Journal of Chromatography, 145 (1978) 393—400
Biomedical Applications

CHROMBIO. 132

QUANTITATIVE ANALYSIS OF 6,11-DIHYDRO-11-OXO-DIBENZ[*b*,*e*] OXEPIN-2-ACETIC ACID (ISOXEPAC) IN PLASMA AND URINE BY GAS—LIQUID CHROMATOGRAPHY

T.A. BRYCE and J.L. BURROWS

Hoechst Pharmaceutical Research Laboratories, Walton Manor, Walton, Milton Keynes, Bucks. MK7 7AJ (Great Britain)

(Received October 10th, 1977)

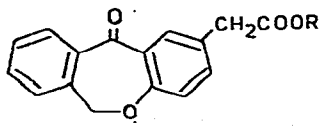
SUMMARY

A method is described for the quantitative analysis of 6,11-dihydro-11-oxo-dibenz[*b*,*e*]-oxepin-2-acetic acid (isoxepac) in plasma and urine. Isoxepac and internal standard are extracted from acidified plasma and urine, converted to the corresponding methyl esters and analysed by gas—liquid chromatography using a flame ionization detector. The method is accurate and precise over the range 0.1—30 μg/ml. The method has been applied to the analysis of plasma and urine from both healthy volunteers and patients receiving therapeutic oral doses of isoxepac.

INTRODUCTION

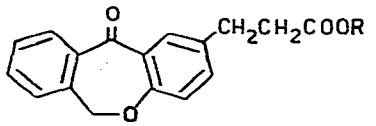
The compound 6,11-dihydro-11-oxo-dibenz[*b*,*e*]oxepin-2-acetic acid, isoxepac (also HP 549, I in Fig. 1), is currently being developed as a new anti-inflammatory agent which also possesses some analgesic activity [1, 2]. In order to obtain detailed pharmacokinetic information from clinical studies, it was necessary to develop an analytical method for isoxepac in plasma and urine. It was expected, from the known metabolism of isoxepac in animals [3], that peak plasma levels in man would be in the range 1—20 μg/ml following a single oral dose of isoxepac at the anticipated therapeutic level of 50—200 mg.

Initial investigations into the gas chromatographic properties of isoxepac established that satisfactory chromatography could be obtained after conversion of isoxepac to its methyl ester (II in Fig. 1), and that adequate sensitivity was available from flame ionization detection.



I R = H

II R = CH_3



III R = H

IV R = CH_3

Fig. 1. Structural formulae of isoxepac (I), P 74 1187 (III) and their methyl esters (II and IV, respectively).

MATERIALS AND METHODS

All solvents and reagents were of analytical grade and were used without further purification.

Preparation of diazomethane

Diazomethane in ether was prepared by a scaled-down version of the method described by de Boer and Backer [4]. As diazomethane is toxic, all work was performed in a fume cupboard. The explosion hazard associated with diazomethane was minimised by keeping the diazomethane at low concentrations and only using glassware fitted with Clearfit joints (Fisons, Loughborough, Great Britain).

The apparatus consisted of a two-necked, round-bottomed flask (100 ml) equipped with a PTFE-coated magnetic stirring bar. A dropping funnel (100 ml) was fitted into one neck of the flask, and a distillation head and condenser into the other. A solution of potassium hydroxide (0.6 g) in 95% ethanol (12.5 ml) was placed in the flask and heated to 65° with stirring. A solution containing N-methyl-N-nitrosotoluene-4-sulphonamide (BDH, Poole, Great Britain) dissolved in diethyl ether (2.2 g in 30 ml) was added slowly from the dropping funnel. Diazomethane and ether co-distilled from the reaction mixture. The resulting yellow distillate of ethereal diazomethane is usable for at least a week if kept refrigerated.

Standard solutions

Standard solutions of isoxepac and P 74 1186 (III in Fig. 1), the internal standard used in the method, were separately prepared by dissolving either compound in the minimum volume of 1 *M* Na_2CO_3 required for solution and diluting to a concentration of 0.1 mg/ml with distilled water.

