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GAS CHROMATOGRAPHIC DETERMINATION OF PEMOLINE AS 5-PHENYL-2,4-OXAZOLIDINEDIONE IN HUMAN URINE

N. P. E. VERMEULEN, D. DE ROODE and D. D. BREIMER

Department of Pharmacology, Subfaculty of Pharmacy, University of Leiden, Wassenaarseweg 72, Leiden (The Netherlands)

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SUMMARY

A simple gas chromatographic assay of the psychostimulant pemoline in human urine has been developed. Instead of extraction of the drug from urine, it is hydrolysed to 5-phenyl-2,4-oxazolidinedione with 1 *N* hydrochloric acid. After the extraction, this compound is methylated with diazomethane and determined by gas–liquid chromatography using a nitrogen-selective detector and a solid injection system. The method has been applied in preliminary human pharmacokinetic studies, by measuring the urinary excretion rate of pemoline following oral administration. At present, the screening procedures for doping control do not involve the detection of pemoline, but the method described can easily be incorporated in such procedures.

INTRODUCTION

Pemoline is a central nervous system stimulant and anorexiant agent and is the major active constituent of many drug preparations (Deadyn, Didascon, Stimul, Tradar, Sigmadyn, etc.). Its structural formula is given in Fig. 1, showing that it can exist in two tautomeric forms, Ia (2-amino-5-phenyl-4-oxooxazoline) and Ib (2-imino-5-phenyl-4-oxazolidinone). Although the therapeutic indication of central nervous system stimulants is seriously questioned at present, it is well known that these agents, including pemoline, are frequently abused in doping during sporting activities. The screening procedures used in doping control, however, do not include the detection of pemoline, because the compound as such is difficult to extract and, when extracted, difficult to analyse; gas chromatography cannot be applied because pemoline is not directly eluted, and when derivatized pemoline forms various compounds in different yields.

Different methods for assaying pemoline have been described, e.g., spectrophotometric[1–3] and radiometric methods[4,5], but they are not specific and sensitive enough for the quantitative determination of pemoline in biological fluids; one meth od, for example, is based on the hydrolysis under alkaline conditions to mandelic acid (Fig. 1, IV), a compound which is a normal constituent of human urine.

Recently a high-performance liquid chromatographic (HPLC) determination of

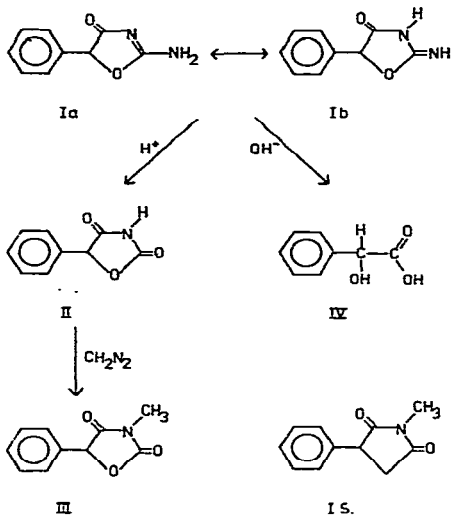


Fig. 1. Structural formulae of compounds used: Ia = 2-amino-5-phenyl-4-oxooxazoline; Ib = 2-imino-5-phenyl-4-oxazolidinone; II = 5-phenyl-2,4-oxazolidinedione; III = N-methyl-5-phenyl-2,4-oxazolidinedione; IV = mandelic acid; I.S. (internal standard) = 3-phenyl-1-methyl-pyrrolidine-2,5-dione (fensuximide).

pemoline in pharmaceutical products and human urine was published[6], but it is questionable whether the investigators determined the intact pemoline quantitatively under the conditions applied.

In this paper, we describe a method, based on acidic hydrolysis of pemoline in urine to 5-phenyl-2,4-oxazolidinedione (Fig. 1, II), followed by solvent extraction and nitrogen-selective gas–liquid chromatographic (GLC) quantitation of the product, which allows the determination of at least 0.5 μg of pemoline per millilitre of urine. The method appears to meet the requirements needed to obtain urinary excretion data in humans following the usual oral doses. The method is also promising for application in screening procedures of doping control, which will be the subject of a subsequent paper.

