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ASSAY OF PEMOLINE IN HUMAN PLASMA, SALIVA AND URINE BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION

N. P. E. VERMEULEN

Departments of Pharmacology and Pharmaceutical Analysis, Subfaculty of Pharmacy, University of Leiden, Gorlaeus Laboratories, Wassenaarseweg 76, Leiden (The Netherlands)

and

M. W. E. TEUNISSEN and D. D. BREIMER

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

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SUMMARY

A simple gas chromatographic assay of the psycho-stimulant pemoline in human urine, plasma and saliva has been developed. Instead of direct extraction of the drug from urine, plasma and saliva, it is hydrolyzed to 5-phenyl-2,4-oxazolidinedione with 1 *N* hydrochloric acid. After extraction this compound is methylated with diazomethane and determined by gas–liquid chromatography using a capillary SCOT column with a mixed stationary phase, a solid injection system and a nitrogen-selective detector. 5-Phenyl-2,4-oxazolidinedione, which was also found to be a metabolite of pemoline, could be determined quantitatively in human urine.

INTRODUCTION

Pemoline (2-imino-5-phenyl-4-oxazolidinone, Fig. 1, I) is a stimulant of the central nervous system and is the major active constituent in many drug preparations. Because of its low lipophilicity and its instability under acidic and basic conditions, it is difficult to extract pemoline from biological fluids. As a consequence, methods such as thin-layer chromatography (TLC)[1] and high-performance liquid chromatography[2], which employ direct extraction of pemoline from human urine, lack the sensitivity and selectivity required for quantitative determination. Gas–liquid chromatography (GLC) cannot be applied because pemoline is not eluted unchanged and derivatization yields various compounds. For these reasons several authors[3–6] have recently described methods where pemoline is hydrolyzed by acidic treatment to 5-phenyl-2,4-oxazolidinedione (Fig. 1, II) prior to extraction. This compound is easily extracted and determined by GLC. However, all these methods neglect the occurrence

of compound II as a metabolite of pemoline. In addition, they are not practical and/or sufficiently sensitive for the assay of pemoline in plasma and saliva.

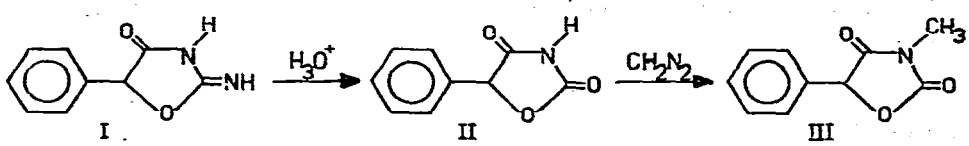

Fig. 1. Derivatization scheme of pemoline (I). First, acidic hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione (II), followed by methylation to N-methyl-5-phenyl-2,4-oxazolidinedione (III).

In this paper an elaboration of our previously published method[5] is reported for human plasma, saliva and urine. The quantitative determination of the oxazolidinedione metabolite in urine is also described.

MATERIALS AND METHODS

Materials

Pemoline and fensuximide (1-methyl-3-phenyl-2,5-pyrrolidinedione, internal standard) 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, Phillipsburg, N.J., U.S.A.), distilled *n*-pentane (J. T. Baker) and concentrated hydrochloric acid.

Apparatus

A Philips GCV gas chromatograph, equipped with a nitrogen-selective detector (rubidium chloride, Model 795014) was used. The capillary SCOT-column (8 m × 0.5 mm I.D., Duran 50 glass) was prepared according to Cramers *et al.*[8], with slight modifications to the stationary phase and solvents. In the first coating step a siliceous support Cap-O-Sil, deactivated with benzyltriphenylphosphonium chloride (BTPPC) and suspended in the polar stationary phase PPE-21 (poly-*m*-phenyl ether ether high polymer, Chrompack, Middelburg, The Netherlands), was used. In the second coating step an additional, mixed stationary phase was coated dynamically on the surface layer of Cab-O-Sil. The mixed stationary phase consisted of 1.6% (w/w) PPE-21 and 2.6% (w/w) OV-17 (Chrompack) in toluene. The solid injection system was a modified pyrolysis sluice system (Becker, Model 767), which has been described for the determination of antiepileptic drugs[9].

