Journal of Chromatography, 310 (1984) 51—59
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2170

GAS—LIQUID CHROMATOGRAPHIC EVALUATION OF LOFEMIZOLE IN BIOLOGICAL SAMPLES FOR PHARMACOKINETIC INVESTIGATIONS

COMPARISON OF TWO ANALYTICAL METHODS

A. MARZO* and E. TREFFNER

B.T.B. Industria Chimica S.p.A., Life Science Division, Laboratory of Drug Metabolism and Pharmacokinetics, Via Paullo 9, 20067 Tribiano, Milan (Italy)

and

P.P. NEGGIANI and G. STAIBANO

Istituto Gentili S.p.A., Chemical Research Department, Via G. Mazzini 112, 56100 Pisa (Italy)

(First received January 23rd, 1984; revised manuscript received March 31st, 1984)

SUMMARY

The present paper reports the analytical conditions allowing lofemizole, a new non-steroidal anti-inflammatory drug, to be evaluated in biological fluids for pharmacokinetic and bioavailability investigations. The first approach led to an N-methyl derivative of lofemizole which could be successfully analysed by gas—chromatography employing a flame-ionization detector, reaching a sensitivity of 2 μg/ml. The second approach led to the N-(2-chlorobenzoyl) derivative of lofemizole which was suitable for pharmacokinetic investigation using gas—liquid chromatography with electron-capture detection, and reaching a much higher sensitivity of 10 ng/ml of plasma. Recovery of the extraction, reproducibility and specificity were all satisfactory with both methods. Since the first method employing flame-ionization detection was suitable for pharmacokinetic investigations in animal species, this paper describes both methods on a comparative basis.

INTRODUCTION

Lofemizole [1H,4-(4-chlorophenyl)-5-methylimidazole, $C_{10}H_9N_2Cl$] has the molecular structure shown in Fig. 1, and its chemical identity was confirmed by mass spectrometry. Lofemizole has proved to possess an interesting anti-phlogistic activity in both animals and humans, associated with a favourable

0378-4347/84/$03.00

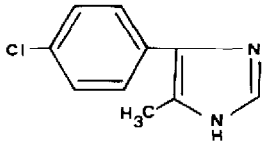

Fig. 1. Molecular structure of lofemizole.

gastric and systemic tolerability [1]. From the point of view of the structure—activity relationship, lofemizole is comparable with other compounds having an imidazole [2, 3], thiazole [4] or oxadiazole [5, 6] structure and possessing anti-inflammatory activity.

The aim of this paper was to investigate the analytical conditions that would allow a pharmacokinetic or bioavailability study to be carried out on lofemizole in both animals and humans.

EXPERIMENTAL

Drugs, chemicals and instruments

Solvents and chemicals, all of analytical grade purity, were supplied by E. Merck (Darmstadt, F.R.G.). 2-Chlorobenzoyl chloride and methyl iodide of an excellent grade purity were prepared at B.T.B. Industria Chimica, Chemical Department; zomepirac methyl ester and miconazole were also supplied by B.T.B. The supports and stationary phases for the gas—liquid chromatography (GLC) columns were supplied by Supelchem (Milan, Italy). A Varian 3700 gas chromatograph and a VG Micromass MS 30/70 mass spectrometer were employed for the analysis. Rats were supplied by Charles River Italia (Calco, Italy). The statistical evaluation was performed on a Hewlett-Packard HP 86 personal computer.

Evaluation by GLC with flame-ionization detection (GLC—FID)

Extraction. A 1-ml volume of sodium hydroxide solution (1 mol/l) and 5 ml of diethyl ether were added to 1 ml of plasma (or urine) in a glass-stoppered test tube. The mixture was vigorously stirred for 5 min and then centrifuged at 2400 *g* for 10 min. Methyl iodide (1 ml of a 10% solution in diethyl ether), 0.2 ml of tetrabutylammonium hydroxide (0.1 mol/l in benzene—methanol, 1:1) and 1 ml of sodium hydroxide solution (1 mol/l) were added to 4 ml of the organic layer in another test tube. The test tube was stoppered and kept at 90°C with stirring for 30 min, after which it was cooled to room temperature and centrifuged. An aliquot of the organic layer containing the N-methyl derivative of lofemizole was dried with anhydrous sodium sulphate, evaporated to dryness and finally dissolved in 100 μl of acetone. Zomepirac methyl ester was added as the internal standard. The sample was then injected into the gas chromatograph.

