Journal of Chromatography, 144 (1977) 201–208

CHROM. 10,206

SENSITIVE AND SPECIFIC GAS CHROMATOGRAPHIC AND EXTRACTION METHOD FOR THE DETERMINATION OF ORPHENADRINE IN HUMAN BODY FLUIDS

J. J. M. LABOUT, C. T. THIJSSEN and W. HESPE
Gist-Brocades NV., Research & Development, P.O. Box 523, Haarlem (The Netherlands)
(Received April 22nd, 1977)

SUMMARY

A gas chromatographic and extraction method for the assay of orphenadrine in plasma and urine has been developed, in which diphenhydramine is used as the internal standard. The procedure involves extraction with isopentane and alkali flame ionization (nitrogen) detection. Orphenadrine N-oxide and N-dealkylated orphenadrine did not interfere with the analysis. Orphenadrine concentrations down to 1 ng/ml can be determined. Application in a pharmacokinetic/bioavailability study is reported.

INTRODUCTION

Orphenadrine hydrochloride (trade names: Disipal, Brocadisipal, Brocasipal) is frequently used in the therapy of Parkinson's disease, or drug-induced parkinsonism. Although it has been used for many years and studies of its metabolic fate in the rat[1,2], dog[2], monkey [2] and man[3,4] have been reported, detailed information on its pharmacokinetics and therapeutically active blood levels is scarce. The extensive study by Khan[5] on orphenadrine pharmacokinetics, based on urine data, involved volunteers on a special dietary regimen (high water-loading; oral dosages of ammonium chloride to acidify the urine) intended to increase renal clearance, hence cannot be regarded as representative of normal clinical conditions.

The pharmacokinetic profile of orphenadrine hydrochloride in man and the relationship between action and blood levels are ill-defined largely because a sufficiently sensitive and specific method for its determination in blood (plasma) is unavailable.

In their description of the determination of diphenhydramine at the nanogram level in human plasma with the aid of a nitrogen-specific gas chromatographic (GC) technique, Bilzer and Gundert-Remy[6] noted that orphenadrine might be measured analogously. However, the method is not sufficiently specific, for two reasons: (i) the orphenadrine metabolite orphenadrine N-oxide, which can be recovered from human urine for *ca.* 5% of the orphenadrine dose[4,5], may interfere; and (ii) the GC separation of orphenadrine from its metabolite N-demethylorphenadrine, which occurs in human plasma and urine in significant quantities, is unsatisfactory.

This paper reports an extraction and GC method by which a kinetic plasma

(and urine) study can be performed in human subjects medicated with orphenadrine.

The application of a sensitive nitrogen detector makes it possible to determine orphenadrine to the desired level of 1 ng/ml. The extraction and GC procedure is such that interference due to the metabolites orphenadrine N-oxide and N-dealkylorphenadrine can be avoided, and it even permits simultaneous quantitative determination of orphenadrine and N-demethylorphenadrine.

MATERIALS AND METHODS

All chemicals were of analytical grade. Orphenadrine, N-demethylorphenadrine, N,N-didemethylorphenadrine and their hydrochlorides and orphenadrine N-oxide were prepared in our laboratory. On the thin-layer chromatograms, all compounds produced single spots at different retention times in different solvent systems. All solvents were distilled twice in glass prior to use. The glassware was treated with nitric acid and carefully boiled out with ethanol. The phosphate buffer of pH 12 was prepared by dissolving 22.25 g of $Na_2HPO_4 \cdot 2H_2O$ and 4.46 g of NaOH in 500 ml of distilled water.

Isolation procedure

To 0.5–2 ml of blood plasma or urine in a glass-stoppered centrifuge tube were added 10–100 ng of internal standard, dissolved in distilled water. Diphenhydramine hydrochloride was chosen as internal standard because of its structural similarity to orphenadrine. The pH was adjusted to 12 by addition of 5 *M* NaOH and 0.5 ml of 0.25 *M* phosphate buffer, and the solution was made up to 3 ml with water. After equilibration for *ca.* 15 min, 3 ml of isopentane were added, and the mixture was extracted for 30 min on a Heidolph apparatus. After centrifugation at 2500 *g* for 10 min, the phases were separated and the aqueous phase was re-extracted with 3 ml of isopentane. The organic phase was collected, and shaken with 1 ml of 0.5 *M* HCl in a clean centrifuge tube for 15 min. The acidic aqueous phase was then washed out with 3 ml of isopentane for 5 min.