Extraction and derivatisation

As solutions of isoxepac are light-sensitive, all operations must be carried out in subdued light. Dichloromethane (5 ml), 1 *M* HCl (0.5 ml) and 10 μg of the internal standard, P 74 1187 (0.1 ml of the 0.1 mg/ml standard solution) are

added to plasma (2 ml) in a screw-capped test tube (Sovirel; V.A. Howe, London, Great Britain). For urine analyses, 20 μg of internal standard (0.2 ml of the 0.1 mg/ml standard solution) is used, other quantities being unchanged. The plasma or urine is extracted for 10 min using an inversion mixer at 20 rpm (Heto Rotamix; V.A. Howe). After the layers have been separated by centrifugation at 2000 *g* for 5 min, the upper aqueous phase is aspirated and discarded. Any emulsions which may have formed are broken by mixing the contents of the test tube briefly with a vortex mixer, and centrifuging once more. The organic phase, after being transferred to a tapered test tube, is heated to 40° in a water bath and removed by a gentle stream of nitrogen. Ethereal diazomethane (0.5 ml) is added to the tube, mixed with the residue using a vortex mixer, and after 5 min removed by a gentle stream of nitrogen at room temperature. The residue is then re-dissolved in ethyl acetate (50 μl for plasma extracts; 100 μl for urine extracts) and aliquots (5 μl) are analysed by gas–liquid chromatography.

Gas–liquid chromatography

Analyses were performed on a Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector. The glass column (1.8 m × 4 mm I.D., Hewlett-Packard configuration 5) was packed with 3% OV-11 on Chromosorb W HP (100–120 mesh). The oven temperature was 265°, and the injection port and detector were maintained at 300°. The carrier gas flow-rate was 60 ml/min of nitrogen. Under these conditions, isoxepac methyl ester and P 74 1187 methyl ester (IV in Fig. 1) eluted after 5.2 and 6.9 min, respectively. Examples of chromatograms obtained from plasma extracts are shown in Fig. 2.

Gas–liquid chromatography–mass spectrometry

An AEI MS 30-DS 50, mass spectrometer computer system coupled via a membrane separator to a Pye 104 gas chromatograph was used to obtain mass spectra. Chromatography was performed at 280° on a glass column (1.5 m × 4 mm I.D.) packed with 3% OV-17 on Chromosorb W HP (100–120 mesh) using helium, flowing at 45 ml/min, as the carrier gas. The mass spectrometer was operated at 45 eV, and 300 μA ionizing current; the ion source temperature was 250° and the separator temperature was 230°.

Liquid scintillation counting

Radioactive samples were counted on a Packard 2450 or 3255 liquid scintillation counter. Values (dpm) were calculated using an external standard channels ratio method. The scintillator used was NE 260 (Nuclear Enterprises, Edinburgh, Great Britain).

Quantification of Isoxepac levels

The concentration of isoxepac was determined from the peak height ratio of isoxepac methyl ester to internal standard methyl ester, and from a response factor obtained by analysing, in parallel with the unknown samples, blank plasma or urine to which had been added isoxepac (10 μg to plasma, 20 μg to urine) as well as internal standard.

During a series of routine measurements over a period of about one year, the

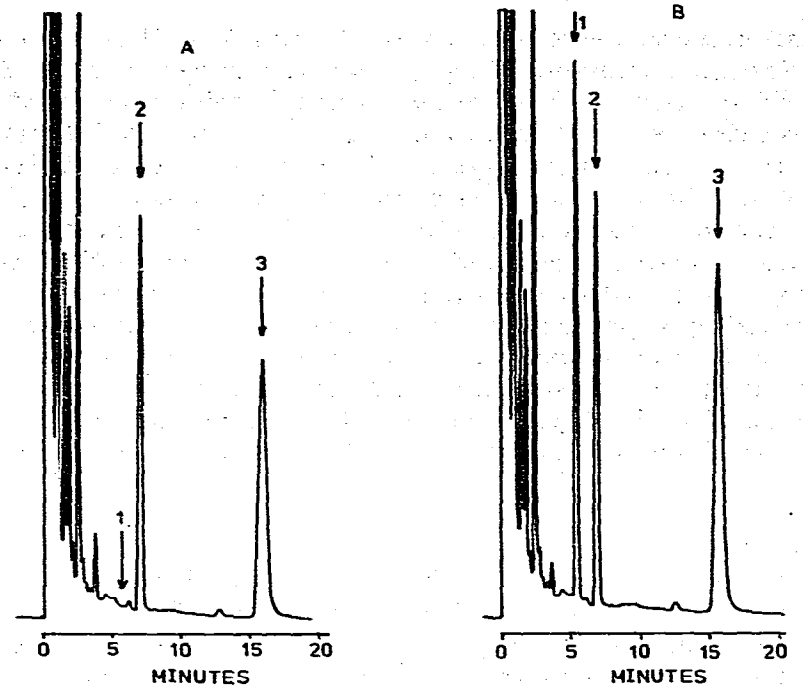


Fig. 2. Examples of chromatograms: A, control plasma to which had been added 5.21 μg/ml P 74 1187; B, control plasma to which had been added 5.25 μg/ml of isoxepac and 5.21 μg/ml of P 74 1187. The arrows 1, 2 and 3 indicate the respective retention times of isoxepac methyl ester, P 74 1187 methyl ester and cholesterol.