MATERIALS AND METHODS

Materials

Pemoline and fensuximide (3-phenyl-1-methylpyrrolidine-2,5-dione; internal standard, Fig. 1) were kindly supplied by Chemische Industrie (Katwijk, The Netherlands). 5-Phenyl-2,4-oxazolidinedione was synthesized by acidic hydrolysis of pemoline according to the method of Traube and Asher[7]. Other reagents were distilled dichloromethane (J. T. Baker, Phillipsburgh, N.J., U.S.A.), distilled *n*-pentane (Baker) and 6 *N* hydrochloric acid.

Standard solutions of pemoline in water

Pemoline is insoluble in non-polar solvents and almost insoluble in water and other polar solvents. For the preparation of standard solutions in water, it is necessary

to dissolve pemoline at pH 10. Immediate neutralization of this solution is advisable because otherwise decomposition under alkaline conditions to mandelic acid will occur.

Apparatus

A Hewlett-Packard Model 5750 gas chromatograph, equipped with a nitrogen-selective detector (rubidium bromide, Hewlett-Packard Model 15161B) was used.

The column (120 × 0.12 cm I.D., borosilicate glass, silanized with 10% dimethyl dichlorosilane in toluene) was prepared according to Driessen and Emonds[8]. The support was Gas-Chrom Q, re-silanized, with a particle diameter of 180–200 μm. The stationary phase was a mixture of 2% (w/w) OV-17 and 1% (w/w) OV-225. The solid injection system was a modified pyrolysis sluice system (Becker, Model 767), which has been used for the determination of antiepileptic drugs[8].

The temperatures were injection port 250°, detector 350° and column 190°. The gas flow-rates were air 180 ml/min, hydrogen 30 ml/min and carrier gas (helium) through the column 5 ml/min; at the end of the column a flow of auxiliary helium was added to ensure a total flow-rate of 60–90 ml/min in the detector, which is required for optimal performance of the nitrogen-selective detector.

An LKB 2091–2130 gas chromatograph–mass spectrometer–computer system was used for the unambiguous identification of the compounds eluting from the gas chromatograph. The column, injection system and other gas chromatographic parameters were as specified above. The following other conditions were used: ion source temperature, 250°; separator temperature, 225°; electron energy, 70 eV; accelerating voltage, 3.5 kV; trap current, 50 μA; and detector, total ion monitor.

The proton magnetic resonance (PMR) spectra of pemoline were recorded on a 100-MHz Jeol JNM-PS-100 instrument in hexadeuterodimethyl sulphoxide with tetramethylsilane as the internal standard.

Extraction procedure for 5-phenyl-2,4-oxazolidinedione

To 5.0 ml of urine in a centrifuge tube were added 1 ml of 6 *N* hydrochloric acid and, after 15-min hydrolysis, 0.10 ml of water containing 50.0 μg of fensuximide as internal standard. After subsequent homogenization the mixture was extracted once with 10 ml of dichloromethane–*n*-pentane (1:1) on a Cenco Whirlmixer for 5 sec. The upper organic phase was removed with a pasteur pipette and, after 3 min centrifuging at 3000 rpm (600 *g*), transferred to a conical evaporation tube. The solvent was evaporated to dryness at 35° in a stream of dry nitrogen. The residue was dissolved in 0.5 ml of freshly prepared (from N-nitrosomethylurea) ethereal diazomethane solution[9]. After at least 15 min, 5 μl of this solution were placed on the needle of the solid injection system and, following evaporation of the solvent, injected into the gas chromatograph.

Hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione

To portions of 5 ml of cold water in glass-stoppered tubes, containing known amounts of pemoline, were added 1 ml of 6 *N* hydrochloric acid and the mixtures were heated in a water-bath at 80°. At different times between 0 and 45 min one sample was cooled in order to stop hydrolysis and immediately extracted and assayed as described above, except for the addition of 1 ml 6 *N* hydrochloric acid. The ratio

of the peak area of compound III (Fig. 1) to that of the internal standard was plotted against the time of hydrolysis. The same procedure was also followed but with the addition of 1 ml of 0.6 *N* instead of 1 ml of 6 *N* hydrochloric acid.

