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Note

Direct thin-layer chromatography—fluorimetric quantification of pharmacological plasma concentrations of an antiarrhythmic steroid (Org-6001)

IB SØNDERGAARD* and EVA STEINESS

Department of Pharmacology and Medical Department B, Rigshospitalet University of Copenhagen, Copenhagen (Denmark)

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Org-6001 (3α-amino-5α-androstan-2β-ol-17-one hydrochloride) is an aminosteroid possessing antiarrhythmic properties without hormonal activity. It has been shown that Org-6001 is effective in the treatment of ventricular tachycardia induced by digitalis [1], and when administered either intravenously or orally is effective in the treatment of experimental arrhythmias induced by coronary occlusion [2]. For future clinical evaluation of this substance a thin-layer chromatography (TLC) method has been developed, which permits specific determination of Org-6001 in plasma to a lower limit of 10 ng/ml with a variability at this concentration of 14%. The fluorescence was developed in situ on thin-layer chromatograms by treatment of the TLC-plate with a solution of perchloric acid using a previously described method for amitriptyline/nortriptyline analysis [3], with a minor modification [4].

MATERIALS AND METHODS

Apparatus

A Zeiss spectralphotometer with TLC scanning equipment KM 3 (Carl Zeiss, Oberkochen, Württemberg, G.F.R.), linked to a Servogor Sb RE 646 recorder

*Present address: The Laboratory for Clinical Allergology, 5903-5 Rigshospitalet, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark

(Goerz Electro, Vienna, Austria), was used with the following settings: Monochromator 345 nm; fluorescence filter cutting at 430 nm; voltage selector 2; scanning speed 120 mm/min.

Chemicals

Sodium hydroxide (analytical grade) was obtained from Elektrokemiska (Bohus, Sweden) and 96% ethanol from DDSF (Copenhagen, Denmark). All other chemicals were analytical grade from E. Merck (Darmstadt, G.F.R.). Org-6001 was from Organon (Oss, The Netherlands) and the filterpaper was from Frisenette (Ebeltoft, Denmark).

Thin-layer chromatography

TLC was performed on 20 × 20 cm pre-coated silica gel 60 thin-layer plates with a layer thickness of 0.25 mm and without fluorescent indicator, from E. Merck. Duplicate samples were always run on separate TLC plates, with 12 samples spotted on each plate, 4 of which were extracts from spiked plasma samples. The chromatography tank was lined with Whatman No. 3 chromatography paper and the solvent was chloroform—methanol—diethylether—25% ammonia (30:15:5:0.25) or 1-butanol—chloroform—25% ammonia (35:15:2.5). Org-6001 has R_F values of 0.22 and 0.50 respectively in these solvent mixtures. The chromatography was conducted with the exclusion of light, then the plates were dried at 50° for 30 min.

Procedures

One ml of plasma was pipetted into a 10-ml PTFE stoppered centrifuge tube, mixed with 0.5 ml glycine—sodium hydroxide buffer, pH 9.9, and extracted twice with 7 ml of chloroform for 15 min. Centrifugation for 5 min at 1500 *g* separated the two phases. The two organic phases were washed twice, each with 3 ml 0.1 *N* NaOH. Five-ml samples from each of the organic phases were then combined and transferred to a conical centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°. The residue was dissolved in 50 μl chloroform and the extracted material was spotted under a stream of nitrogen onto the TLC plate using a 10-μl constriction pipette. An additional 20-μl portion of chloroform was added to the tubes, shaken and then spotted onto the plate. After chromatography, the spots of Org-6001 were oxidized in situ to produce fluorescence as follows: The TLC plates were dipped in a mixture of ethanol—water—perchloric acid, 135 : 135 : 12 (freshly prepared) for 7 sec and any excess of reagent was removed by pressing the wet layer against filter paper No. 617. The TLC plates were then immediately placed in an oven at 90° for 35 min. To ensure uniform heating, the TLC plates were placed on copper plates, 3 mm thick and after heat treatment were left at room temperature for 15 min before scanning, to ensure full development of the spots.

