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GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF THE MAIN URINARY METABOLITE OF DIFTALONE, A NEW ANTI-INFLAMMATORY DRUG

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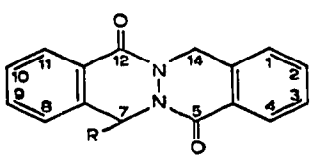
SUMMARY

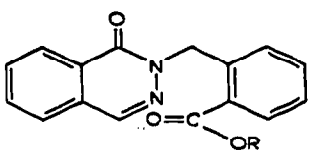
The main metabolite of diftalone in humans is 7-hydroxydiftalone, which is present in urine as the β-glucuronide. Its determination is based on acid hydrolysis in the biological fluid itself to an isomeric carboxylic acid, extraction with ethyl acetate, esterification with diazomethane to the methyl ester and gas–liquid chromatographic analysis of this ester. The method is applicable to the urine of humans, dogs and rats with satisfactory precision and accuracy. The sensitivity is down to 1 μg/ml.

INTRODUCTION

Diftalone*, phthalazino[2,3-*b*]phthalazine-5,12-(7H, 14H)-dione (I), is a new anti-inflammatory drug obtained by synthesis[1,2]. Its main metabolite in man has been demonstrated to be the 7-hydroxy derivative (II), which is present in urine mostly as the β-glucuronide (III)[3]. This paper describes a quantitative method for the determination of the free (II) plus the conjugated (III) metabolite in biological fluids, particularly in urine.



I, R = H
II, R = OH
III, R = O-Gluc.
IV, R = NH–CO–NH_2



V, R = H
VI, R = CH_3

The determination of I in blood serum has been performed by extraction with an organic solvent followed by quantitative thin-layer chromatography with UV detection[4]. This method is, in principle, also applicable to the determination of II in urine,

* Aladione®, Lepetit, Milan, Italy.

but it is not suitable for the determination of III, which is not extractable under such conditions. On the other hand, the acidic hydrolysis of III to II[3] does not seem to be reliable and this conversion seems difficult when performed enzymatically. As it was known that compounds II and III are quantitatively converted into V in acidic media [5,6], urine was hydrolyzed with hydrochloric acid, extracted with ethyl acetate, methylated with diazomethane and the methyl ester (VI) was determined by gas–liquid chromatography (GLC). Compound IV, a metabolite occasionally observed in human urine[3], will be determined together with II and III, because in acidic media it is converted into V[5]. Compound I, if present, will also be determined, as it remains unchanged during the procedure and gives a chromatographic peak well separated from that of VI.

MATERIALS AND METHODS

Reagents

Solvents and hydrochloric acid were of appropriate purities. 5α-Cholestane (purum grade, Fluka, Buchs, Switzerland) was used in methylene chloride solution. Diazomethane was prepared as an ethereal solution by a known procedure[7].

Sample preparation

A 10-ml volume of urine from subjects treated with I was introduced in a 100-ml round-bottomed flask connected to a condenser and 10 ml of 2 *N* hydrochloric acid were added. The solution was refluxed for 30 min, cooled to room temperature and extracted with 20 ml of ethyl acetate. A solution of cholestane, used as the internal standard, at a suitable concentration depending on the expected content of II and III, was added to the organic phase. After evaporation to dryness under vacuum at 50°, the residue was dissolved in 0.2 ml of methanol–methylene chloride (3:7) and treated with an excess of diazomethane for 5 min at room temperature. The solution was concentrated at room temperature under vacuum to a volume of 0.2–1.0 ml and analyzed by GLC.

Apparatus

A Hewlett-Packard Model 5750 gas chromatograph with a flame ionization detector was used under the following conditions: column temparature, 250°; injection port temperature, 280°; detector temperature, 260°; flow-rate of nitrogen carrier gas, 50 ml/min; injection volume, 1–5 μl; column, glass, 3 m long, I.D. 2.6 mm, O.D. 6.0 mm, packed with 10%OV-101 on Diatoport S, 80–100 mesh.

A Perkin-Elmer Model 270 gas chromatograph–mass spectrometer was used for the identification of VI under the following conditions: injection port temperature, 280°; manifold temperature, 250°; ion source temperature, 200°, 70 eV at 100 μA; flow-rate of helium carrier gas, 15 ml/min; column, glass, 2 m long, I.D. 2.6 mm, O.D. 6.0 mm, packed with 3% OV-101 on Chromosorb G, HP, 80–100 mesh, temperature programmed from 200° to 250° at 6.5°/min.

Calculations

Standard mixtures containing different and known amounts of V (and of I) and cholestane in methylene chloride were prepared. The mixtures were treated with

diazomethane under the above conditions and injected into the gas chromatograph in order to obtain the following correction factor:

$$F = \frac{W_s \cdot A}{W \cdot A_s}$$

where W_s = weight of cholestane (μg), A = area of VI (or I) peak, W = weight of V (or I) (μg) and A_s = area of cholestane peak. As the molecular weights of II and V are the same, the quotient of II (or of I) present in the urine sample is obtained by the following equation:

$$W_x\,(\mu\text{g/ml}) = \frac{W_s \cdot A_x}{A_s \cdot F \cdot 10}$$

where W_s = weight of cholestane (μg) added to the organic phase, A_s = area of cholestane peak, A_x = area of VI (or I) peak and F = correction factor for V (or I).

