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Note

Determination of diftalone and its main metabolites in human plasma and synovial fluid by high-performance liquid chromatography

S. ROGGIA, G. GROSSONI, G. PELIZZA, B. RATTI and G. G. GALLO
Laboratori Ricerche Gruppo Lepetit S.p.A., Via Durando 38, 20158 Milan (Italy)
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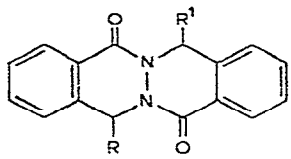
Studies[1–3] on the metabolism of the anti-inflammatory drug phthalazino[2,3-*b*]phthalazine-5,12-(7H,14H)-dione, known as diftalone (I), have shown that the blood of treated patients contains unchanged I and two metabolites, the 7-hydroxy-derivative (II) and the 7,14-dihydroxy derivative (III)[4–6].



I, $R = R^1 = H$
II, $R = OH$, $R^1 = H$
III, $R = R^1 = OH$

Currently available analytical methods do not permit determination of these three compounds in biological fluids. In fact, I has been determined in plasmas from various animal species by thin-layer chromatography[6] and gas–liquid chromatography (GLC)[7], and both I and II have been determined by GLC in guinea-pig plasma[8] and by a combination of GLC with mass fragmentography in human plasma[9]. With this last technique, III can be detected, but not determined, because its transformation into the detectable compound[10] is not quantitative.

The purpose of this work was to devise a method for determining I, II and III in human plasma and synovial fluid by high-performance liquid chromatography (HPLC), a technique suitable for separating polar compounds (such as II and III) without derivatization.

MATERIALS AND METHODS

Reagents

Solvents were of appropriate purities. I, II and III were Lepetit standards and were homogeneous by TLC. Dexamethasone alcohol (purum grade, Fluka, Buchs, Switzerland), used as standard, was added in ethyl acetate solution.

Chromatography

A DuPont Model 830 liquid chromatograph with a UV 254-nm detector was used, connected to a Hewlett-Packard Model 3380 A integrator. The column (1 m × 2.2 mm I.D.) was packed with Permaphase ODS and was operated at 54°. Two elution

conditions were studied: isocratic, with 0.8% aqueous acetonitrile at 200 p.s.i.; and gradient, with an increment of 6% per min from water to 20% aqueous acetonitrile at 500 p.s.i. Determination of the products was achieved with use of an internal standard; for this purpose, dexamethasone alcohol was chosen, because it has a ratio of peak area to weight similar to those of the compounds being studied.

Sample preparation

A 1-ml volume of the biological sample was placed in a 40-ml centrifuge-tube, 1 ml of 0.9% saline solution and 17 ml of ethyl acetate were added, and the mixture was shaken for 10 min and then centrifuged at 800 *g* for 3 min. A 15-ml aliquot of the organic phase was evaporated to dryness under nitrogen at 40°, the residue was dissolved in 3.5 ml of 90% aqueous acetonitrile, and this solution was washed by shaking with 3 ml of *n*-heptane and by centrifuging as described above. A 3-ml portion of the acetonitrile layer was introduced into a conical test-tube, and 3 ml of ethyl acetate containing 3 μg of dexamethasone alcohol were added. After evaporation to dryness under nitrogen at 40°, the residue was dissolved in about 20 μl of acetonitrile–isopropanol (1:1), and about 3 μl of this solution were injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Separation of the compounds under examination was obtained both by isocratic and by gradient elution. The gradient technique (see Fig. 1) is preferred, because compound III is better separated from the polar components of plasma and because of the intrinsic efficacy in washing the column. The reversed-phase chromatographic procedure allows the more polar compounds to be eluted first.

The precision and the accuracy of the method were studied by using samples of

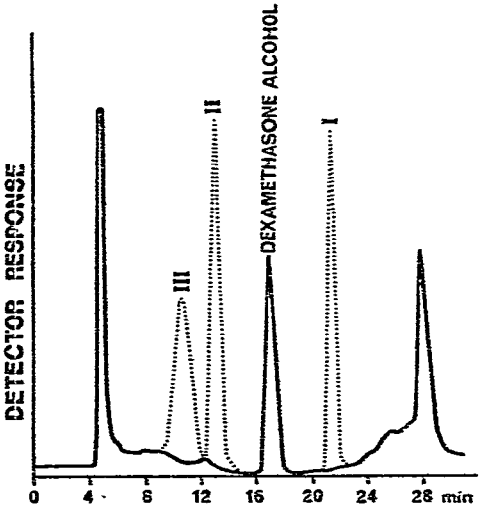


Fig. 1. Chromatograms. ———, From plasma of humans not treated with I (dexamethasone alcohol 1.3 μg/ml); · · ·, from the same plasma with I (1.3 μg/ml), II (1.3 μg/ml) and III (1.5 μg/ml) added.

TABLE I

PRECISION AND ACCURACY OF THE METHOD

Compound	*Added (μg/ml)*	*Found* (μg/ml)*	*Confidence limits (P = 0.05)*	*Recovery (%)*
I	0.5155	0.475	± 0.030	92.1
	1.031	0.978	± 0.017	94.9
	2.062	1.993	± 0.071	96.7
	4.160	4.053	± 0.181	97.4
	8.592	8.322	± 0.592	96.9
II	0.519	0.472	± 0.021	90.92
	1.038	1.127	± 0.085	108.6
	2.076	2.065	± 0.086	99.4
	3.088	4.130	± 0.267	103.0
	8.464	8.241	± 1.000	97.3
III	0.510	0.451	± 0.018	88.45
	1.021	0.811	± 0.033	79.5
	2.041	1.982	± 0.170	97.0
	4.188	3.973	± 0.452	94.8
	9.032	8.396	± 1.266	92.9

* Each result is the average of 5 determinations.

plasma and synovial fluid to which known amounts of I, II and III were added. The results are reported in Table I, from which it can be seen that recovery is satisfactory and that no recovery factor need be applied. The three compounds can be determined in plasma at levels as low as 0.5 μg/ml with an acceptable error.

The method is currently being applied to samples of plasma and synovial fluid from patients receiving treatment with diftalone; the results will be published elsewhere.

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