Calculations

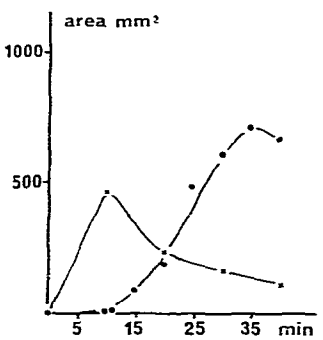
The amount of Org-6001 was calculated by comparison of the peak areas of the samples and standards. Standards with known amounts of Org-6001 were prepared from Org-6001-free plasma spiked with 10 μl methanol per ml of

plasma containing Org-6001. All calculations were corrected to give the free base of Org-6001.

RESULTS AND DISCUSSION

Sample preparation

Extraction of Org-6001 was found to be optimal at pH 9.9. When either the pH was increased or the two washings with 0.1 *N* NaOH were omitted, interference with an unknown substance from the plasma became apparent. Recovery at pH 9.9, by extraction of spiked plasma samples containing 112 and 224 ng/ml of Org-6001, was 90%. Furthermore, it was shown that the recovery by extraction of plasma samples spiked with methanolic Org-6001 solution was constant for an equilibration time between 5 and 240 min. An internal standard was not used because it would have required further clean-up steps. The fluorescence reaction is time and temperature dependent as shown in Fig. 1. Heating for 35 min at 90° was chosen and Fig. 2 shows the development of fluorescence after this heat treatment. The reproducibility of the development of fluorescence, tested with different amounts of pure substance, is seen in Table I.



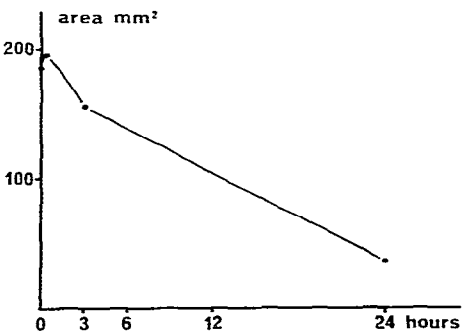


Fig. 1. Effect of heating time on measured areas (•, 90° ; x, 105°) scanning immediately after heating.

Fig. 2. Measured peak area as a function of time after heating for 35 min at 90°.

TABLE I

REPRODUCIBILITY OF THE DIRECT QUANTITATIVE ANALYSIS CARRIED OUT WITH PURE SUBSTANCE

Values from 12 measurements

Amounts applied per spot (ng) of Org-6001	Coefficient of variation (%)
112	5.3
7	11
3.5	19

Specificity, sensitivity and reproducibility

Plasma from subjects who had not ingested Org-6001 showed no interfering peaks when analyzed. An insignificant peak, with the same R_F value as Org-6001, could sometimes be seen if the aqueous phase had not been carefully aspirated. A typical scan is shown in Fig. 3. No interference was found between Org-6001 and other steroids, i.e. aldosterone, cortisol, testosterone, digoxin, and oestrogens. The lower limit for reliable quantitation of Org-6001 using 1 ml of plasma is 10 ng/ml. Reproducibility studies were performed on plasma samples spiked with Org-6001, and are outlined in Table II.

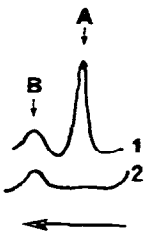


Fig. 3. Chromatogram scan showing 45 ng Org-6001 extracted from 1 ml plasma (1), and 1 ml plasma without Org-6001 extracted in the same manner (2). A is Org-6001 and B is an unknown substance from the plasma sample.

