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# QUANTITATIVE DETERMINATION OF PLASMA OXYPHENBUTAZONE BY GAS–LIQUID CHROMATOGRAPHY WITH SELECTIVE NITROGEN DETECTION

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## SUMMARY

A sensitive and specific gas chromatographic method, using the nitrogen–phosphorus detector for the detection and determination of oxyphenbutazone extracted from plasma is described. The method involves extraction and back-extraction steps followed by derivatization of both oxyphenbutazone and the internal standard with trifluoroacetic anhydride. The procedure permits the rapid and specific routine determination of oxyphenbutazone in plasma with a detection limit of 0.5 $\mu$g/ml. The procedure is linear over the range of concentrations encountered after administration of a single oral therapeutic dose. No interference from the biological matrix is apparent. The suitability of the method for the analysis of biological samples was tested by studying the variation with time of oxyphenbutazone plasma concentrations in normal human volunteers over a period of several biological half-lives.


## INTRODUCTION

Oxyphenbutazone [1-phenyl-2-(*p*-hydroxyphenyl)-3,5-dioxo-4-*n*-butylpyrazolidine] (Fig. 1, I) is a derivative and active metabolite of phenylbutazone (1,2-diphenyl-3,5-dioxo-4-*n*-butylpyrazolidine) with a phenolic group in the *para* position of the benzene ring of the parent compound. The drug exhibits the same spectrum of activity and toxicity as phenylbutazone and is used on its own right for the same therapeutic indications[1]. Oxyphenbutazone is a weak organic acid with a $pK_a$ of about 4.5, has a low aqueous solubility and probably has the same poor wettability properties as phenylbutazone. These combined characteristics are such that this drug may present high potential risks of poor oral bioavailability. Thus, the assay of oxyphenbutazone in biological fluids is important for single-dose pharmacokinetic and comparative systemic availability studies, and also in analytical doping control in horses as many countries have restrictions against the use of anti-inflammatory drugs in horses.

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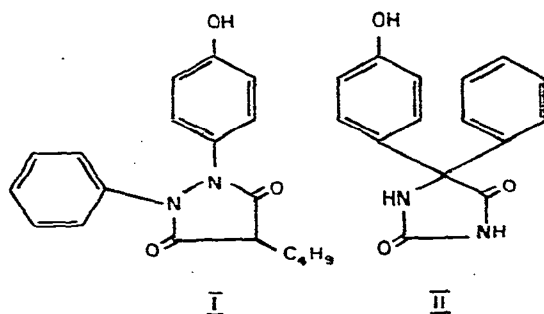


Fig. 1. Structure of (I) oxyphenbutazone and (II) 5-(4-hydroxyphenyl)-5-phenylhydantoin internal standard.

Most published methods are concerned with the assay of phenylbutazone. Ultraviolet spectrophotometry[2,3], gas chromatography with flame-ionization[4,5] and electron-capture[6] detection, high-performance liquid chromatography[7] and mass spectrometry[8] have been proposed. Other studies using gas chromatography with flame-ionization[9,10,11] and electron-capture[12] detection, high-performance liquid chromatography[13] and differential pulse polarography[14] have also been reported for the simultaneous determination of phenylbutazone and oxyphenbutazone (as its major metabolite) extracted from biological fluids.

The objectives of the present study were to develop a simple, rapid, specific and sensitive gas chromatographic assay for the determination of plasma oxyphenbutazone using a selective nitrogen detector and to test its validity for pharmacokinetic and physiological availability studies in man.

## EXPERIMENTAL

### *Extraction and derivatization*

All reagents and solvents were of analytical-reagent grade and were distilled before use. To a 1-ml aliquot of plasma were added the internal standard [0.5 ml of a 40 $\mu$g ml$^{-1}$ solution of 5-(4-hydroxyphenyl-5-phenylhydantoin][11] (Fig. 1, II) and 2 ml of 1 $N$ hydrochloric acid. The solution was extracted with 5 ml of diethyl ether for 10 min on an Eberbach mechanical agitator. After centrifugation at 600 g for 10 min, an aliquot of the organic layer was carefully transferred to another tube and back-extracted with 4 ml of 0.2 $M$ phosphate buffer (pH 11.2). After acidification of the aqueous phase with 1 ml of 4 $N$ hydrochloric acid, the solution was extracted a second time with diethyl ether.