Preparation of calibration graphs and determination of recovery

The concentration of pemoline in urine samples was calculated with the aid of calibration graphs, which were prepared by adding known amounts of pemoline to 5 ml of urine. The samples were analyzed by the procedure described above. The ratio of the peak area of compound III to that of the internal standard was plotted against known concentrations of pemoline. For the determination of the extraction yield of compound II from urine, portions of 5 ml of urine were hydrolysed and, after the addition of various amounts of compound II, carried through the extraction procedure as described above, except for the addition of 1 ml of 6 *N* hydrochloric acid and except that fensuximide was used as an external standard. The ratios found were compared with the ratio of standard amounts of compound II.

RESULTS AND DISCUSSION

Gas chromatographic sensitivity and selectivity

The sensitivity and selectivity of the nitrogen-selective detector for nitrogen-containing compounds allow the relative simple and rapid determination of low concentrations of pemoline as compound II in human urine. The detection limit is about 5 ng per single injection. Methylation of compound II resulted in a shorter retention time, a more symmetrical peak shape and a lower detection limit. The calibration graph, obtained by plotting the ratio of the peak area of compound III to that of the

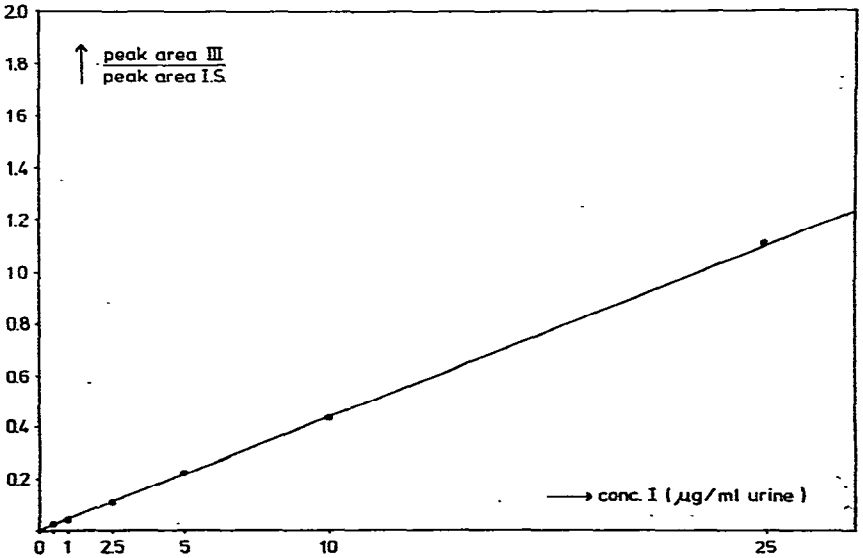
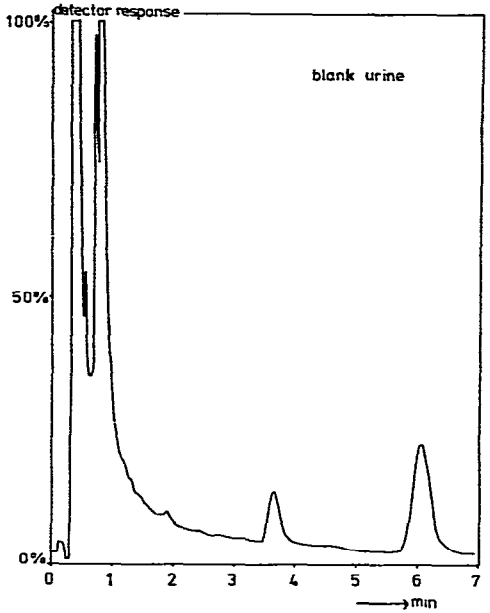


Fig. 2. Standard calibration graph for pemoline added to urine and carried through the analytical procedure.