The temperatures of the injection port, detector and column were 250, 260 and 190°, respectively. The flow-rates of air, hydrogen and the carrier gas (helium) through the column were 300, 35 and 5 ml/min, respectively; at the end of the column an additional flow of nitrogen was used to ensure a total flow-rate of 70–80 ml/min 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 eluted from the gas

chromatograph. The column, injection system and other gas chromatographic conditions were as specified before; the ion source temperature was 200°, the separator temperature 210°, electron energy 70 eV, accelerating voltage 3.5 kV and trap current 50 μA.

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

Hydrolysis of pemoline in human urine to the oxazolidinedione II is rapid and essentially complete in 15 min at 80° in 1 *N* hydrochloric acid[5]. Under the same conditions, complete conversion of pemoline is achieved in human plasma and saliva; this was verified by adding known amounts of the oxazolidinedione II to plasma and saliva and comparing the results with those obtained by adding equivalent amounts of pemoline to similar samples.

Assay of urine samples

Extraction procedure for metabolite II. To 2.0 ml of urine in a centrifuge-tube was added 0.2 ml of concentrated hydrochloric acid. The mixture was immediately extracted once with 5 ml of dichloromethane–*n*-pentane (1:1) on a Cenco Whirlmixer for 15 sec, followed by centrifuging for 3 min at 3000 rpm (600 *g*). The upper organic layer was transferred to a conical evaporation-tube and 0.10 ml of ethanol containing 20.0 μg fensuximide was added as external standard. The solvent was then evaporated to dryness at 35° in a stream of dry air, and the residue was dissolved in 0.5 ml of a freshly prepared (from N-nitrosomethylurea), distilled, ethereal diazomethane solution. After at least 15 min, 5–10 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.

Extraction procedure for the hydrolysis product of pemoline. After the "oxazolidinedione metabolite" pre-extraction described above, the centrifuge-tubes containing the acidic urinary layer were heated in a water-bath at 80° for 15 min. After cooling to room temperature, 0.10 ml of ethanol containing 20.0 μg of fensuximide as internal standard was added and the mixture was extracted and assayed as described for the metabolite II in urine.

Assay of plasma samples

To 3.0 ml of human plasma in a centrifuge-tube were added 0.3 ml of concentrated hydrochloric acid and 5 ml of dichloromethane. The mixture was stirred immediately on a Cenco Whirlmixer for 15 sec, followed by centrifuging for 3 min at 3000 rpm (600 *g*). A 2.0-ml volume of the upper plasma layer was pipetted into another centrifuge-tube, 0.2 ml of concentrated hydrochloric acid were added and the mixture was hydrolyzed at 80° for 15 min. After cooling to room temperature and addition of 0.10 ml of ethanol containing 2.0 μg of fensuximide as internal standard, the mixture was extracted once with 5 ml of dichloromethane on a Cenco Whirlmixer for 10 sec and centrifuged for 3 min at 3000 rpm (600 *g*). The lower organic layer was transferred to a conical evaporation-tube and the solvent was evaporated to dryness at 35° in a stream of dry air. The residue was dissolved in 0.5 ml of a freshly prepared (from N-nitrosomethylurea), distilled, ethereal diazomethane solution. After at least 15 min the ethereal solution was evaporated again and the residue was dissolved in 0.05 ml of absolute ethanol. A 10–15-μl of this solution were placed on the needle of

the solid injection system, and, after evaporation of the solvent, injected into the gas chromatograph.

Assay of saliva samples

To 2.0 ml of saliva in a centrifuge-tube were added 0.2 ml of concentrated hydrochloric acid. After hydrolysis on a water-bath at 80° for 15 min, the extraction was completed as described for the plasma samples.

Preparation of calibration graphs and determination of extraction yields

The concentrations of the "oxazolidinedione metabolite" in urine and of pemoline in urine, plasma and saliva were calculated with the aid of calibration graphs, which were prepared by adding known amounts of the oxazolidinedione II to 2.0 ml of urine and known amounts of pemoline to 2.0 ml of urine, 3.0 ml of plasma and 2.0 ml of saliva, respectively. The samples were analyzed by the procedures described above. The ratio of the peak height of the resulting methylated oxazolidinedione (Fig. 1, III) to that of the internal standard was plotted against the known concentrations of the oxazolidinedione II and pemoline, respectively.

