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GAS CHROMATOGRAPHIC DETERMINATION OF DIFTALONE IN HUMAN PLASMA BY USE OF AN AUTOMATIC SAMPLER

ANTONIO MORRONE and EUGENIO BERETTA
Research Laboratories of Gruppo Lepetit S.p.A., Via Durando 38, 20158 Milan (Italy)
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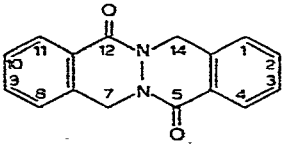
SUMMARY

A method is described for the determination of diftalone in human plasma, based on gas chromatography of a purified diethyl ether extract. The procedure, which has been designed for use with an automatic sampler, allows the assay of diftalone at plasma levels as low as 0.5 μg/ml.

INTRODUCTION

Diftalone*, a new non-steroidal anti-inflammatory agent[1–3], can be measured in biological fluids by gas chromatography (GC)[4–5]. However, with crude extracts of human plasma, especially at low drug concentrations, considerable interference by various endogenous constituents has been observed.



diftalone: phthalazino[2,3-*b*]phthalazine-5,12(7H,14H)-dione

The purpose of the present work was to increase the reliability of the results, and to meet the requirements of an instrument equipped with an automatic sampler and integrator. This was achieved by improving the GC performance and by modifying the entire procedure, as well as by purifying the crude extract, which eliminates most sources of interference.

EXPERIMENTAL

Reagents

Heptane (E. Merck, Darmstadt, G.F.R.), methanol (E. Merck), ethyl acetate

* Lepetit trademark; the compound is also known as Aladione or Diftal in many countries.

(Carlo Erba, Milan, Italy) and diethyl ether (Carlo Erba), of analytical grade, were distilled and stored in brown glass bottles with well-fitting PTFE stoppers in a refrigerator. Since diethyl ether is too unstable to be stored, it must be used within a few hours of distillation. Water was distilled from glass and stored in a glass vessel. Diftalone and 7-ethoxydiftalone, used as internal standard, were synthesized in the Research Laboratories of Gruppo Lepetit[7]. 7-Ethoxydiftalone was dissolved in ethyl acetate to give a concentration of 1 mg/ml. This solution was diluted with diethyl ether, and used as the solvent for the first extraction.

Equipment

Glass centrifuge tubes (100 mm × 10 mm I.D.) were cleaned by heating in a furnace at 550° for at least 3 h. The solvent was dispensed from a 1-ml Oxford pipettor. Newly opened 2-ml disposable vials were used to collect the final extract. A Perkin-Elmer Model F 30 gas chromatograph with a flame ionization detector (FID), connected to a Perkin-Elmer SIP 1 integrator and equipped with a Perkin-Elmer AS 41 automatic sampler, was used.

Preparation of the purified extract

A plasma sample (0.2–0.5 ml), containing at least 0.2 μg of diftalone, was pipetted into a centrifuge tube and 2 ml of diethyl ether containing the internal standard were added. (The amounts used should be such that the ratio of internal standard to diftalone is between 0.5 and 10.) The tube was stoppered with a disposable polythene cap, shaken on a Vortex mixer for at least 1 min and centrifuged for 5 min at 3500 *g*. As much as possible of the ether phase was then transferred with a Pasteur pipette to a test-tube and evaporated* to dryness under a stream of nitrogen in a water-bath at 37°. The residue was taken up in 1 ml of methanol–heptane (4:1); 0.2 ml of heptane was added and the tube was shaken for a few seconds on a Vortex mixer (after which time the residue was completely dissolved).

0.5 ml of water was then added, and the tube was stoppered, shaken on the Vortex mixer for 10–15 sec and centrifuged as before. The heptane phase was removed by suction as completely as possible. It contains lipids, cholesterol and other substances which may cause interference. The water–methanol phase remaining in the tube was exposed to a stream of nitrogen in a water-bath at 37° until its volume was reduced to 0.2–0.3 ml, thus eliminating most of the methanol.

At this stage, 2.0 ml of ethyl ether were added to the tube, which was stoppered and shaken on the Vortex mixer for at least 1 min. After centrifugation, most of the ether phase was transferred to a vial and evaporated to dryness as before. The vial was sealed and stored at −20° until GC analysis of the sample was carried out.