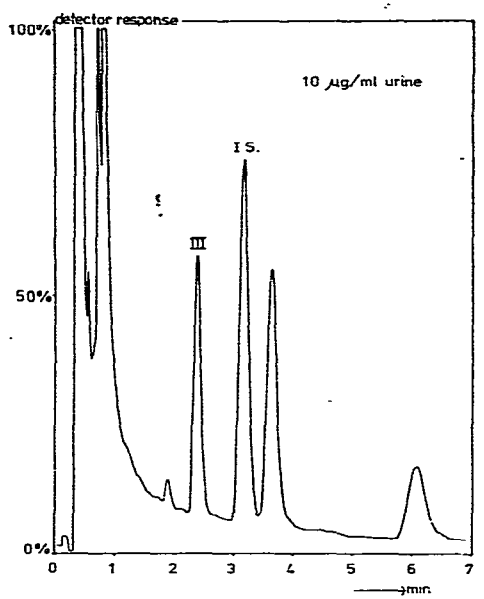


Fig. 3. Gas chromatogram of an extract of 5 ml of human blank urine after acidic hydrolysis and methylation.

Fig. 4. Gas chromatogram of an extract of 5 ml of human urine, containing 10 µg/ml of pemoline after acidic hydrolysis and methylation.

internal standard against known amounts of pemoline added to human urine, is shown in Fig. 2. Good linearity was obtained for concentrations from 500 ng to 25 µg of pemoline per millilitre of urine.

Figs. 3 and 4 show typical gas chromatograms of human blank urine and urine containing 10 µg/ml of pemoline, respectively, both after acidic hydrolysis.

Identification of pemoline and extraction products

The analysis of urine samples by means of GC–MS showed that the peak with a retention time of 3.75 min was identical with methylated hippuric acid and that with a retention time of 6 min was identical with caffeine (Table I). The mass spectra used for the identification of the compounds of interest are presented in Fig. 5, *e.g.*: I, pemoline (direct inlet MS); II, 5-phenyl-2,4-oxazolidine-dione (GC–MS); III, N-methyl-5-phenyl-2,4-oxazolidinedione (GC–MS).

TABLE I

LIST OF 70-eV MASS SPECTRA (ONLY THE MOST ABUNDANT *m/e* VALUES) WITH THEIR RELATIVE INTENSITIES

Compound	*Mol. weight*	*m/e (relative abundances in parentheses)*
Hippuric acid methyl ester	193	150 (100); 77 (40); 193 (20); 134 (20)
Caffeine	194	194 (100); 109 (65); 67 (50); 55 (50)
Mandelic acid methyl ester	166	107 (100); 166 (20); 79 (10)
Fensuximide	189	104 (100); 189 (20); 77 (10); 51 (10)

As already stated in the introduction, for pemoline two tautomeric forms were postulated: Ia (2-amino-) and Ib (2-imino-). Although different workers[10–12] suggested that the 2-amino-compound is the predominant tautomer (based on chemical, UV and IR data), our PMR and nitrogen-decoupled PMR spectra (Table II) did not support this suggestion.

TABLE II

PMR AND NITROGEN-DECOUPLED PMR OF A 10% SOLUTION OF PEMOLINE IN D_6-DMSO

PMR	*Nitrogen-decoupled PMR*
5.37 (s, C_5—H)	5.37 (s, C_5—H)
6.95 (m, C_6H_5)	6.95 (m, C_6H_5)
8.28 (broad resonance, 2 N—H)	8.35 (broad resonance, 2 N—H)