For the determination of the extraction yields from urine, plasma and saliva, the same procedures were followed, except that fensuximide was used as an external standard. The ratios found were compared with the ratios for standard amounts of the methylated oxazolidinedione III.

RESULTS AND DISCUSSION

Gas chromatographic sensitivity and selectivity

The relatively great sensitivity and selectivity of the nitrogen-selective detector and the use of a capillary SCOT-column facilitate the determination of low concentrations of pemoline in plasma and saliva. A prerequisite is the acidic hydrolysis to the oxazolidinedione II prior to extraction and methylation by diazomethane. This results in a short retention time, a better chromatographic behaviour and a detection limit of 1 ng per single injection with a signal-to-noise ratio of 10:1.

In Fig. 2 typical gas chromatograms are shown of a blank urine extract and a urine extract obtained from a human volunteer, having previously received 50 mg of pemoline. The lowest detectable concentration of pemoline in urine is *ca.* 100 ng/ml, which is about five times lower than obtainable by the method previously described[5]. This higher sensitivity is a direct consequence of the use of a capillary SCOT-column with a total number of 10,000 theoretical plates (calculated for the methylated oxazolidinedione III), compared with a micropacked column which has a considerably lower total number of theoretical plates ($N = 1000$ for the same compound).

In Figs. 3 and 4 gas chromatograms of extracts of plasma are shown, containing 0.61 μg pemoline per ml, and of saliva, containing 0.64 μg pemoline per ml, respectively, obtained after oral administration of 50 mg of pemoline to a human volunteer; gas chromatograms of blank plasma and saliva extracts from the same volunteer are also shown. There is no interference from endogenous urine, plasma or saliva constituents. The retention times of the compounds to be measured are short and the total time for one GC run is 5 min.

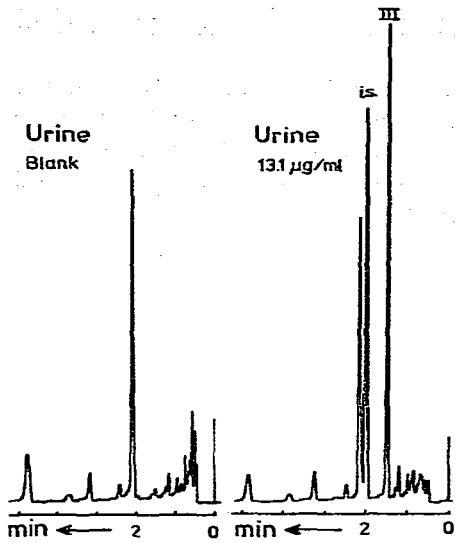

Fig. 2. Gas chromatogram of a 2.0 ml blank extract of human urine and of human urine containing 13.1 µg/ml pemoline.

Extraction procedure and precision

Because of the high lipophilicity of the oxazolidinedione II (a weak acid with $pK_a = 5.5$) only short extraction times (15 sec for urine and 10 sec for plasma and saliva) are needed, immediately after the acidic hydrolysis of pemoline at pH 0. For urine a short "oxazolidinedione metabolite" pre-extraction was necessary. In plasma