Analytical conditions. A glass column (1.5 m × 6 mm O.D., 2 mm I.D.) filled with 3% SP-2250 DB on 100—120 mesh Supelcoport was used. The temperatures of the injection port, oven and flame-ionization detector were maintained at 280°C, 245°C and 350°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 35 ml/min.

Evaluation by GLC with electron-capture detection (GLC—ECD)

Extraction. A 1-ml aliquot of plasma (or urine) was placed in a glass-stoppered test tube with 1 ml of sodium hydroxide solution (1 mol/l) and 5 ml of diethyl ether. The test tube was vigorously stirred for 5 min and then centrifuged at 2400 *g* for 10 min. An aliquot (4.5 ml) of the organic layer was added to 0.5 ml of 2-chlorobenzoyl chloride (400 μg/ml in diethyl ether) and to 1 ml of sodium hydroxide solution (1 mol/l) in another test tube. The mixture was vigorously stirred for 5 min and then centrifuged at 2400 *g* for 10 min. An aliquot of the organic layer was dried over anhydrous sodium sulphate and evaporated to dryness under a gentle nitrogen stream at 60° C. The residue was redissolved in 0.5 ml of benzene. Miconazole was added as the internal standard. The solution was then ready for GLC analysis.

Analytical conditions. A glass column (1 m × 6 mm O.D., 2 mm I.D.) was filled with 10% OV-11 on 100—120 mesh Supelcoport. The temperatures of the injection port, oven and electron-capture detector were maintained at 380° C, 300° C and 380° C, respectively. Nitrogen was employed as the carrier gas at a flow-rate of 35 ml/min.

RESULTS

GLC—FID method

The retention times observed were 58 sec for the N-methyl derivative of lofemizole, the chemical identity of which was confirmed by mass spectrometry, and 2 min 4 sec for the internal standard (zomepirac methyl ester, I.S.). Linearity was investigated in the range of 2.5—200 ng for each injection (Table I) by means of the detector response factor (d.r.f.) evaluated as follows:

$$\text{d.r.f.} = \frac{\text{lofemizole weight}}{\text{I.S. weight}} \times \frac{\text{I.S. peak area}}{\text{lofemizole peak area}}$$

This factor allows the different response of the detector to the analytical substance and the internal standard to be counterbalanced. Table II shows the d.r.f. with a lofemizole derivative/internal standard ratio ranging from 1:4 to 4:1.

TABLE I

LINEARITY OF THE DETECTOR RESPONSE TO N-METHYLLOFEMIZOLE EVALUATED BY GLC—FID

Linearity was confirmed by a constant detector response factor (d.r.f.). The lofemizole derivative and the internal standard were injected at a 1:1 ratio.

Lofemizole injected (ng)	Mean d.r.f. ± S.D. (n = 4)
2.5	1.10 ± 0.07
5	1.08 ± 0.01
10	1.08 ± 0.03
20	1.07 ± 0.02
50	1.09 ± 0.03
100	1.08 ± 0.03
200	1.08 ± 0.02

TABLE II

DETECTOR RESPONSE FACTOR AT DIFFERENT LOFEMIZOLE DERIVATIVE/I.S. RATIOS (GLC—FID METHOD)

Lofemizole/I.S. weight ratio	Mean d.r.f. ± S.D. (n = 4)
1:4	1.15 ± 0.11
1:2	1.12 ± 0.07
1:1	1.06 ± 0.02
2:1	1.04 ± 0.03
4:1	1.04 ± 0.05

TABLE III

RECOVERY AFTER THE EXTRACTION PROCEDURES FOR ANALYTICAL EVALUATION OF LOFEMIZOLE (GLC—FID METHOD)