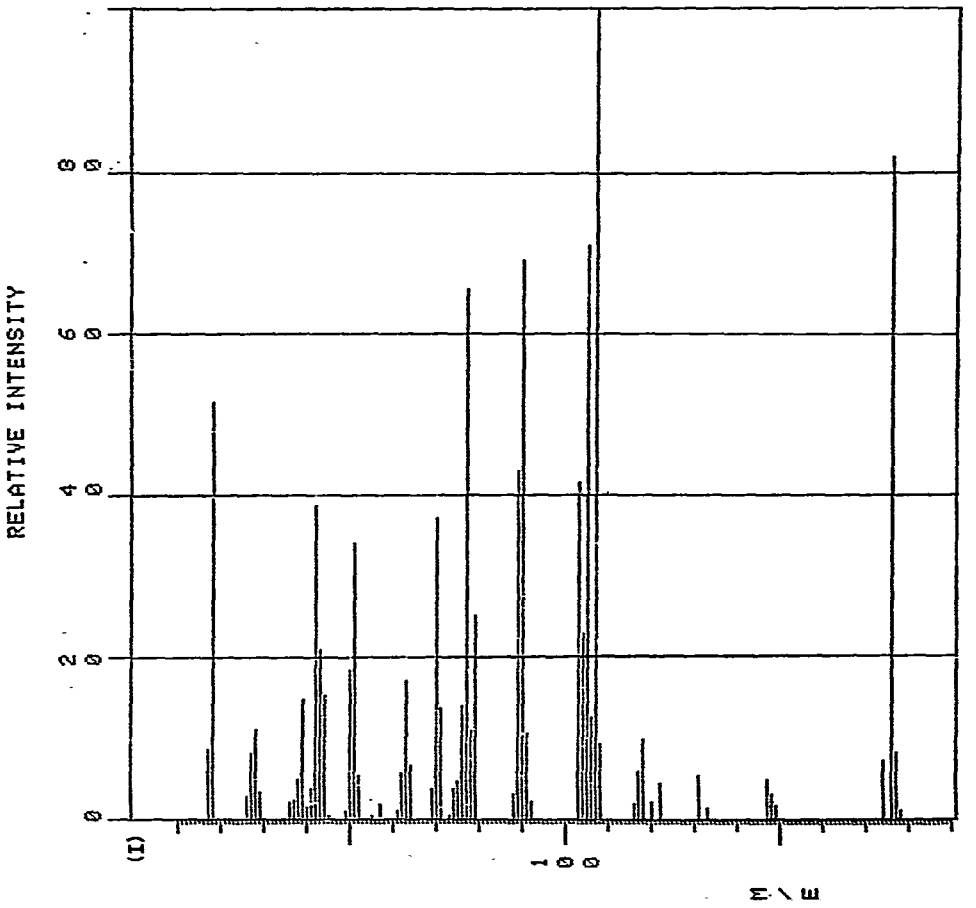


Fig. 5 (I).

Hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione

In Fig. 6 the ratio of the peak area of compound III to that of the internal standard is plotted against reaction time in 1 *N* and 0.1 *N* hydrochloric acid. A constant level is reached rapidly in both instances, but slightly faster in 1 *N* hydrochloric acid. The level in 1 *N* hydrochloric acid is also slightly higher due to a higher extraction yield of compound II, probably caused by higher ionic strength of this solution. Complete conversion of pemoline is achieved in 15 min at 80° in 1 *N* hydrochloric acid; completeness was checked by adding known amounts of compound II and comparing the results with those from equivalent amounts of pemoline.

Extraction procedure and precision

The extraction of compound II ($pK_a = 5.5$) from urine with dichloromethane–*n*-pentane (1:1) after acidic hydrolysis at pH 0 proved to be suitable. No interfering constituents are extracted and the extraction yield is satisfactory, being about 60% and constant over a large concentration range. Standard deviations did not exceed 5%. Extraction times longer than 5 sec did not result in significantly higher extraction