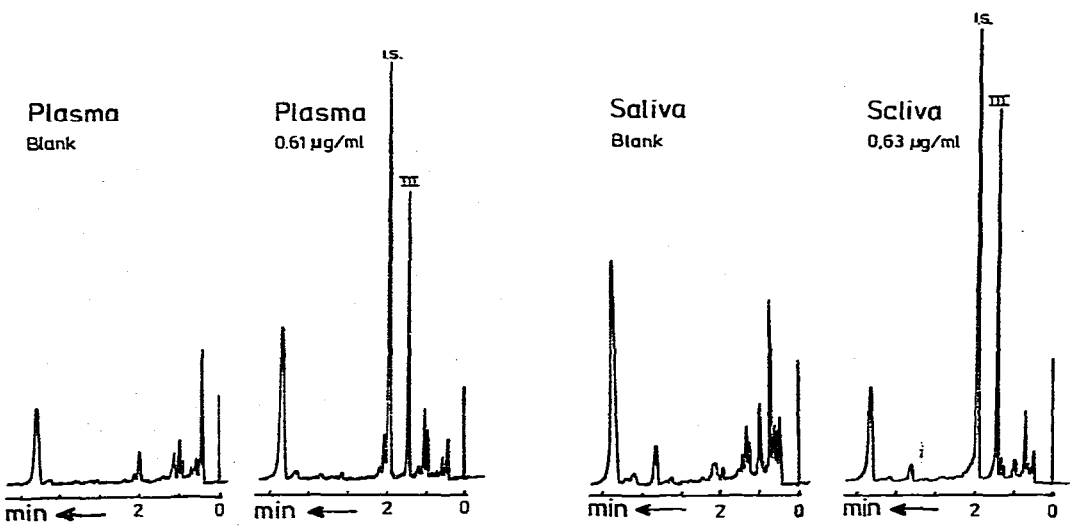

Fig. 3. Gas chromatogram of a 2.0 ml blank extract of deproteinized human plasma and of an extract of plasma containing 0.61 µg/ml pemoline.

Fig. 4. Gas chromatogram of a 2.0 ml blank extract of human saliva and of saliva containing 0.63 µg/ml pemoline.

a short pre-extraction with dichloromethane resulted in a lower detectable concentration of pemoline.

According to the standard curves (Fig. 5), there is a linear relation between the ratio of the peak height of the methylated oxazolidinedione III to that of fensuximide and the concentration of pemoline between 0.05 and 1.0 μg/ml of plasma and saliva. For urine samples, good linearity was obtained for concentrations of pemoline and the "oxazolidinedione metabolite" between 100 ng and 25 μg/ml.

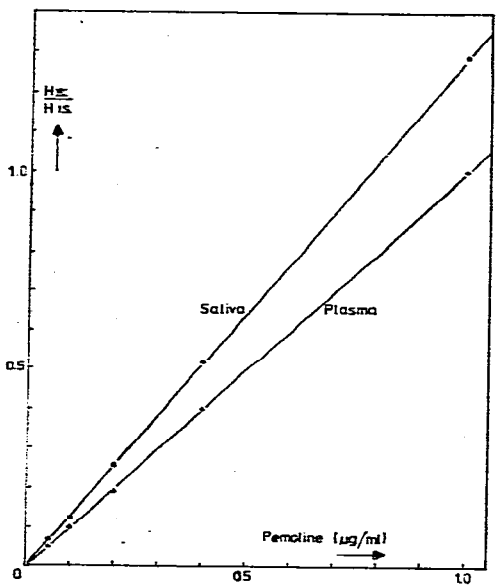

Fig. 5. Sta ndard calibration graphs for pemoline added to plasma and saliva, carried through the analytical procedure.

The reliability and accuracy of the assay procedure was assessed with the aid of five calibration curves each for urine, plasma and saliva. The corresponding correlation coefficients were in all cases better than 0.999, clearly establishing the linearity of the calibration curves. In addition, the mean recoveries were determined for the same concentration range as used to construct the calibration curves, using fensuximide as an external standard. The extraction yield of 67% for urine with a standard deviation at each concentration of $\leqslant 3\%$ ($n = 5$) is only slightly higher than the results obtained previously[5], due to the three times longer extraction time (15 sec) and the "oxazolidinedione metabolite" pre-extraction. For plasma and saliva, extraction times of 10 sec gave satisfactory and reproducible extraction yields of 82 and 100% with a standard deviation at each concentration of $\leqslant 3$ and $\leqslant 2\%$ ($n = 5$), respectively.

Identification of the extraction products and the "oxazolidinedione metabolite"

The analysis of urine, plasma and saliva samples by means of a GC–MS–computer system showed that the methylated oxazolidinedione III and fensuximide are eluted from the gas chromatograph without chemical alteration under the con-

ditions applied. Fig. 6 shows a reconstructed mass chromatogram for three specific *m/e* values obtained from a saliva extract. The retention times, at which the characteristic masses [*m/e* 191 (100%) and *m/e* 105 (90%) for the methylated oxazolidinedione III, and *m/e* 189 (20%) and *m/e* 105 (8%) for fensuximide[5]] appear simultaneously, are equal to the values for authentic methylated oxazolidinedione III and fensuximide. Furthermore, the peak having a retention time of 2.1 min (Fig. 2) appeared to correspond to methylated hippuric acid[5], and that having a retention time of 4.8 min to that of caffeine[5] (Figs. 2–4).

