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GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF 1,3-DIHYDRO-3-PHENYLSPIRO[ISOBENZOFURAN-1,4-PIPERIDINE], HP 505, IN BIOLOGICAL FLUIDS USING A NITROGEN-SPECIFIC DETECTOR

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SUMMARY

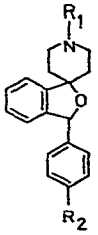
A gas chromatographic method for the determination of 1,3-dihydro-3-phenylspiro[isobenzo-1,4-piperidine], HP 505, in plasma, red blood cells and urine has been developed. HP 505 and internal standard are extracted from basified fluid with hexane and then back extracted into acetic acid. After re-extraction into hexane, HP 505 and internal standard are analysed by gas–liquid chromatography as the N-propionyl derivatives using a nitrogen-specific detector. Concentrations of HP 505 can be measured over the range 2–100 ng/ml plasma.

The method has been applied to the analysis of biological fluids from volunteers receiving oral doses of HP 505.

INTRODUCTION

The compound 1,3-dihydro-3-phenylspiro[isobenzofuran-1,4-piperidine], HP 505 (I in Fig. 1), is currently being developed as a new drug acting on the central nervous system.

In order to examine its bioavailability and pharmacokinetics in man, it was necessary to have an analytical method to determine its concentration in biological



	R_1	R_2
I	H	H
II	$COCH_2CH_3$	H
III	H	CH_3
IV	$COCH_2CH_3$	CH_3

Fig. 1. Structural formulae of HP 505 (I), HP 1197 (III) and their N-propionyl derivatives (II and IV, respectively).

fluids. It was anticipated, from the known metabolism of HP 505 in animals[1], that the levels of HP 505 in plasma resulting from the projected oral dose of 5–50 mg would be very low (*ca.* 10–50 ng/ml). Preliminary investigations indicated that neither fluorimetry nor spectrophotometry would be sufficiently sensitive, but that gas–liquid chromatography using a nitrogen-specific detector would be sufficiently sensitive and specific after conversion of HP 505 to the corresponding N-propionyl derivative (II in Fig. 1).

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade and were used without further purification, unless otherwise indicated.

Hexane ("Distol" grade, Fisons, Loughborough, Great Britain) was allowed to stand over concentrated sulphuric acid for 24 h, and then over an acidic solution of potassium permanganate (0.5% in 1 *N* sulphuric acid) for a further 24 h; it was then washed with water, dried over sodium sulphate and distilled. A reagent mixture in hexane containing 0.5% (v/v) propionic anhydride (G.P.R. grade; Hopkins and Williams, Chadwick Heath, Great Britain) and 1% (v/v) pyridine was freshly prepared for each batch of samples. Acetic acid (1 *M*) was prepared by diluting glacial acetic acid with distilled water. Cyclohexane was "Distol" grade (Fisons).

Standard solutions

A solution of 1 mg/ml HP 505 was prepared by dissolving HP 505 in the minimum amount of 1 *M* acetic acid and making up to the required volume with distilled water. This solution was then diluted with distilled water to provide the stock solution containing 1 μg/ml HP 505.

A stock solution containing 1 μg/ml of the internal standard HP 1197 (III in Fig. 1) was prepared in exactly the same way. The stock solutions were stable for at least a month if stored below 5°.

Extraction and derivatization from plasma and urine

Hexane (10 ml), 1 *M* sodium hydroxide (0.5 ml) and 100 ng of the internal standard, HP 1197, (0.1 ml of the 1 μg/ml stock solution) are added to plasma (2 ml) [or urine (1 ml)] in a screw-capped test tube. The plasma is extracted for 15 min using a mechanical rotary inversion mixer at 20 rpm (Heto Rotamix, V.A. Howe) and the layers are separated by centrifugation at 2000 *g* for 5 min. The hexane phase is transferred to a clean test tube containing 1 *M* acetic acid (1 ml) and is then extracted for 15 min using the inversion mixer. After centrifugation at 800 *g* for 2 min to separate the layers, the upper hexane phase is aspirated and discarded. After the aqueous phase has been washed with hexane (1 ml), it is made alkaline by the addition of 1 *M* sodium hydroxide (1.5 ml), and extracted with hexane (5 ml). The layers are separated by centrifugation at 800 *g* for 2 min and the hexane phase is transferred to a clean test tube. A freshly prepared hexane solution (0.5 ml) of 0.5% propionic anhydride and 1% pyridine is added to the test tube; the contents are mixed and reacted for 1 h at 60° in a water-bath.