The organic solvent was discarded and the aqueous phase made alkaline (pH 12) with 5 *M* NaOH and 0.5 ml of phosphate buffer. Subsequently, a 15-min extraction with 3 ml of isopentane and centrifugation were carried out. The organic phase was transferred to a clean tapered tube, the inner wall of which had been carefully rinsed with ethanol. Finally, the sample was evaporated to dryness under a stream of nitrogen, and the residue dissolved in 10 μl of ethanol. A 1–2 μl sample was injected onto the GC column.

Reduction of orphenadrine N-oxide with $TiCl_3$

To 1 ml of a solution of orphenadrine N-oxide in water (100 ng/ml), or to 1 ml of plasma, were added 1 ml of 2 *M* HCl and 0.2 ml of $TiCl_3$ (15%, w/v), and the mixture was kept in a glass-stoppered tube in the dark at room temperature for 10 min. Immediately after the reaction, 100 ng of the internal standard diphenhydramine were added, and the mixture was made alkaline and extracted for quantitative analysis, as described above.

Gas chromatography

Equipment. A Hewlett-Packard gas chromatograph Type HP 5750, fitted with a sensitive nitrogen-flame ionization detector (rubidium bromide crystal) Model 14161B plus a selective detector to improve the signal to noise ratio were used[7].

Very recently we have also used the dual nitrogen–phosphorus flame ionization detector Model HP 18789 A, installed in a gas chromatograph Type HP5730A. This detector, with a new collector design, enables highly selective (it produces virtually no response to hydrocarbons) and highly sensitive detection of a wide range of compounds containing nitrogen and phosphorus atoms[8].

Column. Glass columns (1.3 and 1.5 m × 2.3 mm I.D.), packed with 3% KOH + 3% Carbowax 20 M on 100–120 mesh Gas-Chrom Q, were used.

Operating conditions. The carrier gas had a flow-rate of 30 ml/min. For optimal operation of the HP 14161B detector auxiliary helium gas (16 ml/min) had to be introduced directly into the detector system. The hydrogen flow was adjusted very carefully to 28 ± 0.5 ml/min. The air flow-rate was 200 ml/min. Oven, detector and injection port temperatures are indicated in the legends to the Figs. 1 and 3.

A high sensitivity and selectivity require optimal ionization current and, therefore, the distance between the flame and the collector containing the RbBr crystal had to be adjusted carefully[7,9]. The response of the detector varied with the cleanliness of the crystal.

The hydrogen flow-rate in the HP 18789A detector was 3 ml/min, and the air flow-rate was 50 ml/min. Voltage control was set at 16 V.

Calibration graphs and recovery. Quantitative data were derived from calibration curves, obtained by the addition to blank plasma of diphenhydramine hydrochloride as internal standard in constant concentration and orphenadrine hydrochloride in different concentrations. After extraction of the plasma as outlined above, the ratio of the GC peak areas of orphenadrine and diphenhydramine was plotted against their weight ratio. The same procedure was followed for N-demethylorphenadrine. The recovery at different concentrations was measured by extraction of plasma to which known amounts of orphenadrine hydrochloride had been added; following extraction, a known amount of diphenhydramine was added as external standard, and the ratio of the GC peak areas of the two bases was calculated and compared with those of corresponding amounts from standard solutions.

RESULTS

Fig. 1 shows typical gas chromatograms obtained by injection of part of a control human plasma extract and an extract of plasma to which, before extraction, known amounts of diphenhydramine, orphenadrine, N-demethylorphenadrine and N,N-didemethylorphenadrine had been added. The column used had 1800 plates. As can be seen from the chromatogram, there was sufficient separation between the internal standard, orphenadrine and its two metabolites.

When the method is used in pharmacokinetic studies with orphenadrine, it is of great importance to find out whether the metabolite orphenadrine N-oxide interferes with the determination of orphenadrine. It was established that orphenadrine N-oxide decomposes to orphenadrine and other products at the ambient temperature in the injection port of the gas chromatograph (unpublished results). Thus, without