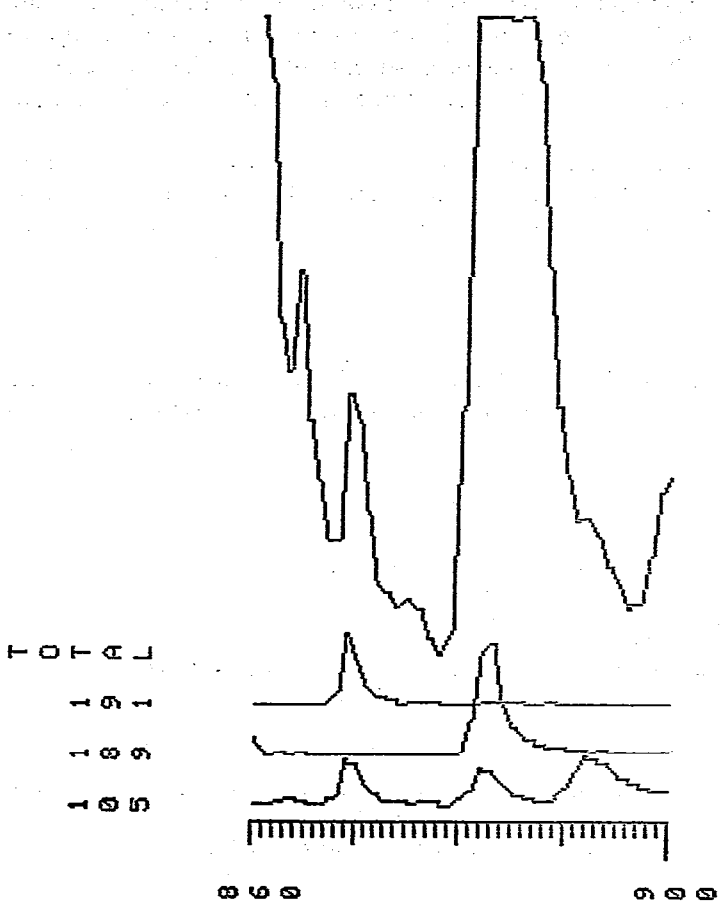

Fig. 6. Total ion current and mass chromatogram of three selected *m/e* values, characteristic of the methylated oxazolidinedione III and fensuximide: *m/e* 191 and *m/e* 105 for III, and *m/e* 189 and *m/e* 105 for fensuximide.

To investigate whether the "oxazolidinedione metabolite" is a real metabolite or an artefact of the acidic extraction procedure the following experiment was performed. Urine, plasma and saliva, spiked with 25 μg/ml pemoline, were extracted as described before except that fensuximide was used as external standard and except acidic hydrolysis. Since the peak height ratio found with GLC–AFID and GLC–MS did not significantly exceed that of the blanks, it must be concluded that the "ozazoli-

dinedione metabolite" which is extracted prior to acidic hydrolysis is indeed a real metabolite. This metabolite was identified only in the urine of human volunteers after oral administration of 50 mg of pemoline.

CONCLUSIONS

The assay described for the quantitative determination of pemoline in human urine, plasma and saliva is rapid and relatively simple. It takes account of the occurrence of 5-phenyl-2,4-oxazolidinedione itself as a metabolite of pemoline, while mandelic acid, another metabolite of pemoline, or other endogenous substances do not interfere with the method. The method has been successfully applied to the study of pharmacokinetics of pemoline and its "oxazolidinedione metabolite" in human urine, plasma and saliva[10].

The SCOT-columns (with mixed stationary phases) are not yet in common use but they appear to be preferable to packed and micropacked columns when it is required to determine low concentrations of drugs or metabolites in biological fluids for pharmacokinetic studies.

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