Lofemizole added to 1 ml of plasma (μg)	Lofemizole found (μg/ml) (mean ± S.D., n = 4)	Recovery (%)
1.0	0.92 ± 0.22	92.0
2.5	2.44 ± 0.32	97.6
5.0	4.93 ± 0.27	98.6
10.0	9.82 ± 0.40	98.2
25.0	24.24 ± 0.78	97.0
50.0	45.80 ± 2.15	91.7
100.0	93.70 ± 4.39	93.7
	Mean ± S.D.:	95.5 ± 3.0

The recovery of lofemizole was investigated in the range 1—100 μg/ml of plasma, the analysis being performed in quadruplicate at each concentration (Table III). The mean recovery in the whole range investigated was 95.5%. The amount of lofemizole added correlated well with that found, as shown by the following relationship obtained by linear regression: $\text{lofemizole}_{\text{found}}$ (μg/ml) = 0.221 + [0.932 × $\text{lofemizole}_{\text{added}}$ (μg/ml)], r = 0.9999, $p < 0.0001$. The slope of the resulting linear function can be considered as a significant expression of the extraction recovery; in this respect the slope (0.932) is very near to the mean recovery value.

Reproducibility was evaluated by the standard deviation (S.D. %). It was 2.5% when 1 ml of solution containing 50 ng of N-methyllofemizole and 50 ng of internal standard was injected seven times. Reproducibility for the entire analytical procedure, including the extraction, was about 4—5% in the range 5—100 μg/ml of plasma and rose to 13% at a concentration of 2.5 μg/ml and to 24% at a concentration of 1 μg/ml. On this basis the sensitivity of the method appears to be about 2 μg/ml.

GLC—ECD method

Retention times were 3 min 42 sec for the 1-N-(2-chlorobenzoyl) derivative of lofemizole, the chemical identity of which was ascertained by mass spectrometry (Fig. 2), and 6 min 15 sec for the internal standard (miconazole

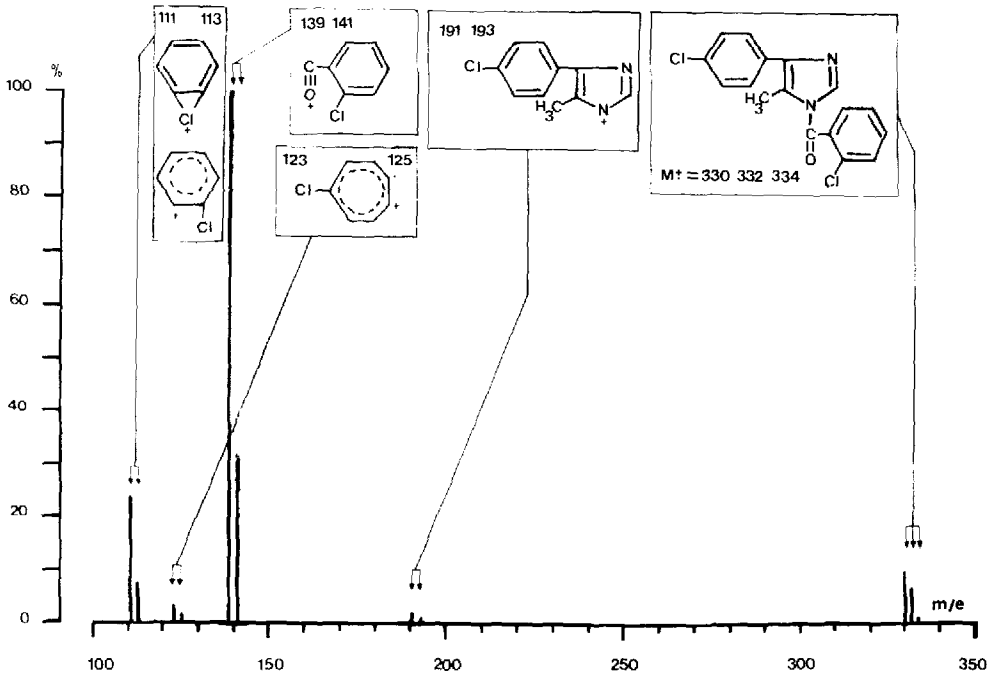

Fig. 2. Mass spectrum of 1-N-(2-chlorobenzoyl)lofemizole obtained in the electron-impact ionization mode (70 eV), the product being introduced into the source through the gas chromatograph.

