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Note

Quantitative gas–liquid chromatographic determination of pemoline using trimethylanilinium hydroxide

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Pemoline [2-amino-5-phenyl-2-oxazolin-4-one (I)] is a central nervous system stimulant and is the major constituent of many drug preparations (Cylert, Deadyn, Stimul, Tradon, etc.). It is also a dopant and its use in sport is banned by the Internal Olympic Commission. Known procedures for the determination of pemoline, except for a high-performance liquid chromatographic (HPLC) method[1,2], are based on the measurement of the products of its alkaline[3–5] or acidic[6–10] hydrolysis.

As mandelic acid (III) and benzaldehyde (IV) are also endogenous urine components, methods based on the detection of III and IV cannot be considered to be specific. Procedures based on acidic hydrolysis of I are sufficiently specific, but it is necessary to carry out preliminary methylation of 5-phenyl-2,4-oxazolidinedione (II) and to detect N-methyl-5-phenyl-2,4-oxazolidinedione (V) in order to achieve a high sensitivity of gas–liquid chromatographic analysis. A major drawback of these methods[6–9] is use of diazomethane, which is volatile, unstable and very toxic, as a methylating agent.

In this work the possibility of using trimethylanilinium hydroxide for the methylation of II was studied; this method has been applied previously for the analysis of barbiturates[11]. Methylation with help of TMAH [$(CH_3)_3C_6H_5N^+$ OH^-] is carried out in the injection port of the chromatograph, so that the stage of preliminary derivatization is virtually eliminated and the total analysis time is decreased.

EXPERIMENTAL

Apparatus

For GLC, a Hewlett-Packard 5710A gas chromatograph, equipped with a nitrogen–phosphorus specific detector (NPD), was used. The glass chromatographic column (0.8 × 1 mm I.D.) was packed with 5% OV-17 on 80–100-mesh Chromosorb 750 (Johns-Manville, Celite Division, Greenwood Plaza, Denver, CO, U.S.A.). The carrier gas was helium at a flow-rate of 40 ml/min, and the flow-rates of air and hydrogen to the detector were 60 and 3.2 ml/min, respectively. The injection port temperature was maintained at 200°C and the detector temperature at 300°C. The temperature of column was programmed from 100°C at the rate 30°C/min to a final temperature of 250°C, which was held for 4 min.

For GLC–mass spectrometry (MS), a Hewlett-Packard 5985 instrument was

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used. The glass chromatographic column (1.2 m × 2 mm I.D.) was packed with 3% OV-101 on 80–100-mesh Chromosorb W HP (Hewlett-Packard, Avondale, PA, U.S.A.) and the helium flow-rate was maintained at 40 ml/min. The mass spectrometric conditions were ionization beam energy 70 eV, electron multiplier 2600 V and temperatures of jet separator and ion source 250 and 200°C, respectively.

Standards and reagents

N-Methylphthalimidine was prepared by diazomethane alkylation of phthalimide. The latter was obtained from phthalic anhydride and ammonia by a published procedure[12]. Sublimation provided the analytical sample of N-methylphthalimide with m.p. 128–130°C (lit.[13] m.p. 130°C).

5-Phenyl-2,4-oxazolidinedione was prepared by acidic hydrolysis of pemoline (1 *M* hydrochloric acid, 120°C, 15 min). The acidic solution was extracted twice with methylene chloride. The combined organic extract was dried with sodium sulphate and evaporated at 40°C under vacuum. Recrystallization from water afforded the desired 5-phenyl-2,4-oxazolidinedione with m.p. 105°C (lit.[14] m.p. 109°C).

N-Methyl-5-phenyl-2,4-oxazolidinedione was prepared by methylation of 5-phenyl-2,4-oxazolidinedione with diazomethane. Recrystallization from ether provided the analytical sample with m.p. 112–113°C (lit.[14] m.p. 114°C). Calculated for $C_{10}H_9NO_3$: C 62.82, H 4.76, N 7.32%. Found: C 62.95, H 4.86, N 7.22%.

For the methylation reaction TMAH was used as a 0.2 *M* solution in methanol ("MethElute"; Pierce, Rotterdam, The Netherlands).

Standard mixtures

Mixtures for the determination of detector response factors and conversion of 5-phenyl-2,4-oxazolidinedione into N-methyl derivative were prepared by dissolving the corresponding substances (3 mg) and internal standard (N-methylphthalimide) in a suitable volume of methanol.