Gas chromatography

The contents of the vial were dissolved in ethyl acetate–methanol (1:1) and an aliquot portion of this solution, containing not less than 200–300 ng of the internal standard and corresponding to no more than 0.3 ml of plasma, was injected. GC was performed on a column of 3% SP 2250 coated on Supelcoport (80–100 mesh) contained in a silanized glass tube (1.8 m × 2 mm I.D.). The working conditions were:

* Ether extracts must be evaporated without delay (see Reagents).

nitrogen carrier gas flow-rate, 40 ml/min; column temperature, 255°; injection block temperature, 270°; detector temperature, 300°. The retention times were: 8.5 min for diftalone and 10 min for 7-ethoxydiftalone (Figs. 1 and 4). The analysis time was 13 min (when the automatic sampler is used, it should be extended to 22 min).

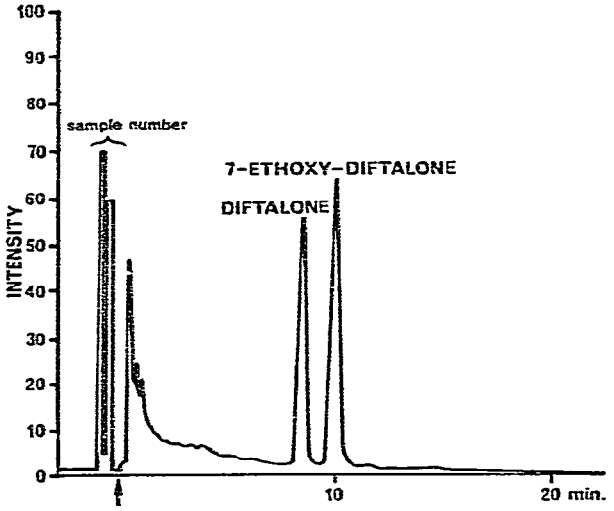


Fig. 1. Gas chromatogram of a diftalone–7-ethoxydiftalone (2:3) mixture. The arrow indicates the starting time, and the two narrow columns to the left of it represent the sample number in analog form (in this case 76).

RESULTS

Linearity, reproducibility and sensitivity of the method

Known amounts of diftalone were added to plasma from untreated subjects to produce concentrations ranging from 0.5 to 16 μg/ml. All of the samples having concentrations $\geqslant 1$ μg/ml gave recoveries of diftalone ranging from 93 to 103% (Table I), demonstrating that 7-ethoxydiftalone is a suitable internal standard. The linearity and reproducibility of the data also appeared to be satisfactory. The limit of sensitivity was *ca.* 0.1 μg of diftalone per ml of plasma. At this concentration, the purified extract produced a definite peak in the gas chromatogram, which was clearly distinguishable from any interference.

Automatic sampling

In the AS 41 injection system, the aliquot portion of the extract (up to 25 μl) is placed in an aluminium capsule where the solvent may be totally evaporated under vacuum. The capsule is then sealed, and at programmed time intervals is introduced automatically into the injection block, perforated and "washed" by the carrier gas. The injection of the solid residue provides a baseline which falls rapidly because of the elimination of the interference of the solvent in the initial phase of the chromatography. Non-volatile material from the plasma does not enter the column and is ex-

TABLE I

RECOVERY OF DIFTALONE FROM HUMAN PLASMA

Substance in 1 ml of plasma (μg)			*Recovery (%)*
Int. standard added	*Diftalone added*	*Diftalone found*	
6.00	0.50	0.46	92.0*
6.00	0.50	0.40	80.0*
6.00	0.50	0.43	86.0*
6.00	1.00	0.96	96.0
6.00	1.00	0.93	93.0
6.00	2.00	1.93	96.7
6.00	2.00	1.85	92.7
6.00	4.00	3.94	98.7
6.00	4.00	3.84	96.0
24.00	8.00	8.07	100.9
24.00	8.00	7.57	94.6
24.00	8.00	7.90	98.7
24.00	16.00	16.51	103.1
24.00	16.00	15.59	97.4

* Addition of a smaller amount of the internal standard would probably result in higher recoveries.

pelled with the capsule at the end of the analysis, thus avoiding contamination of the column with pyrolysis products.