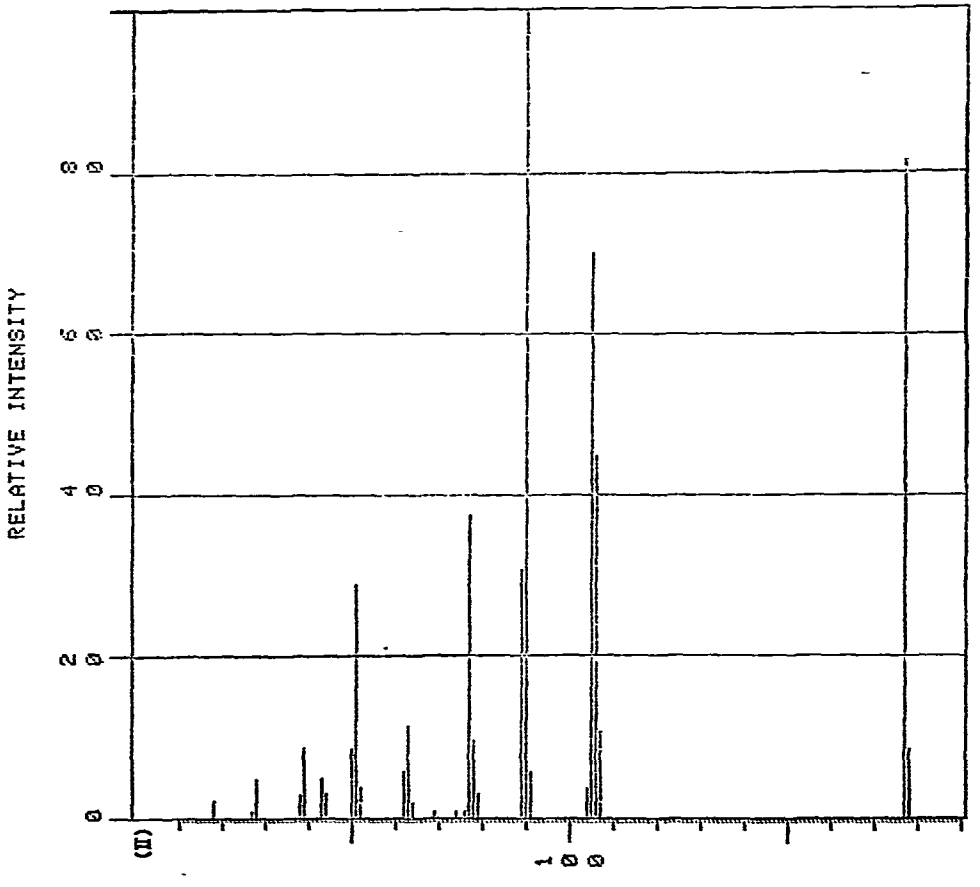


Fig. 5 (II).

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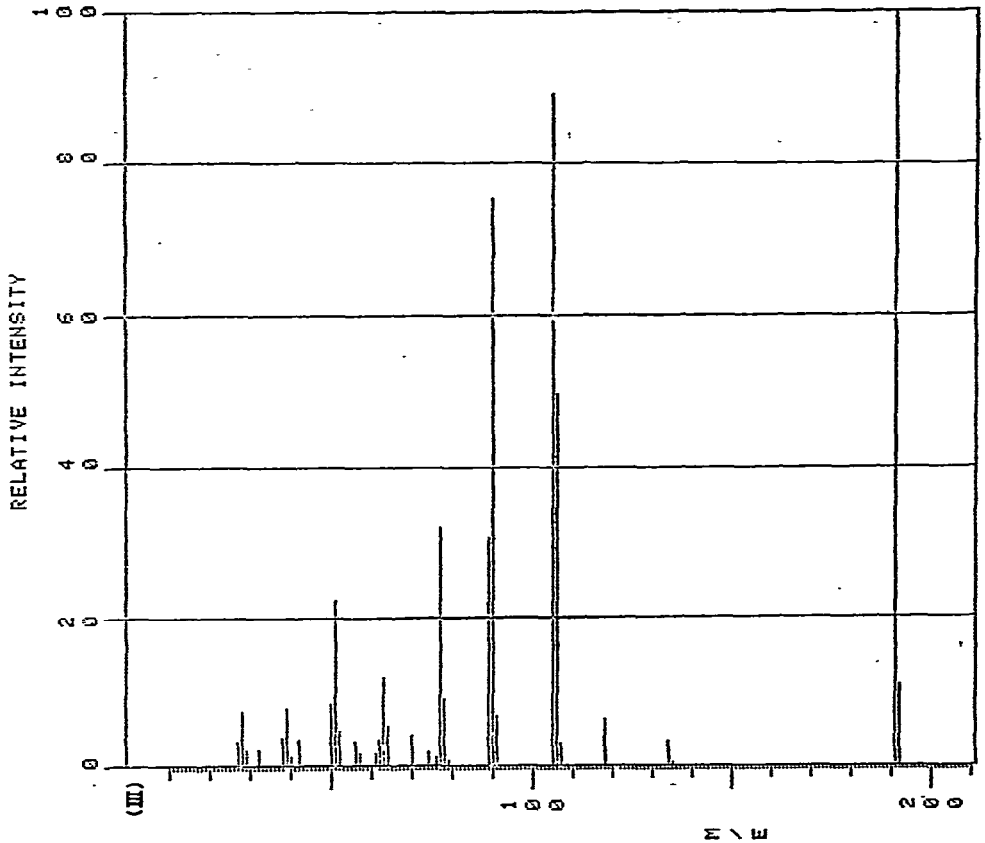