One *M* acetic acid (1 ml) is added to the cooled reaction mixture, and the contents are extracted for 15 min using the inversion mixer. After centrifugation at 800 *g* for 2 min, the hexane phase is transferred to a tapered test tube. The tubes are immersed in a water-bath at 40°, and the solvent is removed by a gentle stream of nitrogen. Cyclohexane containing 5% of ethyl acetate (100 μl) is used to wash the walls of the tube and concentrate the residue in the tapered tip of the test tube. The samples can then be stored below 5° until needed for analysis, at which time the solvent is removed by a nitrogen stream at room temperature. The dry residue is then taken up in the mixture of cyclohexane and ethyl acetate (25 μl) and aliquots (5 μl) are analysed by gas–liquid chromatography.

Extraction and derivatization from packed red blood cells

For determinations in packed red blood cells, about 1 g of cells is accurately weighed into a test tube and diluted with water (1 ml) before proceeding in exactly the same way as for plasma.

Gas–liquid chromatography

Analyses were performed on a Perkin-Elmer F17 gas chromatograph equipped with a Perkin-Elmer nitrogen–phosphorus detector which has a nitrogen:carbon selectivity of at least 5000:1 (ref. 2). The coiled glass column (2 m × 1.75 mm I.D.) was packed with 3% OV-25 on Chromosorb W-HP (100–120 mesh). The carrier gas flow-rate was 20 ml/min of helium and the oven temperature was 275°. The injector and detector were maintained at 300°. The hydrogen and air flow-rates to the detector were 3 ml/min and 60 ml/min, respectively.

Because of the high temperatures required to achieve short analysis times, only the thermally stable silicone phases were considered suitable. Of the phases investigated, the relatively polar OV-25 was chosen, rather than OV-1 or OV-17, as this gave better separation of N-propionyl HP 505 from other peaks in the chromatogram arising from endogenous plasma constituents. Under these conditions the retention times of N-propionyl HP 505 and N-propionyl HP 1197 were 5.5 and 6.5 min, respectively. Typical chromatograms obtained from plasma are shown in Fig. 2.

Gas chromatography–mass spectrometry

Mass spectra were determined on an AEI MS 30 mass spectrometer coupled to a Pye 104 gas chromatograph (Pye Unicam) via a membrane separator. The mass spectometer was operated at 45 eV, and 300 μA ionizing current; the source temperature was 250° and the separator temperature was 230°. Chromatography was performed at 280° using a glass column (1.5 m × 4 mm) packed with 3% OV-17 on Chromosorb W-HP (100–120 mesh). Helium flowing at 45 ml/min was the carrier gas.

Quantification of HP 505 levels

HP 505 levels in biological fluids were calculated using a response factor obtained by analysing blank samples of the fluid to which 100 ng of HP 505 (0.1 ml of the 1 μg/ml stock solution) had been added. These calibration samples were analysed in parallel with the unknown samples, and the response factor was calculated for each