The organic layer was transferred into a clean, dry culture tube and evaporated to dryness in a Buchler vortex/evaporator. To the residue were then added successively 100 $\mu$l of ethyl acetate and 100 $\mu$l of trifluoroacetic anhydride (TFAA). After rapid vortex agitation, the mixture was heated at 65° for 15 min on an aluminium block. A second evaporation was then effected as indicated above and the residue taken up into 50 $\mu$l of ethyl acetate and a 3-$\mu$l aliquot was injected into the gas chromatograph.

### *Gas chromatography*

All analyses were performed using a Hewlett-Packard Model 5730 gas–liquid

chromatograph modified with a side-mounted Perkin-Elmer nitrogen–phosphorus (N–P) detector operated in the N/P mode. The chromatographic conditions were as follows: a 122 cm × 2 mm I.D. coiled glass column containing 3% OV-17 on DMCS-treated and acid-washed high-performance Gas-Chrom W (80–100 mesh) (Chromatographic Specialties, Brockville, Canada) was heated at 180°, then programmed at 16°/min to a final temperature of 280°, which was held for 4 min. The injector temperature was 250°, the detector temperature 300°, with the bead current adjusted for maximal operating conditions. The flow-rate of helium carrier gas through the column was 30 ml/min and the air and hydrogen flow-rates of the N–P detector were 150 and 3.5 ml/min, respectively. The instrument was linked to a laboratory data system (Hewlett-Packard, Model 3352B) which allowed numerical reduction of the data and through which computerized analytical reports were obtained. The identities of the oxyphenbutazone and internal standard peaks were ascertained by gas chromatography–mass spectrometry.

Calibration graphs were constructed in the following manner: Twenty-five drug-free plasma samples were supplemented with 20 μg of the internal standard and to each of five sets of five samples were added weighed amounts of oxyphenbutazone to give concentrations of 2.5, 5, 10, 15 and 20 μg/ml. These samples were carried through the complete extraction and derivatization procedure described above. The average of each five measurements of peak-area ratios [oxyphenbutazone derivative to 5-(4-hydroxyphenyl)-5-phenylhydantoin derivative] were then plotted against oxyphenbutazone concentration. For quantitation of plasma extracts, a relative response factor ($F_r$), calculated in the following way

$$F_r = \frac{\text{(internal standard peak area) (oxyphenbutazone concentration)}}{\text{(internal standard concentration) (oxyphenbutazone peak area)}}$$

was given to the computer. This factor takes into account simultaneously the percentage recovery of oxyphenbutazone from plasma and the yield of the derivatization step.

### *Human studies*

Ten-millilitre blood samples were drawn from the antecubital vein at various times from volunteers who had taken a single 200-mg dose of oxyphenbutazone under controlled conditions to be described elsewhere. Collection was effected into 10-ml evacuated heparinized tubes and the blood was immediately transferred into a glass culture tube before centrifugation at 600 g for 6 min. The plasma was then harvested, transferred into another tube and frozen at −20° until analysed. This procedure was followed in order to avoid contact with the tip of the evacuated tube which causes contamination of the sample with tri-(2-butoxyethyl) phosphate, as was found by others[15] and identified in this laboratory by gas chromatography–mass spectrometry[16].

## RESULTS AND DISCUSSION

Typical gas chromatograms are shown in Fig. 2. Fig. 2A shows the chromatogram of a drug-free sample carried through the extraction and analysis procedure. At maximal detector sensitivity and minimal computer threshold, no detectable signal