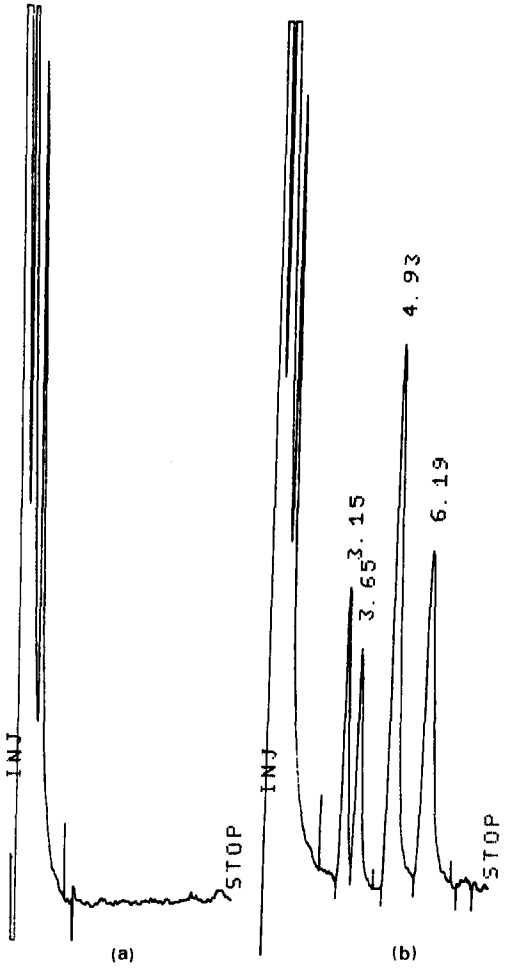

Fig. 1. Typical gas chromatograms of blank plasma extract (a) and an extract of 1 ml of plasma (b), to which 5 ng of diphenhydramine (t_R 3.15 min), 4.8 ng of orphenadrine (t_R 3.65 min), 21.5 ng of N-demethylorphenadrine (t_R 4.93 min) and 36.4 ng of N,N-didemethylorphenadrine (t_R 6.19 min) had been added. Nitrogen detector HP 18789A. Integrator HP 3380. Electrometer: $5 \cdot 10^{-12}$ A/mV. Attenuation, 1. Column length, 1.3 m. The temperatures of the oven, the detector and the injection port were 200°, 300° and 250°, respectively. Samples were injected with an automatic sampler HP 7671A; injection volume: 2 μl.

further precautions, orphenadrine N-oxide would partly be determined as orphenadrine and thus affect the specificity. By appropriate choice of extraction solvent, this risk can be avoided.

Orphenadrine N-oxide can be extracted from plasma and urine with polar organic solvents such as chloroform or, to a lesser extent, with diethyl ether. On the other hand, no detectable amounts of the N-oxide are extracted when isopentane is used as described in the isolation procedure. Even the addition of a 100-fold excess of orphenadrine N-oxide does not contribute significantly to the orphenadrine level in

the 10–500 ng/ml concentration range. Such an excess is not observed in practice (see applications), and thus isopentane is the solvent of choice.

A representative calibration graph is shown in Fig. 2. The lower detection limit, defined as the amount giving a signal three times greater than the noise at the maximal sensitivity of the nitrogen detector, is 0.05 ng orphenadrine or *ca.* 1 ng in 1 ml of plasma (see Fig. 1). The method has a good precision. The standard deviations of the analysis are $\pm$ 6% ($n = 4$) in the concentration range 10–200 ng/ml of plasma. The average total recovery of orphenadrine from human plasma in the 20–200 ng/ml concentration range is 77%, with a standard deviation *ca.* 5% ($n = 5$). At lower concentrations (down to 1 ng/ml), recovery is lower (*ca.* 60%).

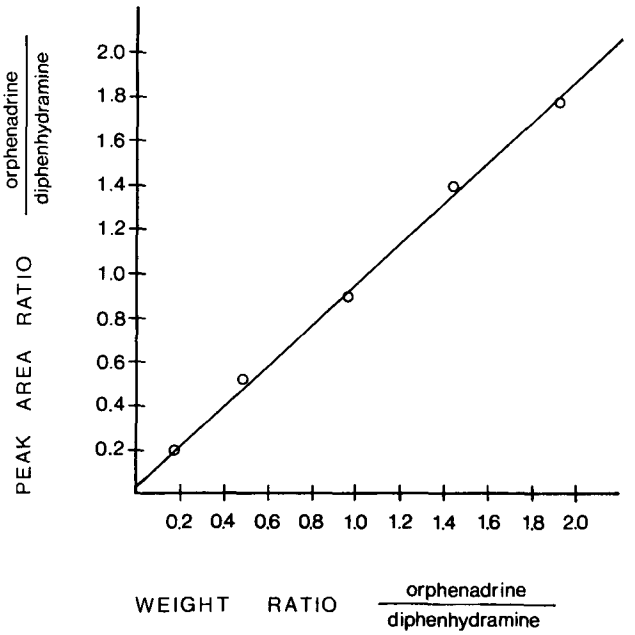

Fig. 2. Standard curve for the determination of orphenadrine concentrations.