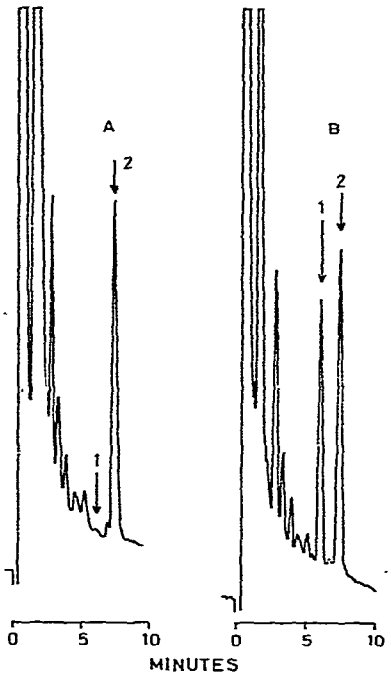


Fig. 2. Examples of chromatograms: A, extract of plasma (2 ml) taken from a volunteer prior to dosing; B, extract of plasma (2 ml) taken from the same volunteer 8 h after a 50-mg oral dose of HP 505. The arrows 1 and 2 indicate the retention times of the N-propionyl derivatives of HP 505 and HP 1197, respectively. The calculated amount of HP 505 in B was 35 ng/ml.

batch of samples. The concentration of HP 505 was calculated from the following formula

$$\text{concentration of HP 505} = \text{response factor} \times \frac{\text{peak height of N-propionyl HP 505}}{\text{peak height of N-propionyl HP 1197}} \times \times \text{concentration of added HP 1197}$$

$$\text{where: response factor} = \frac{\text{peak height of N-propionyl HP 1197}}{\text{peak height of N-propionyl HP 505}} \times \frac{\text{concentration of added HP 505}}{\text{concentration of HP 1197}}$$

Over a number of months during which the method was applied to the analysis of HP 505 in plasma, the average value of the response factor was 1.18 (S.D. $\pm$ 0.04, 32 observations).

RESULTS AND DISCUSSION

Low levels of primary and secondary amines cannot usually be analysed quantitatively by gas–liquid chromatography because of high adsorptive losses on the column and poor peak shape. However, the gas chromatographic properties of amines can be considerably improved by formation of N-acyl derivatives which can be prepared in good yield by reacting the amine with anhydrides, often in the presence

of pyridine as a catalyst. Four derivatives of HP 505 were prepared, namely, N-heptafluorobutyryl, N-acetyl, N-trifluoroacetyl and N-propionyl. Of these, the N-propionyl was found to have the longest retention time on OV-25 and this resulted in better separation of the drug and internal standard from other compounds present in the final extract.

As it was anticipated that a very sensitive assay would be required for this drug, a preliminary study was carried out using the N-heptafluorobutyryl derivative and electron capture detection (Hewlett-Packard ^{63}Ni). Although the electron capture detector was extremely sensitive to N-heptafluorobutyryl derivative of HP 505, this approach was abandoned because the detector response was found to be non-linear over a wide concentration range and because there was considerable interference from contaminants in the final extract arising from either solvent residues or from compounds co-extracted from plasma. These problems were overcome by using a nitrogen–phosphorus detector; backgrounds were considerably lower and the detector response was found to be linear over at least two orders of magnitude. In addition, this nitrogen–phosphorus detector has been shown to have high stability and reliability approaching that of a conventional flame-ionization detector[2].

EVALUATION OF THE METHOD

Optimization of the extraction

The procedure for isolating HP 505 from plasma was optimized by using [^{14}C]-HP 505. The maximum yield of HP 505 in the first hexane extract, obtained at pH 14 and after 20 min extraction, was found to be about 70%; lower pH resulted in lower recovery, and longer extraction times did not significantly increase the amount of HP 505 extracted. About 50% of the HP 505 remained after the subsequent extraction into 1 *M* acetic acid and back extraction into hexane. Further losses in the remaining steps of the method were found to be negligible and thus the overall recovery for the entire method is about 50%.

Accuracy and precision

The accuracy of the method was established by analysing blank plasma to which had been added HP 505. The results of four separate determinations are summarised in Table I. They show that the accuracy is satisfactory over the range 2–100 ng/ml. In all control samples, a small peak equivalent to about 1 ng/ml of HP 505 was found at the retention time of N-propionyl HP 505. The presence of this peak adversely affects the accuracy of the method for concentrations of less than 2 ng/ml of HP 505.

The precision of the method was determined from duplicate analyses of 2-ml portions of plasma from volunteers who had taken HP 505 orally. The method of Snedecor[3] was used to analyse the data and the results are shown in Table II.