average value of the response factor was 1.24 (standard deviation, ± 0.06; 94 observations) for plasma and 1.31 (± 0.07; 48 observations) for urine.

RESULTS

Optimization of the extraction

The efficiency of extraction of isoxepac from plasma buffered to various pH values into dichloromethane, diethyl ether and diethyl ether—*n*-pentane (50:50, v/v) at pH <1 only, was determined using [^{14}C] isoxepac as follows. To plasma (2 ml) was added *ca.* 10 μg of [^{14}C]isoxepac (100 μl of a solution containing 600 dpm/μl), solvent (5 ml), and 1 *M* HCl (0.5 ml) or buffer (1.0 ml). After the plasma had been extracted and centrifuged (see Materials and methods), an aliquot (3.0 ml) of the organic phase was transferred to a counting vial and taken to dryness in a stream of nitrogen. Scintillator was added, and the amount of radioactivity in each vial was measured by liquid scintillation-counting.

The results (Table I) show that, although the extraction efficiency falls off rapidly with increasing pH, all of the isoxepac is recovered from plasma after acidification with 1 *M* HCl.

TABLE I

EFFICIENCY OF EXTRACTION OF [^{14}C]ISOXEPAC FROM BUFFERED PLASMA

Results are from duplicate determinations. The buffer was 0.1 *M* glycine—sodium chloride except for pH <1 which was 1 *M* HCl. The values quoted have not been corrected for any change in volume of the organic phase during extraction.

pH of added buffer	Recovery of [^{14}C]isoxepac (%)		
	Dichloromethane	Diethyl ether	Ether—pentane (50:50)
<1	99	106	97
	99	110	96
1.7	40	76	
	39	75	
2.0	24	59	
	24	60	
2.5	14	44	
	14	45	
3.0	8	29	
	8	29	
3.5	4	17	
	4	17	

Accuracy and precision

The accuracy and precision of the method were measured in the following experiment.Three standard solutions containing 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml of isoxepac were prepared. Aliquots of these solutions were then added to blank plasma samples (20 ml) such that the concentration of added isoxepac ranged from 0.03—31.2 μg/ml of plasma. The plasma samples were analysed on six occasions, and the results obtained are shown in Table II. Except for the two lowest concentrations, the precision is ± 3% or better and the results are accurate to within 5%. Although isoxepac could still be detected at the lowest concentration (0.03 μg/ml), interference from co-extracted material resulted in much poorer accuracy and precision.

Specificity

The specificity of the assay was established by combined gas—liquid chromatography—mass spectrometry of a plasma extract. The mass spectra of the peaks at the retention times of isoxepac methyl ester and P 74 1187 methyl ester were identical with the spectra from authentic standards.

TABLE II

DETERMINATION OF ISOXEPAC ADDED TO BLANK PLASMA

Each result is the mean of 6 measurements.

Isoxepac added (μg/ml)	Isoxepac found (μg/ml)	Standard deviation (μg/ml)	Percentage of theoretical
0.03	0.02	0.01	67 ± 50
0.10	0.09	0.01	90 ± 11
0.31	0.29	0.01	95 ± 3
1.01	0.98	0.02	97 ± 2
3.07	3.07	0.03	100 ± 1
10.2	9.96	0.04	97 ± 0.4
31.2	30.9	0.1	99 ± 0.3

DISCUSSION

Despite the hazards associated with its use, diazomethane was chosen for the esterification of isoxepac because its reaction with isoxepac is rapid, quantitative and free from by-products.

On-column methylation with a 0.2 *M* trimethylanilinium hydroxide solution in methanol (Methelute; Pierce and Warriner, Chester, Great Britain) was investigated, but was found to cause extensive decomposition of isoxepac.