The column used in our study proved to be satisfactory and showed no evidence of contamination even after the analysis of *ca.* 1000 plasma extracts. The separation capacity even increased after the injection of the first hundred samples. The use of the automatic sampler requires certain precautions. For instance, when manual intervention is impossible, the analysis time should be extended from 13 to 22 min. This eliminates the possibility that a large amount of cholesterol (retention time, 17 min), accidentally remaining in the extracts of high cholesterol plasmas in spite of the purification, causes interference with the next chromatogram, such as displacement of the baseline and/or baseline slope. For the same reasons, injection of excessively large amounts of the extract should also be avoided. Insertion after every 10 test samples of a capsule containing the external standard mixture [diftalone and 7-ethoxydiftalone (2:3)] helps to adjust the baseline to the correct level and provides additional time for column "washing" and checking the response of the GC system.

DISCUSSION

Diftalone levels in human plasma encountered in clinical and bioavailability studies[8] have been satisfactorily evaluated by a thin-layer chromatographic (TLC) method[9]. The GC procedure described here gives improved sensitivity in the drug determination and reduces the error when the drug level is below 3 μg/ml.

GC determination of diftalone in crude plasma extracts from rat, guinea-pig and monkey presents no difficulties, but the same technique when applied to human

plasma requires a purification step. In the GC determination of various biologically active substances, considerable interference from constituents of human plasma has been noted by a number of workers. Because of the great variability in the composition of human plasmas, due to the timing of collection, the state of health, age and diet of the subject, etc., one must eliminate substances which cause interference when making measurements of relatively small amounts of drugs.

Among the solvents capable of extracting diftalone from plasma, diethyl ether is particularly suitable, since it provides a good yield of the drug with reduced extraction of those substances which cause interference. Also, it does not form an emulsion with the plasma and evaporates very rapidly. However, crude ether extracts contain endogenous substances which may cause: (1) superimposition of peaks with those of diftalone and of the internal standard; (2) temporary deterioration in the resolving power of the column, reduction in sensitivity (Fig. 2) and incomplete separation of diftalone from the internal standard; (3) a considerable increase in the analysis time to allow for the removal of cholesterol, which often produces an enormous and tailed peak (Fig. 2a); (4) excessive contamination of the column and reduction in its useful life and (5) difficulties in automatic operation and integration due to excessive baseline slope or shift in a long sequence of samples.

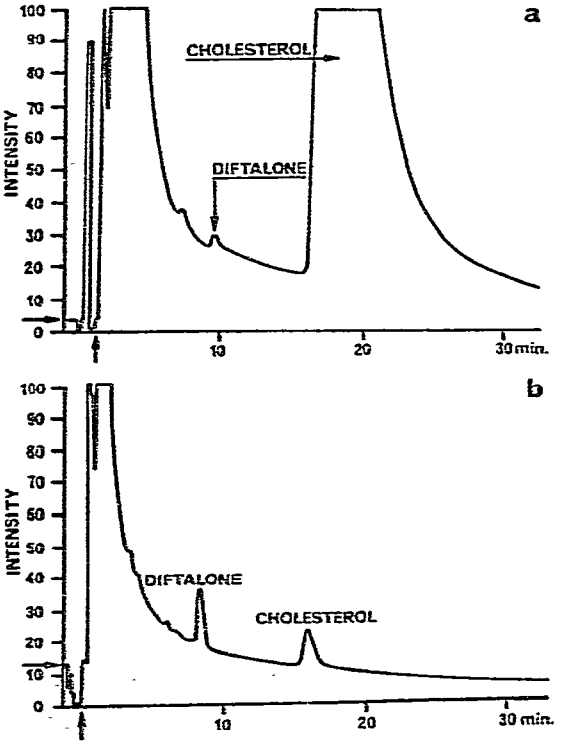


Fig. 2. Gas chromatogram of a diethyl ether extract of human plasma, to which diftalone had been added at 1 μg/ml, from a patient suffering from rheumatoid arthritis: (a) without purification; (b) the same extract after purification. The horizontal arrows indicate the baseline level just before injection. Note: (1) the substantial reduction of the cholesterol peak; (2) the better definition of the diftalone peak and (3) the improved return of the baseline to the starting level.

