents

Isonicotinylhydrazone of testosterone was prepared by the procedure of Umberger[2]. Other chemicals were obtained from commercial sources. A 16 m*M* INH solution was prepared by dissolving INH in methanol, and a 80 m*M* aluminium chloride solution by dissolving $AlCl_3 \cdot 6H_2O$ in methanol. The solutions were stable for several weeks at room temperature in brown bottles.

Liquid chromatography

Fig. 1 shows the complete liquid chromatographic system. Instruments and materials constituting the system were obtained from Kyowa Seimitsu Co. (Tokyo, Japan) unless otherwise stated.

The column for the separation of cortisol (F), corticosterone (B), deoxycorticosterone (DOC), 4-androstene-3,17-dione (AD), and progesterone (P) was 500 × 2 mm I.D. Hitachi Gel 3011 (Hitachi, Tokyo, Japan), a porous styrene–divinylbenzene copolymer of average particle diameter *ca.* 10 μm. The resin was packed in a stainless steel tube by a slurry technique at 2000 p.s.i. The steroids were eluted with *n*-hexane–methanol (5:95) at a flow-rate of 0.26 ml/min. The column inlet pressure was 780 p.s.i.

A Zorbax-Sil column, 250 × 2.1 mm I.D. (DuPont, Wilmington, Del., U.S.A.), and the eluent reported by Trefz *et al.*[3] were employed with a slight modification to

* Present address: Toyama Medical and Pharmaceutical University, Sugitani, Toyama-shi 930, Japan.

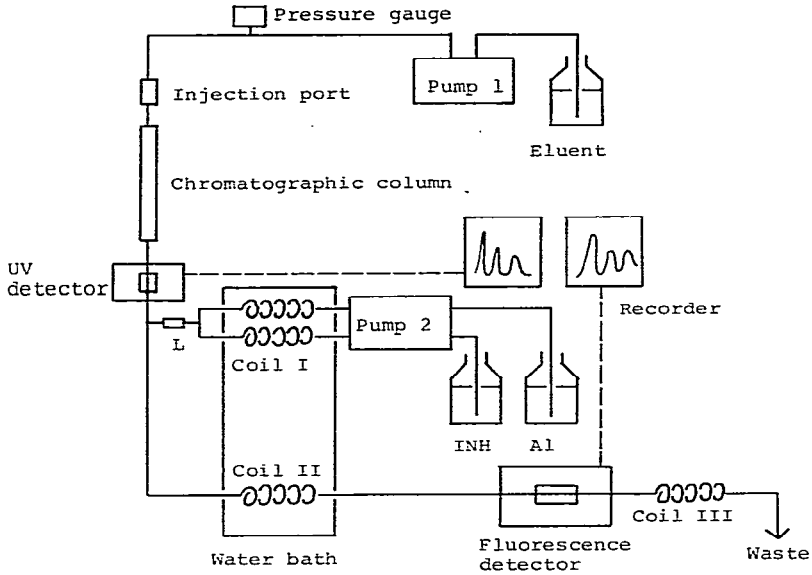

Fig. 1. Schematic diagram of the chromatographic system.

separate B, cortisone (E), F, and prednisolone (PL). The eluent was prepared as follows: 7 ml of distilled water and 12 ml of ethanol in a volumetric flask were made up to 500 ml with dichloromethane. The mixture was stirred vigorously for 1 h at 18° with a magnetic stirrer and allowed to stand overnight; the separated organic phase was used. The flow-rate for elution was 0.42 ml/min at 1200 p.s.i.

Each column was fitted with an on-column injection port, and sample solutions were injected through a PTFE rubber septum using a 5-μl high-pressure Hamilton syringe. The columns were operated at 18°.

Detection system

The column effluent was first monitored by a UV detector (Yanaco UV-212, Yanagimoto Co., Kyoto, Japan) at 254 nm, then added to a mixture of INH and aluminium chloride solution. Each reagent solution, heated for *ca.* 3.7 min in a PTFE tube (Coil I, 0.5 mm I.D.) immersed in a water bath maintained at 70°, was delivered separately by a double plunger pump (Pump 2) at half the flow-rate of the effluent. The solutions were combined just before the mixing with the effluent. The reaction mixture was passed through a reaction coil (Coil II, 20 m × 0.25 mm I.D. PTFE tube) in the same water bath and was monitored by a FLD-1 fluorescence detector (Shimadzu, Kyoto, Japan), which was equipped with a low-pressure mercury lamp of 360 nm and a secondary filter that transmits above 450 nm. The outlet of the detector was attached to Coil III (10 m × 0.25 mm I.D. PTFE tube), which provided enough back pressure to prevent bubble formation in the flow-cell. The recorders were EPR-2TC Electronic Polyrecorders (Toa Electronics, Tokyo, Japan).

The reaction times for the systems of Hitachi Gel 3011 column (System I) and Zorbax-Sil column (System II) were about 2.0 and 1.2 min, respectively.