During development of the method, solvents other than dichloromethane, and stationary phase other than OV-11, have been used. Extractions were carried out with diethyl ether and diethyl ether—*n*-pentane (50:50). As both of these solvents are less dense than plasma, it is easier and less time-consuming to remove them than it is for dichloromethane. However, it was found that they extracted more cholesterol and other lipophilic material from plasma and that the number of samples which emulsified on extraction was greater.

An advantage of using OV-17 rather than OV-11 as the stationary phase is that the retention time of cholesterol is about 4 min shorter on OV-17 under the conditions used. Its main disadvantage is that the background from co-extracted plasma constituents at the retention time of isoxepac methyl ester is greater. Thus, OV-11 can be used with greater sensitivity and accuracy, but with a possible drop in the through-put of samples; if a higher through-put of samples is needed, OV-17 can be used, but with some sacrifice of sensitivity.

Application of the method

The method has been in routine use for more than a year. It has been applied to the analysis of plasma and urine from healthy volunteers who had taken single, oral doses of 50, 100 and 200 mg isoxepac as well as to plasma and urine from patients suffering from rheumatoid arthritis who had taken 25, 50 or 150 mg isoxepac three times daily for 21 days.

In a typical experiment, a number of healthy volunteers were given a capsule

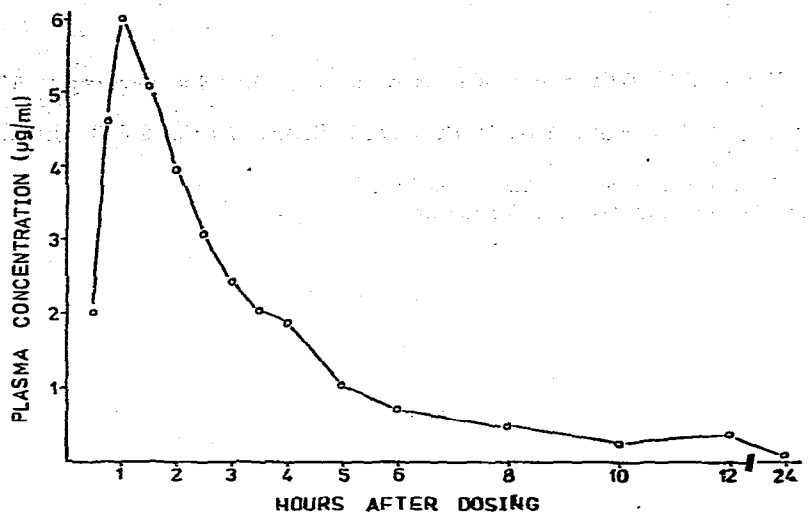


Fig. 3. Plasma level of isoxepac in a volunteer after a 50-mg oral dose of isoxepac.

TABLE III

URINE CONCENTRATIONS OF ISOXEPAC AFTER A SINGLE ORAL DOSE OF 50 mg ISOXEPAC

Total recovery of isoxepac, 6.01 mg.

Time after dosing (h)	Volume of urine collected (ml)	Conc. of isoxepac in urine (µg/ml)	Amount of isoxepac recovered (mg)
0—2	145	8.21	1.19
2—4	290	6.80	1.97
4—8	525	4.02	2.11
8—12	234	0.90	0.21
12—24	1500	0.25	0.38
24—36	848	0.08	0.07
36—48	670	0.12	0.08

containing 50 mg of isoxepac. Blood samples were withdrawn at various times during the next 24 h, and urine collected for various periods during the next 48 h. The average peak plasma level of six volunteers taking a 50 mg dose was 5.63 ± 1.03 µg/ml and the average recovery of isoxepac in urine was 7.3 ± 2.4 mg. Results from one of the volunteers are shown in Fig. 3 and Table III.

ACKNOWLEDGEMENT

The excellent technical assistance of Mrs. B.A. Bedford in the development and application of the method is gratefully acknowledged.

REFERENCES

1 H.B. Lassmann, R.E. Kirby, J.C. Wilker and W.J. Novick, Jr., The Pharmacologist, 17 (1975) 226.
2 D.E. Aultz, G.C. Helsley, D. Hoffman, A.R. McFadden, H.B. Lassman and J.C. Wilker, J. Med. Chem., 20 (1977) 66.
3 H.P.A. Illing and J.M. Fromson, personal communication.
4 Th. J. de Boer and H.J. Backer, Org. Syn., 4 (1963) 250.