Applications

The applicability of the analytical method was demonstrated by measuring as a function of time the concentrations in plasma from a volunteer given a single oral dose of 100 mg of orphenadrine. Fig. 3 shows a gas chromatogram of plasma extracted 24 h after administration. Blood was collected in heparinized tubes, and centrifuged at 2500 *g*. The samples were separated and frozen until analysis.

Fig. 4 shows the variation with time of the orphenadrine and N-demethylorphenadrine plasma concentrations. There appears to be a considerable rise in orphenadrine concentration *ca.* 1 h after administration, indicating rapid absorption. The linear time course on a log scale during the elimination phase (monitored until 48 h after dosage) indicates a first-order kinetic process. In this example, the biological half-life was calculated as *ca.* 16 h, consistent with the results of radio-tracer studies in man[3]. N-Demethylorphenadrine has a longer half-life. When orphenadrine N-

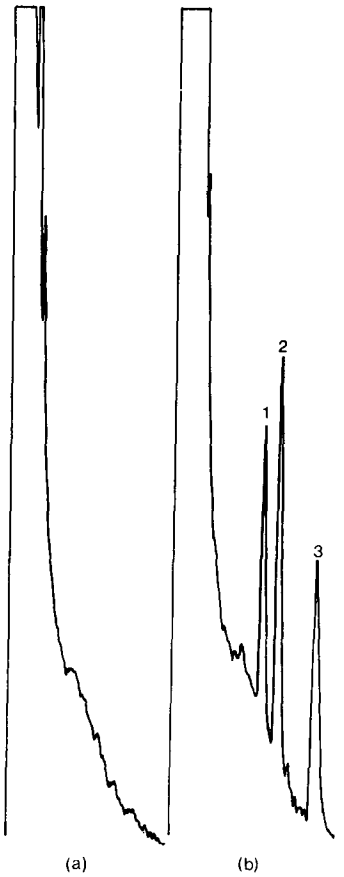

Fig. 3. Gas chromatogram of a control plasma extract (a) and a plasma extract from a volunteer's blood 24 h after treatment with 100 mg of orphenadrine hydrochloride (b). Peak 1, diphenhydramine (internal standard); peak 2, orphenadrine; peak 3, N-demethylorphenadrine. N,N-didemethylorphenadrine was not found. Nitrogen detector: HP 15161B. Electrometer: 10^{-11} A/mV. Attenuation, 4. Column length, 1.5 m. The temperatures of the oven, detector and injection port were 180°, 400° and 220°, respectively. Recorder chart, 0.5 cm/min.

oxide is added to human plasma, it is reduced quantitatively to orphenadrine within 10 min by $TiCl_3$ in hydrochloric acid, a medium in which orphenadrine itself is not converted to a measurable degree.

In various plasma samples, including some from patients treated with a daily 300-mg oral dose of orphenadrine over periods up to a few years, reduction with $TiCl_3$ never increased the orphenadrine concentration by more than 7%. Since a 100-fold excess of N-oxide (added to plasma) failed to increase the response of orphenadrine significantly on application of our isolation procedure, it can be concluded that the amount of N-oxide present in the plasma as a metabolite does not interfere with the determination of orphenadrine concentrations.