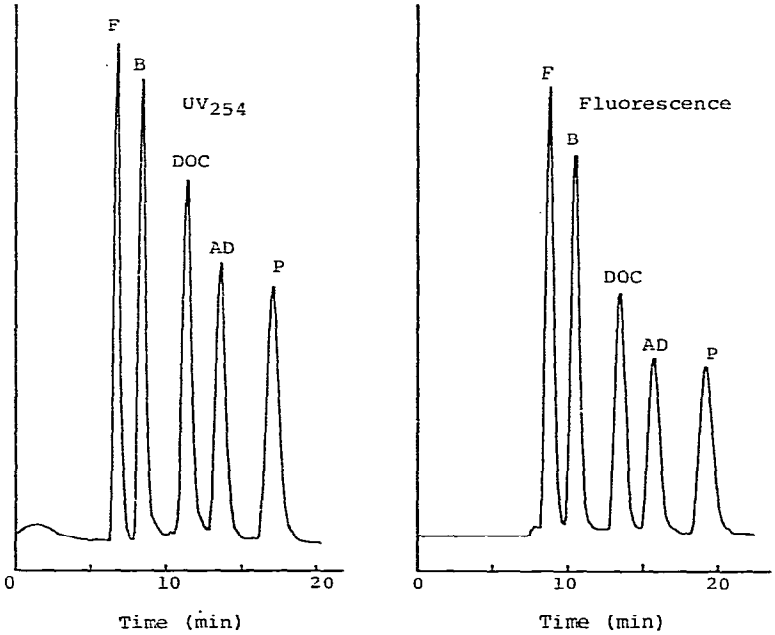

Fig. 2. Chromatograms of Δ^4-3-ketosteroids by UV and fluorescence detection. Column: Hitachi Gel 3011 (500 × 2 mm I.D.). 1 nmol of each steroids was injected.

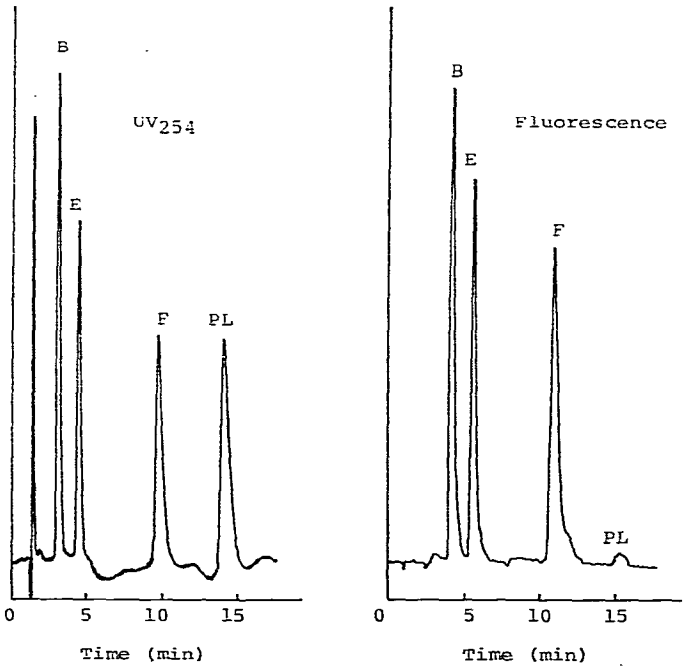

Fig. 3. Chromatograms of Δ^4-3-ketosteroids and prednisolone by UV and the fluorescence detection. Column: Zorbax-Sil (250 × 2.1 mm I.D.). The amount of each steroid injected was 0.5 nmol.

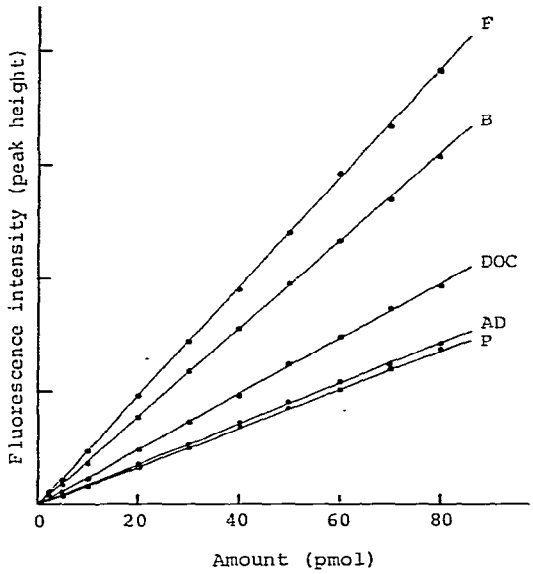

Fig. 4. Calibration curves of Δ^4-3-ketosteroids by fluorescence detection. Column: Hitachi Gel 3011 (500 × 2 mm I.D.).

RESULTS AND DISCUSSION

The steroids were derivatized to the fluorophors without serious distortion of the peak shapes (Figs. 2 and 3). The hydrazone of Δ^4-3-ketosteroid seemed to be formed quantitatively in both Systems I and II under the reaction conditions, as 50 pmol methanolic solutions of testosterone and isonicotinylhydrazone of testosterone injected into the reaction device by a line sample injector (L in Fig. 1) both gave equal fluorescence intensities in each system. The very weak response of the fluorescence detector to prednisolone (PL in Fig. 3) compared with those to Δ^4-3-ketosteroids was mainly due to the slower hydrazone formation of $\Delta^{1,4}$-3-ketosteroid with INH.

In System I, the calibration curves of peak heights against amounts of Δ^4-3-ketosteroids by the fluorescence detection were linear up to several nmol, and 2.5 pmol of cortisol could be detected (Fig. 4). The peak heights were reproducible and the coefficient of variation was 0.9% with 50 pmol of cortisol for five determinations. In System II, the fluorescence intensity of Δ^4-3-ketosteroid decreased to less than one-sixth of that in System I owing to the solvent effect of dichloromethane on the fluorescence[1] and to the greater flow-rate of eluent.

Though the sensitivity of the fluorescence detection is no better than that reported for UV detection[3,4], the selectivity of the fluorescence reaction will be useful for the analysis of the steroids in complex biological samples.

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