Fig. 5. Mass spectra at 70 eV of (I) pemoline, (II) 5-phenyl-2,4-oxazolidinedione and (III) N-methyl-5-phenyl-2,4-oxazolidinedione.

yields; other solvents (*e.g.*, diethyl ether) and solvent mixtures (*e.g.*, diethyl ether–*n*-pentane) gave better extraction yields, but interfering constituents of the urine were co-extracted.

Preliminary results of renal excretion

Preliminary results with the present method were obtained with three human volunteers who received 20 mg of pemoline orally, their urine being collected during the 72 h following administration of the drug.

Fig. 7 shows a typical example of the renal excretion curve obtained in one of the volunteers. As the excretion rate is proportional to the plasma concentration of a drug under linear pharmacokinetic circumstances[13], it can be deduced from this curve that the absorption of pemoline is slow, as the maximum rate is reached only after 5 h. After this maximum there is a relatively rapid decrease in the excretion rate, which may be due to a distribution process or to a capacity-limited protein-binding phenomenon. Further, it can be derived that pemoline is eliminated slowly

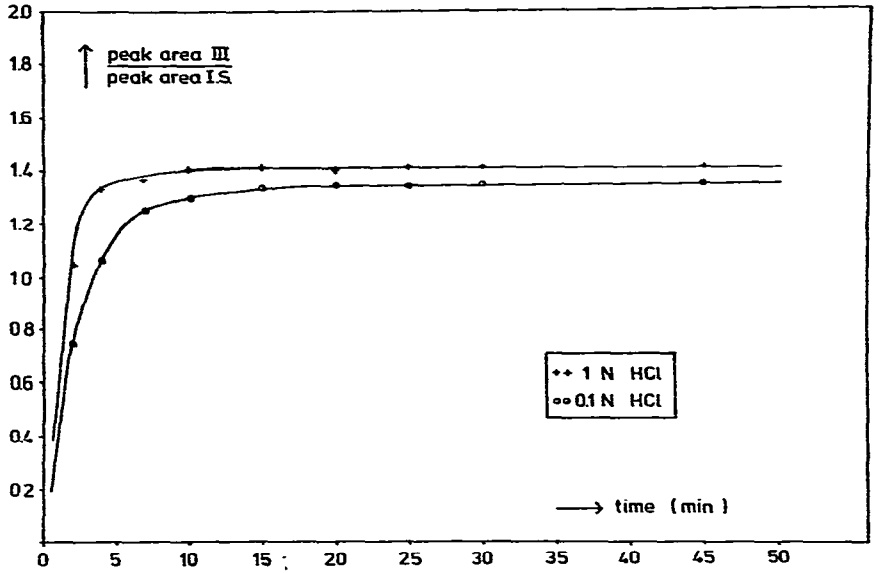

Fig. 6. Hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione under acidic conditions at 80°.