To obtain the calibration graph, pemoline (3–4 mg) was dissolved in 10 ml of methanol and 1 ml of the solution was diluted with urine to a concentration 10 μg/ml. Standards of pemoline were prepared by adding the appropriate volume of pemoline solution (10 μg/ml) to urine to give final concentrations of 1, 2, 4, 6, 8 and 10 μg/ml.

Sample preparation

To samples of 5 ml of urine was added 0.5 ml of concentrated hydrochloric acid, then they were hydrolysed in an autoclave at 120°C for 20 min. 5-Phenyl-2,4-oxazolidinedione was extracted with 5 ml of methylene chloride containing 1.5 ppm of internal standard (N-methylphthalimide) (2 min; Vortex-Evaporator). After centrifugation (900 *g*) the organic layer was separated and dried over sodium sulphate. The solvent was evaporated in stream of nitrogen. The residue was dissolved in 200 μl of absolute methanol, 20 μl of 0.2 *M* of trimethylanilinium hydroxide solution were added and the mixture was analysed by GLC.

RESULTS AND DISCUSSION

The response factor (*K*) of the NPD to N-methyl-5-phenyl-2,4-oxazolidinedione compared with a standard was determined by the analysis of mixtures of these sub-

stances in methanol at concentrations from 10 to 500 ng/μl. Each mixture was analysed 3–5 times, and an average value of $K = 0.78 \pm 0.05$ was obtained. The detector worked in the linear range at concentrations from 10 to 500 ng on injection.

Conversion of 5-phenyl-2,4-oxazolidinedione into the N-methyl derivative was determined by analysing mixtures of II and the internal standard after a sufficient amount of TMAH had been added. On the basis of the data obtained, the concentration of the methylation product and its percentage yield were calculated with respect to the response factor of the detector, which had been calculated previously.

Depending on the conditions, the yield of the N-methyl derivative varied from 65 to 80%. It was established that changes of the injection port temperature significantly affected the yield of the main product, which appeared to be maximal when the temperature was 200°C.

An increase in the excess of methylating agent relative to 5-phenyl-2,4-oxazolidinedione from 2- up to 10-fold did not affect the yield of the methylation product. However, when the excess of trimethylanilinium hydroxide was considerable the yield of N-methyl-5-phenyl-2,4-oxazolidinedione (retention time = 2.45 min, Fig. 1) decreased and an extra peak appeared on the chromatogram (retention time = 2.20 min), and it corresponded to peak V when an 80-fold excess of TMAH was achieved.

Fig. 2 shows the mass spectrum of N-methyl-5-phenyl-2,4-oxazolidinedione (retention time = 2.45 min) and Fig. 3 that of compound VI which appears with an excess of methylating agent (retention time = 2.20 min).

Comparing these mass spectra, it can be concluded that VI is the result of methylation of V with an excess of trimethylanilinium hydroxide. This is shown by difference between molecular masses of the compounds: V, $m/z = 191$; VI, $m/z =$

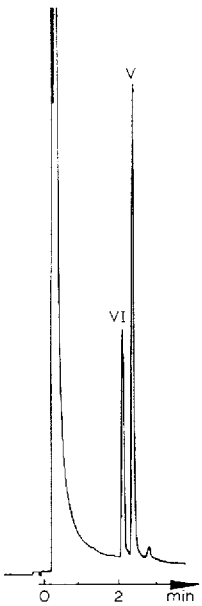

Fig. 1. Gas chromatogram of a solution V with an 80-fold excess of TMAH.

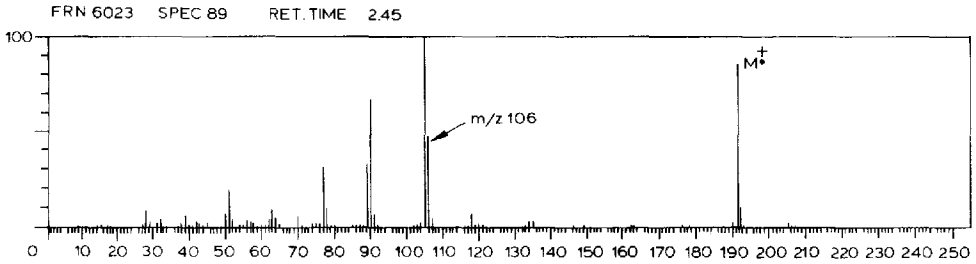

Fig. 2. Mass spectrum at 70 eV of N-methyl-5-phenyl-2,4-oxazolidinedione (V).