RESULTS AND DISCUSSION

The chemical conversions during the procedure were checked on a standard mixture of I and II in aqueous acidic solutions by ultraviolet spectroscopy[3], thin-layer chromatography[3], GLC and polarography*. It was found that, by refluxing II in 0.1–

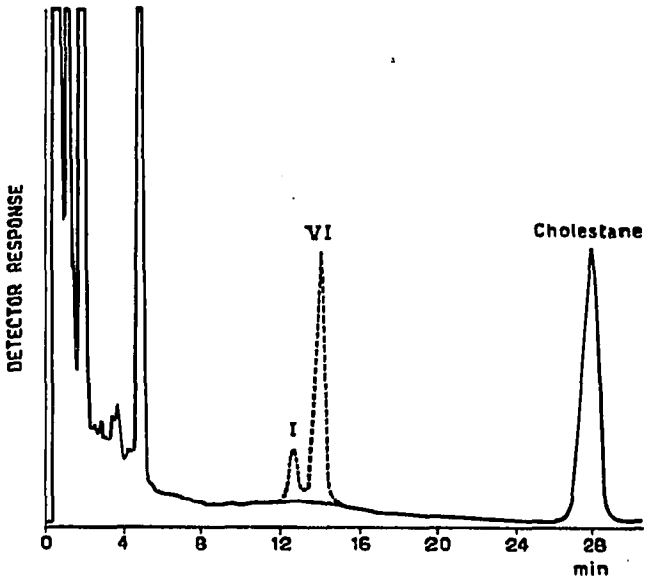


Fig. 1. Gas chromatograms: ———, from the urine of a patient not treated with I nor with other drugs; — — —, from the same urine with I and II added. Both samples were treated according to the present procedure.

* In methanol–acetate (1:1) buffer of pH 5.4, V gives a reduction wave with $E_{\frac{1}{2}} = -1.4$ V *vs.* saturated calomel electrode.

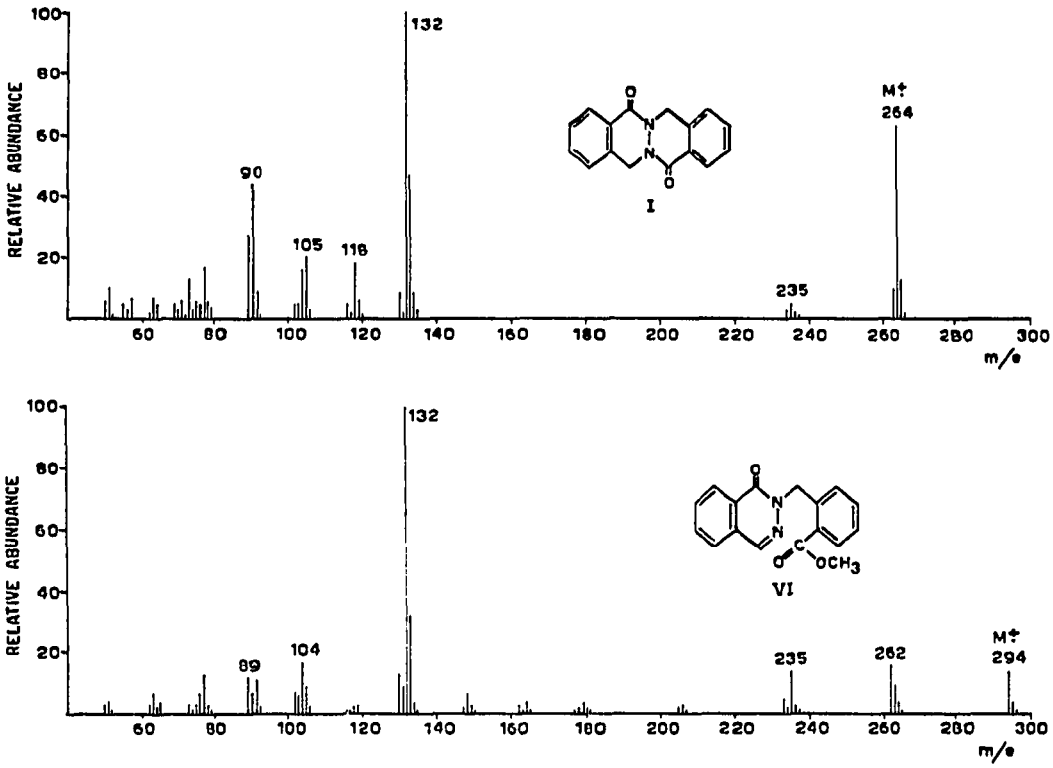


Fig. 2. Mass spectra obtained by GLC–mass spectrometry of I and VI.