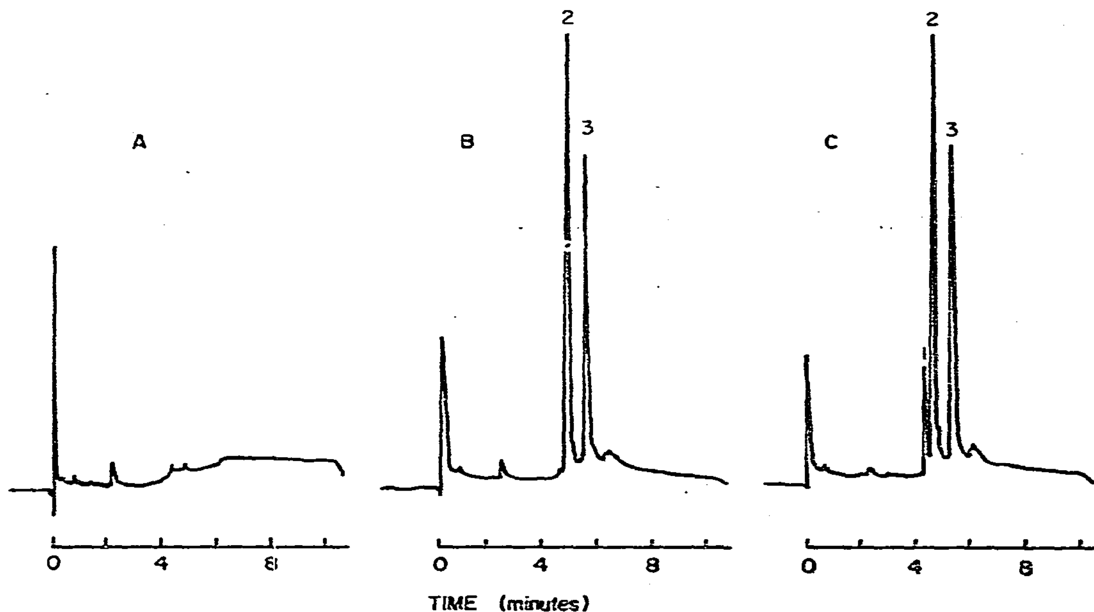


Fig. 2. Chromatograms of plasma extracts. (A) The 0-h sample of a volunteer before receiving oxyphenbutazone; (B) the 96-h sample of the same subject after ingestion of a 200-mg oral dose of oxyphenbutazone; (C) the 2-h sample of the same subject. Peaks: 1 = non-interfering peak resulting from the contamination by the tip of the evacuated tube; 2 = TFA-derivatized oxyphenbutazone; 3 = TFA-derivatized 5-(4-hydroxyphenyl)-5-phenylhydantoin.

appeared on the chromatograms and computer print-outs at the retention times of oxyphenbutazone derivative and the internal standard derivative. An interesting and constant feature of chromatograms resulting from such analyses under the specified operating conditions was the virtual absence of solvent peaks. In addition, even though temperature programming without a compensating column was used, the baseline in chromatograms of actual biological samples was stable throughout the analyses. This is mostly due to the selectivity of the N–P detector towards nitrogen-containing compounds and discriminating effects against biological matrix interferences. Further, no meaningful contamination of the detector assembly was observed, allowing experimental conditions to be maintained constant and stable over the duration of a study involving the analysis of several hundred samples. This is a distinct advantage of using trifluoroacetic anhydride as the derivatization reagent[17].

Fig. 2B shows a typical chromatogram of a plasma sample of the same subject 96 h after administration of a single oral dose of 200 mg of oxyphenbutazone. The computer print-out giving integration parameters and analytical results corresponding to Fig. 2B is given in Fig. 3. No peak corresponding to the tri(2-butoxyethyl) phosphate contaminant was observed in most of the chromatograms. This compound, which apparently leaches out of the tip of the evacuated tube into blood, has been observed by us[16] and other investigators[15]. In some chromatograms in the present study, this compound was present and eluted before the oxyphenbutazone derivative (Fig. 2C) and thus did not interfere in the quantitative analysis, having a retention time of 0.82.

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REPORT NO. 1315                                   SYSTEMIC AVAILAB.

CHAN#1        METHOD:  OXYPHE          SAMPLE: PLASMA

MIN AR  MV/M    DLY.     DVT      DIL-FTR%      ID-LVL  REF-RTW  %RTW
300      .200   1.00    0.00       100.00         300      .15     2

ISTD = 20.000,R PPM

RT       ITM      FACTOR                AREA          PPM       NAME

4.69     4.69      .38023               5165 BV      8.398      OXYPHEN
4.90              1.00000               .449 VB      1.919
5.36     5.36#    1.00000               4677 BB                 &ISTD

TOTAL AREA =      10291

STORED AS FILE  30  PLASMA 1

METHOD:OXYPHEN         SAMPLE:PLASMA        CANAL 1
       RT                RRT        NOMS
       4.691            .876        OXYPHEN
       4.902            .915
       5.357           1.000        &ISTD
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Fig. 3. Computer print-out corresponding to Fig. 2B showing integration parameters of the assay and analytical results.

The response of the detector to increasing amounts of oxyphenbutazone derivative relative to the internal standard derivative is shown in Fig. 4 to be linear over the range 2.5–20 $\mu$g/ml ($r = 0.98$). Similarly, the validity of the peak-area ratio for quantitation is shown over the same range with results from drug-free samples supplemented with increasing amounts of oxyphenbutazone and a constant amount of internal standard carried through the analytical procedure. The linearity of this plot ($r = 0.99$) indicated constant recoveries of both compounds and ascertained the validity of the method.