Separation of the substances which cause interference, mainly lipids, from diftalone was attempted on the basis of differences in their solubilities. Chlorinated solvents, which are most suitable for diftalone, could not be used because they are also good solvents for lipids. Diftalone is practically insoluble in both water and heptane but relatively soluble in methanol; heptane–water–methanol (4:5:8) was suitable, since the upper layer (heptane) preferentially extracts the unwanted substances. The choice of aqueous methanol as the second phase was a compromise dictated by the need to have a biphasic system, essential for separation, and to minimize the dissolution of lipid material in the more polar phase and of diftalone in the upper phase. The complete removal of heptane is essential for successful purification.

7-Ethoxydiftalone seemed to be suitable as internal standard for the following reasons: (1) its solubility in organic solvents and polarity characteristics are similar to those of diftalone; (2) it gives a peak quite distinct from that of diftalone; (3) its retention time is not excessively long and (4) it peaks in the interference-free zone of

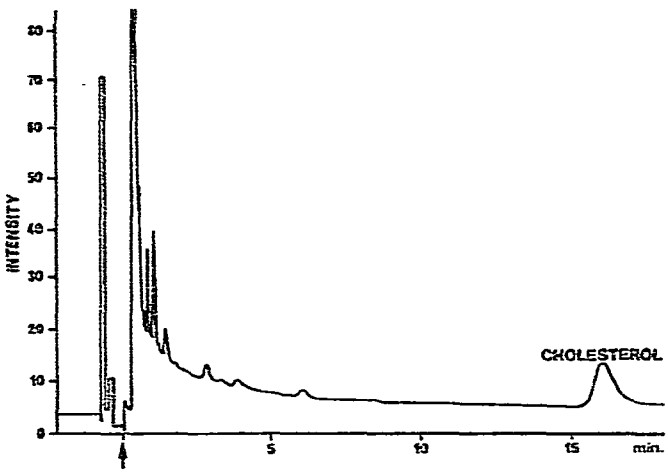


Fig. 3. Typical GC pattern of a purified extract of plasma from an untreated subject.

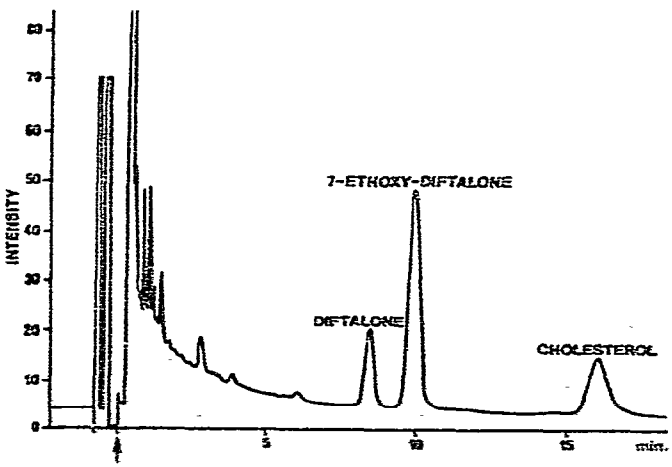


Fig. 4. Gas chromatogram of a purified extract of human plasma containing diftalone (0.9 μg/ml).

the chromatogram (Fig. 3). As regards choice of the amount of the internal standard to be added to the test samples, we noted that amounts of less than 200–300 ng may be partly adsorbed on the column and give abnormally low correction factors. On the other hand, in order to avoid interference from residual substances, the injected sample should correspond to $\leqslant 0.3$ ml of plasma. In practice, when samples contained very low levels of drug (< 1 μg/ml) we could no longer employ an extract containing similar amounts of diftalone and of the internal standard.

The metabolites of diftalone in human plasma, *i.e.*, 7-hydroxydiftalone and 7,14-dihydroxydiftalone, do not interfere with the determination of this drug, even when they are present in amounts up to 20 μg/ml. Finally, plasma samples re-assayed after 2 months storage at $-20°$ did not show significant changes in the concentrations of the drug. Moreover, occasional freezing and thawing did not result in deterioration of the diftalone in the samples.

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