TABLE II

REPRODUCIBILITY OF THE ANALYSIS WHEN SPIKED PLASMA SAMPLES WERE ANALYSED ON DIFFERENT DAYS

Amount (ng) of Org-6001 in plasma sample	Coefficient of variation (%)	*n*
9	14.2	5
22	14.8	6
45	7.4	5
90	4.3	5
112	7.0	7

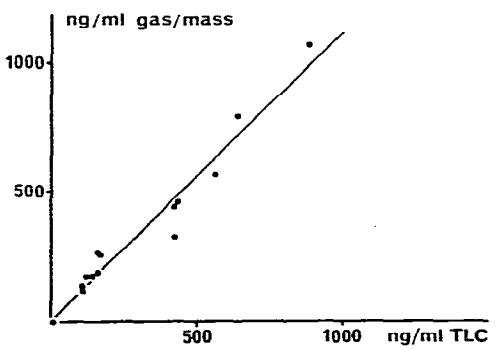


Fig. 4. Comparison of a GC—MS method (see text) with the present TLC method. Linear regression showed that the slope of the line is 1.1 and r^2 = 0.95.

Plasma samples from two patients actually treated with Org-6001 were measured both with gas chromatography—mass spectrometry (GC—MS)* and with the present TLC method. The two methods correlate well, as shown in Fig. 4. Fig. 5 shows the plasma concentrations of Org-6001 in two patients following intravenous administration of 350 mg. The dose was given in three parts (100 mg, 100 mg, 150 mg) at two-minute intervals. A rough estimate of the plasma half-life (Fig. 5) in cases A and B suggests a half-life of 10 h and 22 h, respectively. The relatively long half-life suggests that the plasma concentrations should be followed for a long period in future studies.

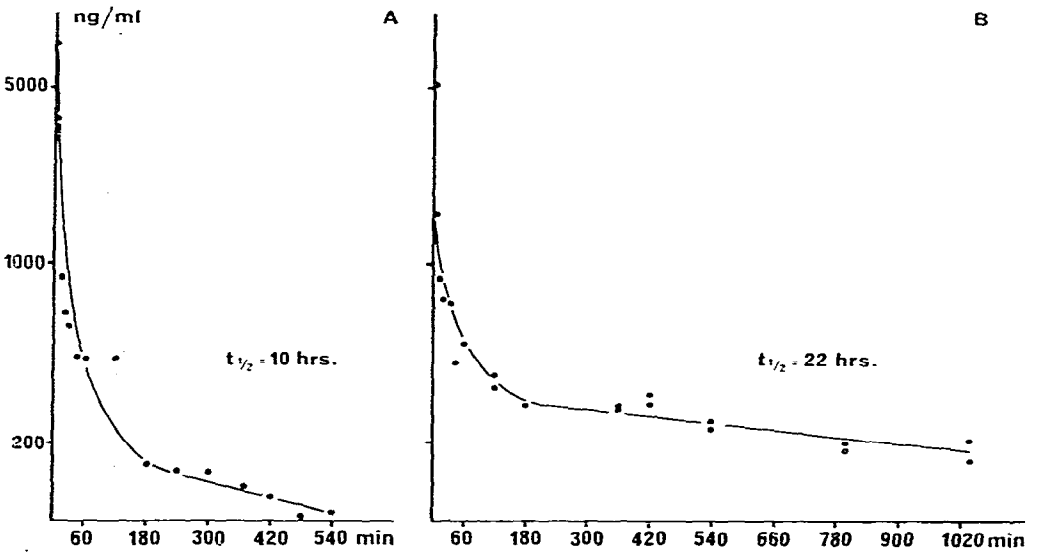


Fig. 5. Plasma concentrations of Org-6001 in two patients following intravenous administration of 350 mg Org-6001.

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4 J. Christiansen, personal communication.

*The GC—MS procedure can be outlined as follows: extraction, several clean-up steps, derivatization with tertiary butyl-dimethylchlorosilane—imidazole in dimethyl formamide, using [9,11,16-$^2H_3$]—Org-6001 as internal standard (Organon), GC and finally measurement of the peak heights at *m/e* 390 and *m/e* 393.