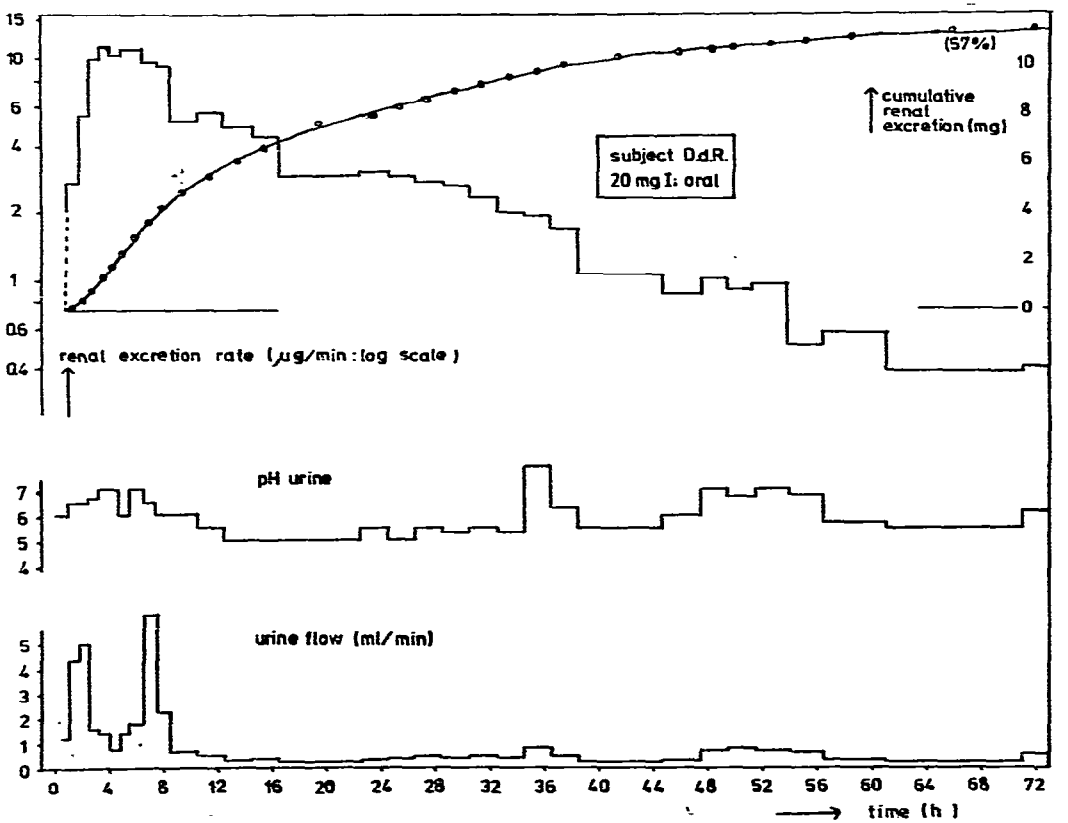

Fig. 7. Renal excretion rate (µg/min), urine pH, urine flow-rate (ml/min) and cumulative urinary excretion (mg) after oral administration of 20 mg of pemoline to a healthy human volunteer.

TABLE III

CUMULATIVE RENAL EXCRETION AND BIOLOGICAL HALF-LIFE OF PEMOLINE AFTER ORAL ADMINISTRATION TO HEALTHY HUMAN VOLUNTEERS

Subject	*Dose (mg)*	*Proportion of dose excreted unchanged (%)*	*Half-life (h)*
D. de R.	20.0	63.4	16.0
D. de R.	20.0	57.0	15.5
D. B.	20.0	53.6	15.0

from the human body; the biological half-life, calculated from the terminal part of the excretion rate curve, is 15.5 h. Variations in the urinary pH between 5.0 and 7.5 seem to have no influence on the excretion rate of unchanged pemoline in urine. The cumulative renal excretion shows that 11.40 mg of pemoline are excreted in 72 h, corresponding to 57% of the administered dose.

In Table III the results of the three experiments with the healthy human volunteers are given.

CONCLUSION

The method described for the qualitative and quantitative determination of pemoline as 5-phenyl-2,4-oxazolidinedione is rapid and simple. Owing to its high sensitivity and specificity, the method is suitable for the study of the pharmacokinetics of pemoline and also for the detection and identification of pemoline during doping control. The application of the method for the latter purpose will be the subject of a subsequent paper.

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