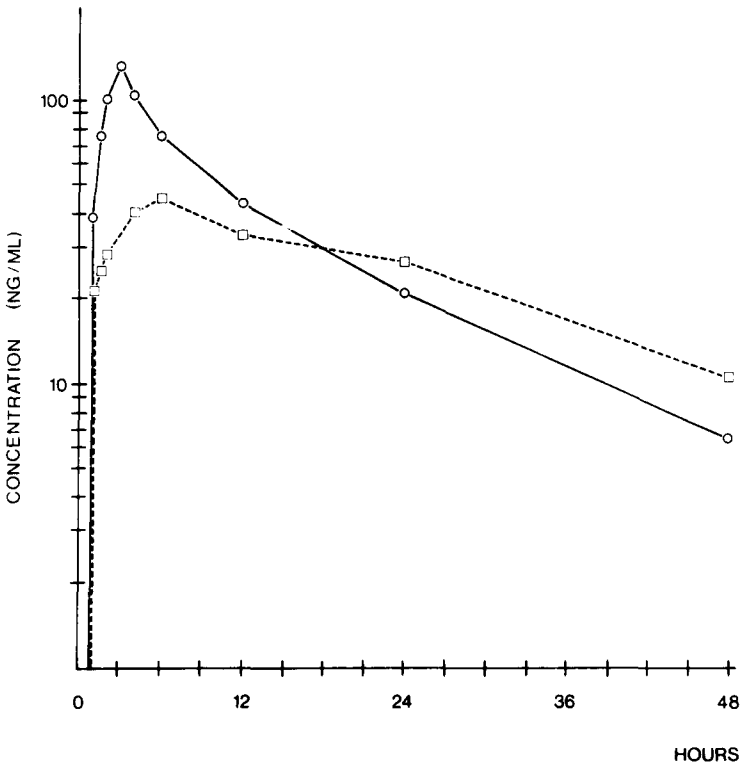

Fig. 4. Plasma concentration–time curves of orphenadrine (○) and N-demethylorphenadrine (□) after a single oral dose of orphenadrine (100 mg as a tablet) to a healthy volunteer.

DISCUSSION

As Fig. 4 shows, the maximal plasma concentration after an oral 100 mg dose of orphenadrine (in clinical practice, such a dose is applied for multimedication) is *ca.* 150 ng/ml. For a study of the pharmacokinetic profile of orphenadrine the concentration should be followed for at least three times the half-life. This implies accurate measurement in the 5–150 ng range, which is made possible by an isolation method based on extraction with isopentane and sensitive nitrogen detection after separation on a KOH–Carbowax column. The method is selective and specific enough to separate orphenadrine from its N-dealkylated metabolites and its N-oxide*.

Although with normal flame ionization it is possible to detect concentrations down to 5 ng/ml, the technique requires a thorough clean-up procedure to eliminate contaminants from blood plasma, extraction solvents, glassware, and rubber stoppers as supplied together with vacutainer tubes. When a nitrogen detector is used, re-extraction at pH 3 and washing with isopentane (it is essential that all the washing liquid should be removed) suffices. Interfering peaks in the gas chromatogram are not

* Other metabolites containing nitrogen and having similar chromatographic properties were not found, in agreement with current knowledge about metabolic pathways.

observed, and the time elapsing between two individual injections can be limited to *ca.* 10 min. The method is, therefore, suitable for routine purposes.

An important factor that may influence the reliability and accuracy of the method, especially at low concentrations, is adsorption of orphenadrine onto the glass walls. Such adsorption can be reduced considerably by careful rinsing of the inner wall of the glass centrifuge tube with ethanol just before the final isopentane extract is introduced.

In this manner, an acceptable recovery (50–60 %) is possible even at the lowest concentrations. Adsorption on the column can be minimized by periodic injection of some extra base (*e.g.* 1 μg). It is recommended that solutions in water, plasma, alcohol, etc., should be stored for the shortest possible time, as loss on standing due to adsorption cannot be avoided. The above considerations also hold for N-demethylorphenadrine if determined quantitatively, alone or simultaneously with orphenadrine.

CONCLUSION

The method reported is suitable for routine determinations of low orphenadrine concentrations in human plasma and urine. It separates orphenadrine from its N-dealkylated metabolites and its N-oxide, thus providing a useful tool in pharmacokinetic and bio-availability experiments.

ACKNOWLEDGEMENT

The technical assistance of Mrs. A. Baars-de Boer and Mr. Th. R. M. Cornelissens is gratefully acknowledged.

REFERENCES

1 W. Hespe, A. M. de Roos and W. Th. Nauta, *Arch. Internat. Pharmacodyn.*, 156 (1965) 180.
2 T. Ellison, *Arch. Internat. Pharmacodyn.*, 195 (1972) 213.
3 T. Ellison, A. Snijder, J. Bolger and R. Okun, *J. Pharmacol. Exp. Ther.*, 176 (1971) 284.
4 A. H. Beckett and F. Khan, *J. Pharm. Pharmacol.*, 23 (1973) 222S.
5 F. Khan, *Thesis*, Chelsea College, University of London, 1972.
6 W. Bilzer and U. Gundert-Remy, *Eur. J. Clin. Pharmacol.*, 6 (1973) 268.
7 *Operating note, N-FID conversion kit, 15161-90003*, Hewlett-Packard, Avondale, Pa., 1973.
8 C. A. Burgett, D. H. Smith, H. B. Bente, J. C. Wirfel and S. E. Goodhart, *Chromatographic capabilities of the nitrogen–phosphorus selective detector, ANGC 2-76*, Hewlett-Packard, Avondale, Pa., 1976.
9 M. Donike, L. Jaenicke, D. Stratmann and W. Hollmann, *J. Chromatogr.*, 52 (1970) 237.