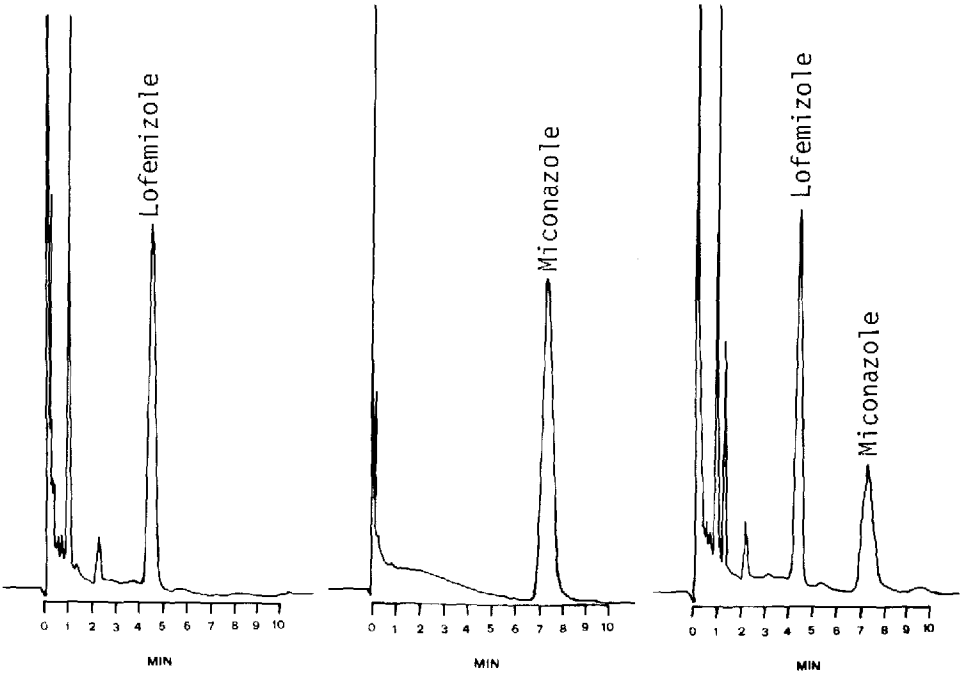

Fig. 3. Gas chromatograms of lofemizole, miconazole (I.S.) and, in the right panel, of a mixture of lofemizole and miconazole. The peaks appearing in the first 2.5 min are due to the reactants employed for derivatization and are missing in the case of miconazole, which had not undergone derivatization.

as such). The related gas chromatograms are shown in Figs. 3 and 4. Linearity was investigated in the range 20—4000 pg injected. Table IV shows the good linearity verified in the range 20—1000 pg. The d.r.f. obtained by varying the lofemizole/I.S. ratio is shown in Table V. Recovery of lofemizole was investigated in the range 10—5000 ng/ml of plasma and proved to be 94.5% on average (Table VI). The linear relationship between lofemizole added and that

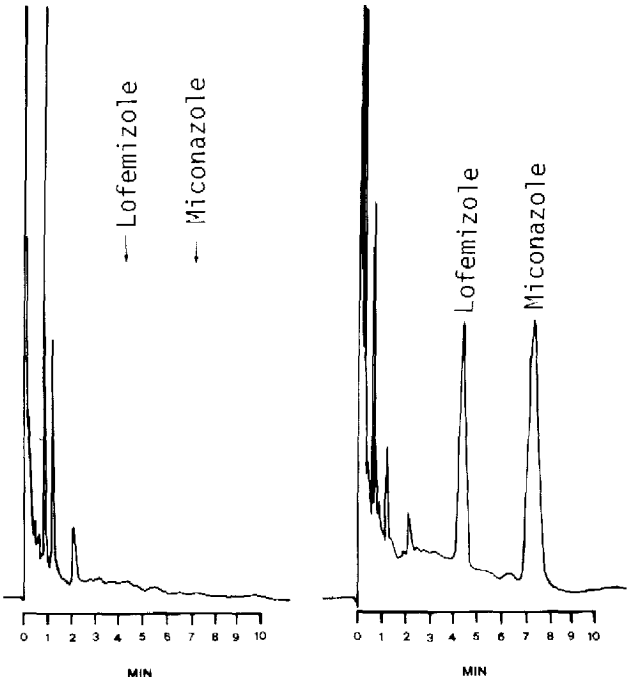

Fig. 4. Left: gas chromatogram of a blank plasma with neither lofemizole, nor miconazole, after being taken through the whole analytical procedure. Right: gas chromatogram of an analysis performed on a plasma sample from a volunteer treated with the drug, carried through the entire analytical procedure. No interference by endogenous peaks could be detected in the blank sample.