Analysis of variance showed no significant differences between calibration plots constructed at different periods and a constant response factor of 0.38 was therefore assigned to the computer for quantitation. Day-to-day variations in absolute retention times of oxyphenbutazone and internal standard derivatives were negligible and without consequence as they were automatically corrected for by the auto-calibration routine of the data system. The relative retention time of oxyphenbutazone derivative was 0.87 throughout the study.

The reliability of the quantitative results was tested by injecting daily, throughout the study, a mixture containing 10 $\mu$g/ml of oxyphenbutazone and 20 $\mu$g/ml of the internal standard derivatives. Peak integration on samples having identical concentrations injected at different times showed little variation. For example, a coefficient of variation of 7.9% was obtained from the average of daily injections made

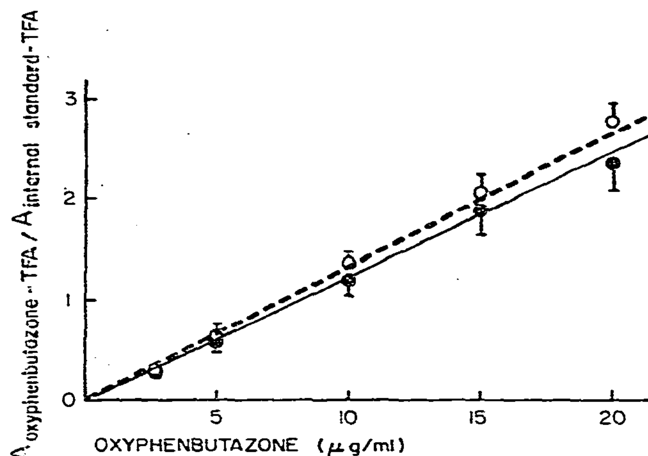


Fig. 4. Calibration plot for oxyphenbutazone (○) derivatized standards in ethyl acetate and (●) carried through the complete analytical procedure. Vertical bars indicate one standard deviation of the average of five determinations. A = peak areas in absolute computer counts.

over a period of 24 days. The lower limit of sensitivity of the procedure as described permits the quantitation of oxyphenbutazone at an acceptable precision of 500 ng/ml in plasma with a signal-to-noise ratio of a least 20, thus allowing the assay of oxyphenbutazone over a period of several biological half-lives, after oral administration of a single therapeutic dose.

The average plasma concentrations (as a function of time) found in 10 normal volunteers after a single 200-mg oral dose of oxyphenbutazone are illustrated in Fig. 5. The observed variability in plasma concentrations of individuals who had

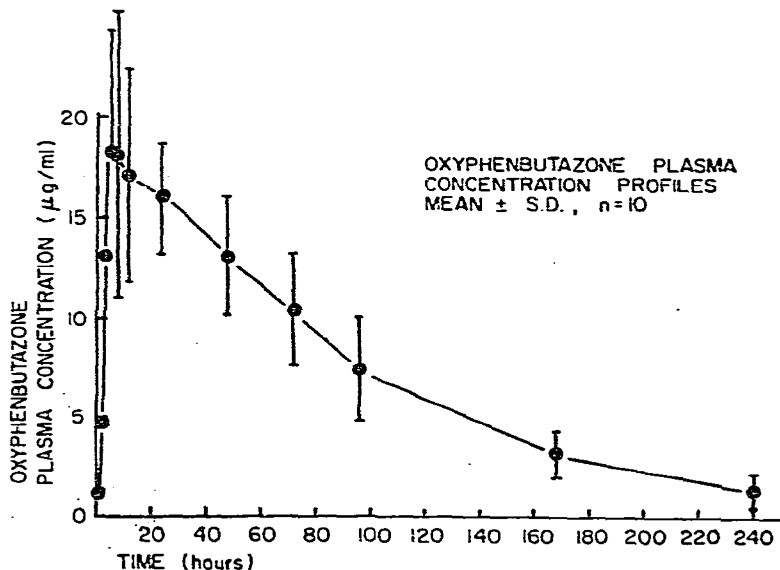


Fig. 5. Average (±S.D.) of plasma concentrations after administration of 200 mg of oxyphenbutazone. Standard deviations not shown graphically: 1 h, 2.23; 2 h, 5.01; 3 h, 6.03.

received identical doses under controlled conditions is consistent with those found and reported after administration of phenylbutazone[18]. These variations may be ascribed to individual differences in the metabolic rate of elimination. Peak concentrations as high as 43.7 and as low as 11.4 $\mu$g/ml were observed from 2 to 12 h post-administration, indicating that the fate of this drug is very different in individuals standardized with respect to their age, weight and health status. Further studies are in progress to evaluate the effect of an alkaline buffer on oxyphenbutazone absorption in man.

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