Specificity

The small endogenous peak at the retention time of the N-propionyl derivative of HP 505 could not be eliminated completely by solvent purification or glassware cleaning. However, in the analysis of predose plasma samples from thirty volunteers, this peak never amounted to more than about 1 ng/ml of HP 505.

TABLE I

DETERMINATION OF HP 505 ADDED TO BLANK PLASMA

Each result is the mean of 4 determinations.

HP 505 added (ng/ml)	*HP 505 found (ng/ml)*	*Standard deviation (ng/ml)*	*Coefficient of variation (%)*
0	1.1	0.1	9
0.6	1.5	0.4	27
1.1	1.9	0.6	32
2.6	3.1	0.5	16
5.1	5.4	0.4	7
10.0	10.3	0.5	5
26.7	27.7	0.7	3
51.4	54.1	0.3	0.5
76.2	79.3	2.3	3
100.2	104.7	3.0	3

TABLE II

ESTIMATE OF THE PRECISION OF THE METHOD FROM DUPLICATE DETERMINATIONS

Concentration range of HP 505 (ng/ml)	*Number of samples analysed in duplicate*	*Mean concentration (ng/ml)*	*Estimated standard deviation (ng/ml)*	*Coefficient of variation (%)*
1–10	17	4.8	0.80	16.7
10–20	27	14.8	0.77	5.2
20–30	17	25.8	0.80	3.1
30–50	14	35.2	0.53	1.5

Combined gas chromatography–mass spectrometry was carried out on a urine extract. The spectra obtained from the compounds at the retention times of the N-propionyl derivatives of HP 505 and HP 1197 were identical to the spectra of the authentic standards.

Application of the method

The method has been applied to the analysis of plasma, urine and red blood cells from volunteers who had taken single or multiple oral doses of HP 505. In a typical experiment, a volunteer was given 50 mg of HP 505 orally in capsule form and at various times during the next 24 h blood samples were collected. Immediately after withdrawal, the blood was added to a heparinized tube and the plasma and red blood cells separated by centrifugation. The plasma and red blood cell profiles from one of these experiments are shown in Fig. 3.

In a similar study, a volunteer was given 25 mg of HP 505 orally in capsule form. Urine was collected at various intervals during the next six days and analysed for HP 505. The results are given in Table III.

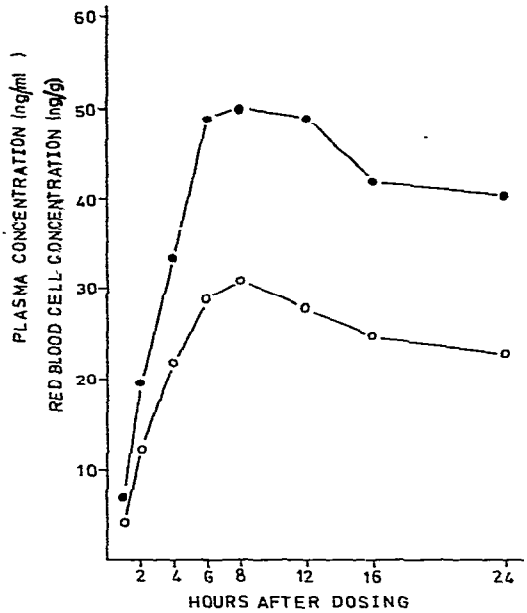


Fig. 3. Plasma (○) and red blood cell (●) levels of HP 505 in a volunteer after a 50-mg oral dose of HP 505.

TABLE III

URINE LEVELS OF HP 505 IN A VOLUNTEER AFTER A SINGLE ORAL DOSE OF HP 505 (25 mg)

Total amount of HP 505 recovered = 4.85 mg.

Time after dosing (h)	*Volume of urine collected (ml)*	*Concentration of HP 505 in urine (μg/ml)*	*Amount of HP 505 recovered (mg)*
0– 4	1145	0.09	0.10
4– 12	2010	0.28	0.56
12– 24	515	0.86	0.44
24– 48	1600	0.76	1.22
48– 72	1400	0.68	0.95
72– 96	1555	0.42	0.65
96–144	5450	0.17	0.93

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