TABLE IV

LINEARITY OF DETECTOR RESPONSE TO 1-N-(2-CHLOROBENZOYL)LOFEMIZOLE EVALUATED WITH THE GLC—ECD METHOD

Linearity was confirmed by a constant detector response factor (d.r.f.). The lofemizole derivative and the internal standard were injected at a 1:1 ratio.

Lofemizole injected (pg)	Mean d.r.f. ± S.D. (n = 4)
20	0.97 ± 0.08
50	0.98 ± 0.04
100	0.98 ± 0.02
200	0.98 ± 0.03
500	0.94 ± 0.03
1000	0.97 ± 0.02
2000	1.22 ± 0.08
4000	1.75 ± 0.07

TABLE V

DETECTOR RESPONSE FACTOR AT DIFFERENT LOFEMIZOLE DERIVATIVE/I.S. RATIOS (GLC—ECD METHOD)

Lofemizole/I.S. weight ratio	Mean d.r.f. ± S.D. (n = 4)
1:4	0.92 ± 0.08
1:2	0.94 ± 0.03
1:1	0.94 ± 0.02
2:1	0.98 ± 0.04
4:1	0.87 ± 0.08

TABLE VI

RECOVERY OF THE EXTRACTION PROCEDURES FOR THE ANALYTICAL EVALUATION OF LOFEMIZOLE (GLC—ECD METHOD)

Lofemizole added to 1 ml of plasma (ng)	Lofemizole found (ng/ml) (mean ± S.D., n = 4)	Recovery (%)
10	9.4 ± 1.5	94.0
20	18.9 ± 1.8	94.5
50	47.0 ± 2.0	94.0
100	95.0 ± 6.3	95.0
200	187.0 ± 8.4	93.5
500	480.0 ± 16.4	96.0
1000	940.0 ± 31.0	94.0
5000	4753.0 ± 177.0	95.0
	Mean ± S.D.: 94.5 ± 0.8	

found, using linear regression, was $\text{lofemizole}_{\text{found}}$ (μg/ml) = $-1.209 + [0.951 \times \text{lofemizole}_{\text{added}}$ (μg/ml)], $r = 1.000$, $p < 0.0001$. In this case too the slope of the resulting linear function agrees with the mean recovery. Reproducibility, evaluated as S.D. % on repeated assays, ranged between 2% and 3% when standards of lofemizole derivative and I.S. were injected into the gas chromatograph in the range 100—1000 pg without extraction, the test being repeated four times. Over the whole analysis, including the extraction procedures, reproducibility ranged between 3.3% and 6.6% in the concentration range 50—5000 ng/ml, and increased to 9.5% at a concentration of 20 ng/ml and to 16% at 10 ng/ml. The sensitivity of this method therefore seems to be around 10 ng/ml.

DISCUSSION

In the very first approach lofemizole was analysed without derivatization on non-polar stationary phases such as SP-2100 DB. The tailing, low sensitivity and poor reproducibility prompted us to develop an appropriate N-derivatization of the molecule, as others have done with other 1H-imidazoles [7]. This in fact proved to be a very difficult process, because the more aggressive reactants such as anhydrides or acyl chlorides cause imidazole ring cleavage in lofemizole.

An extractive N-methylation, like that introduced by Ervik and Gustavii [8] for sulphonamide diuretics, led to a stable derivative with a quantitative yield. Despite the aromatic chlorine in the lofemizole molecule it could not be revealed by using the electron-capture detector, and the thermoionic-specific detector did not attain a sensitivity high enough to be suitable for our purposes. Thus the first analytical method employing a flame-ionization detector was performed with the aim of starting the pharmacokinetic studies on the rat. The distribution volume data obtained in the rat clearly indicate that the sensitivity achieved with the FID method was not high enough for pharmacokinetic studies to be carried out on human beings. A new N-derivatization method with a chlorinated reactant therefore needed to be developed.