TABLE I

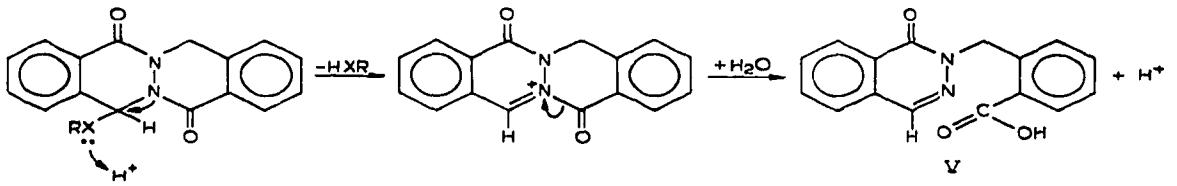
AMOUNT OF II (μg/ml) FOUND IN URINE OF PATIENTS TREATED WITH I USING DIFFERENT ACID CONCENTRATIONS WITH A REFLUXING TIME OF 30 min

Sample	*0.2 N HCl*	*1 N HCl*	*5 N HCl*
1	23	21	22
2	73	72	74
3	150	147	151
4	183	195	204
5	258	262	252
6	318	333	323
7	700	729	705

5 *N* hydrochloric acid for 15–20 min, it is converted quantitatively into V, while under the same conditions I remains unchanged. The same treatment was applied to human urine samples containing known amounts of I and II and GLC was shown to be the most suitable technique for their determination. I and II can be determined in urine because the peak of VI is well separated from that of I and those of the common substances in urine (Fig. 1). Furthermore, the peaks of I and VI were checked by GLC–mass spectrometry in order to confirm their identity, by comparing their mass spectra, shown in Fig. 2, with those of authentic samples[8].

In order to establish the best conditions for the hydrolysis, the acid concentration and the reflux time were studied using reference samples of urine containing I and II. The conversion of III into V could not be studied directly because a standard sample of III was not available. However, by applying the complete procedure to samples of urine from patients treated with I using different acid concentrations, results that were not significantly different were obtained (Table I).

We concluded that the glucuronide was quantitatively hydrolyzed by the acid at all concentrations, and this conclusion was confirmed by the results of studies carried out on animals treated with ^{14}C-labelled I[6]. The conversion of III (and IV) into V under acidic conditions can be considered to occur by a direct reaction, as in the conversion of II into V[5], as shown in the following scheme:



II, XR = OH
III, XR = O-Gluc.
IV, XR = NH–CO–NH_2

The reflux time for the transformation was studied and the results are summarized in Table II.

TABLE II

AMOUNT OF II (μg/ml) FOUND IN URINE OF PATIENTS TREATED WITH I USING 1 *N* HYDROCHLORIC ACID WITH DIFFERENT REFLUX TIMES

Reflux time (min)	*Amount of II (μg/ml)*
15	132
30	136
60	136
120	138

The accuracy of the method was studied using urine samples to which known amounts of I and II were added. The recovery (Tables III and IV) can be considered to be satisfactory and therefore a recovery factor does not need to be applied. However, owing to the complexity of the method, it is advisable to check the recovery for each series of samples. In Table IV, some precision results are also reported. Compounds I and II can be determined in urine at a level as low as 1 μg/ml with an acceptable error, as shown in Table III. Experiments with high concentrations of I were not performed because the content of I in urine, if present, is generally very low.

The method has been applied successfully to urine samples from humans[9], dogs and rats. The application of the method to urine samples from other animal species appears possible in principle, but the components of the urines of different animal species or other metabolites[10] might interfere in different ways.

TABLE III

RECOVERY OF I AND II ADDED TO URINE SAMPLES

Amount added (μg/ml)		*Amount found (μg/ml)*			*Error (μg/ml)*		$\frac{Found}{Added} \cdot 100$	
I	*II*	*I*	*II*		*I*	*II*	*I*	*II*
1.33	1.46	1.1	1.3		−0.23	−0.16	83	89
2.66	2.92	2.5	2.8		−0.16	−0.12	94	96
6.65	7.3	6.4	7.4		−0.25	+0.10	96	101
13.3	14.6	13.7	13.7		+0.40	−0.90	103	94
				Mean error	−0.06	−0.27		
				Relative error	−1.00%	−4.11%		

TABLE IV

RECOVERY OF II ADDED TO URINE SAMPLES

Amount added (μg/ml)	*Amount found (μg/ml)*	*Error (μg/ml)*	$\frac{Found}{Added} \cdot 100$	*Relative standard deviation (%)*
40.0	42	+2	105	
46.0	41	−5	89	7.4
46.0	42	−4	91	
46.0	47	+1	102	
57.0	54	−3	95	
120.0	116	−4	97	
142.0	131	−11	92	
567.0	539	−28	95	2.2
567.0	522	−45	92	
567.0	533	−34	94	
567.0	550	−17	97	
895.0	868	−27	97	
895.0	877	−18	98	
	Mean error	−14.8		
	Relative error	−4.2%		

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