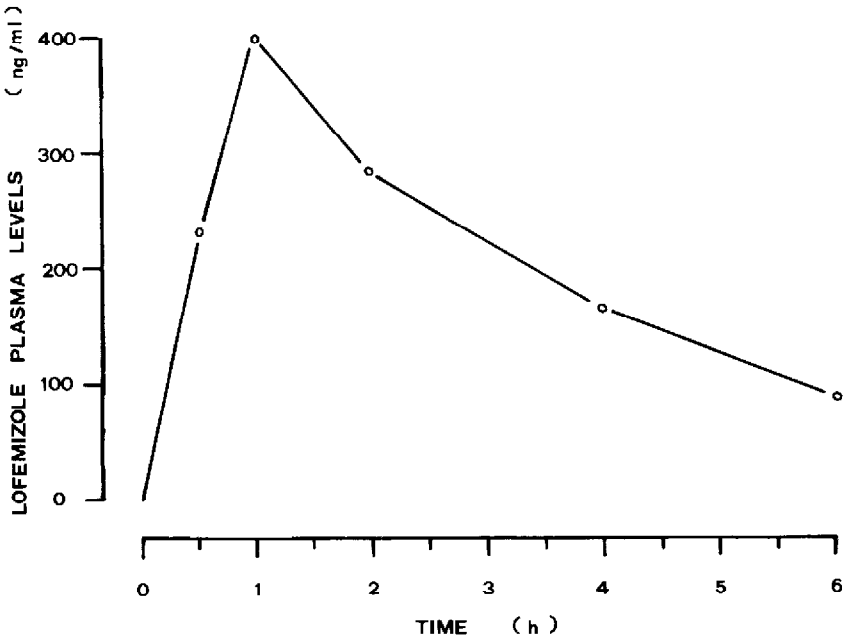

Fig. 5. Lofemizole plasma levels evaluated in a human volunteer orally treated with 100 mg of the drug.

The acyl chloride of 2-chlorobenzoic acid showed rather slow reaction kinetics, thus any undesired cleavage of the imidazole ring was avoided. The derivative was obtained in an extractive acylation process with a quantitative yield and could be revealed by ECD with a very high degree of sensitivity. This method allowed quantitative evaluation to be performed on humans who had been orally or rectally treated with 50 or 100 mg of lofemizole for bioavailability investigations. Fig. 5 shows the plasma levels of lofemizole in one subject treated with a 100-mg dose of lofemizole per os. An extensive investigation is now in progress using this method on both animal species and human beings in order to clarify the pharmacokinetic behaviour of lofemizole.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. Renzo Viscardi from

B.T.B. Chemical Research Department for synthesizing 2-chlorobenzoyl chloride and for supplying other chemical substances, to Mr. Giovanni Meroni and Mr. Marco Ripamonti from B.T.B.'s Laboratory of Drug Metabolism and Pharmacokinetics for their invaluable technical assistance, and to Mr. Crispian Piggott for reviewing the English manuscript.

REFERENCES

1 S. Rosini, unpublished results, 1983.
2 T. Corell and G. Hasselmann, Acta Pharmacol. Toxicol., 53 (1983) 288.
3 M. Grau, Drugs of the Future, 7 (1982) 740.
4 J.P. Tarayre, V. Caillol, M. Barbara, G. Villanova, M. Bru, M. Aliaga and H. Lauressergues, Agents Actions, 14 (1984) 93.
5 B. Silvestrini and C. Pozzatti, Brit. J. Pharmacol., 16 (1981) 209.
6 B. Silvestrini and C. Pozzatti, Arzneim.-Forsch., 13 (1963) 798.
7 R.M. Ward, M.J. Cooper and B.L. Mirkin, J. Chromatogr., 231 (1982) 445.
8 M. Ervik and K. Gustavii, Anal. Chem., 46 (1974) 39.