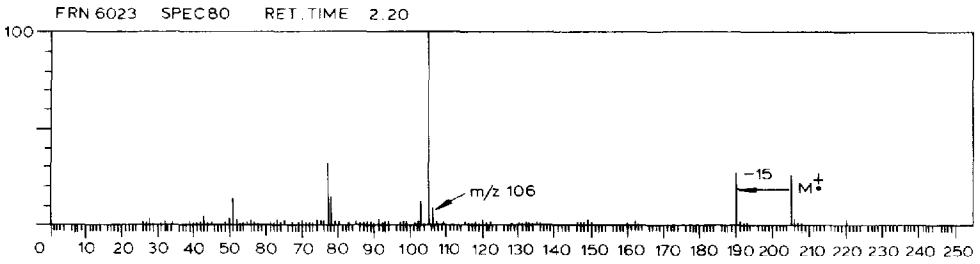

Fig. 3. Mass spectrum at 70 eV of the methylation product of V (VI).

205. There is also an intense ion ($M - 15^+$) in the mass spectrum of VI, which is absent from the mass spectrum of V. Taking into account that in compound V keto–enol tautomerism is highly possible, we can assume that the hydroxy group of the enol form of V can be methylated. It is worth mentioning that the mass spectrum of V the intensity of the ion at *m/z* 106 ($PhCH=O^+$) is medium, and in the mass spectrum of VI the intensity of the ion at *m/z* 106 corresponds only to that of the isotope ion at *m/z* 105.

Thus, the maximum yield of N-methyl-5-phenyl-2,4-oxazolidinedione is obtained when the temperature of the injection port is 200°C and the excess of TMAH is 2–10-fold. Under these conditions the yield of V is 80 ± 3%. It should be noted that the appearance of the peak of VI when a considerable excess (50-fold) of methylating agent is achieved can be utilized as additional information in the identification of pemoline.

When analysing biological samples (*e.g.*, urine), it should be taken in account that there are other compounds that can be methylated and extracted. It was found that when the concentration of pemoline in urine is 1–30 μg/ml, for 5-ml aliquots of urine it is necessary to add 20 μl of 0.2 *M* TMAH.

In order to establish the influence of pH on the extraction of II from urine and on the amount of contaminants registered on the chromatograms, several urine samples with a pemoline concentration of 10 μg/ml were hydrolysed and then extracted at pH 0, 3, 4, 5, 6 and 7 and analysed by GLC. The dependence of the degree of extraction of V on pH was determined from the ratio between the peak areas of V and the internal standard, S_V/S_{st}. An increase in pH from 0 to 5 did not influence significantly S_V/S_{st}. At pH 6 and 7, S_V decreased 2- and 4-fold, respectively. It should be noted that the amount of contaminants on the chromatogram did not change greatly. Thus it is reasonable to extract immediately after hydrolysis at pH 0.

Those endogenous components present in urine and partly detected with the NPD detector (Fig. 4) do not influence the determination of pemoline and the limit of determination of pemoline in the sample is 0.1 μg/ml. The chromatogram of a urine sample containing pemoline at a concentration of 8 μg/ml is shown in Fig. 5. Thus the sensitivity and selectivity of the developed method for the determination of pemoline are similar to those of the known GC (0.5 μg/ml[7], 0.1 μg/ml[6]) and HPLC (0.1–0.05 μg/ml[2] procedures. Moreover, the present method is more rapid than other GC procedures, and it is not necessary to use diazomethane as a derivatization agent.

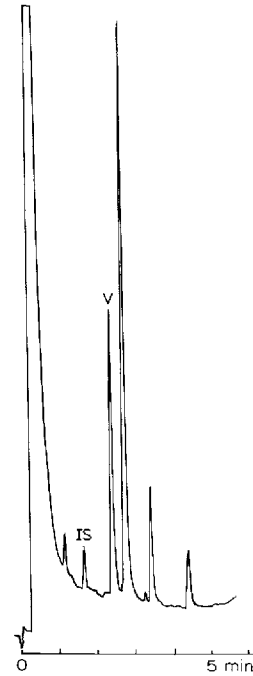

Fig. 4. Gas chromatogram of an extract from 5 ml of pure urine. IS = internal standard (N-methylphthalimide).

Fig. 5. Gas chromatogram of an extract from 5 ml urine containing 8 μg/ml of pemoline.

For the purpose of quantitative analysis using all of the preparation procedures, several urine samples containing pemoline at concentrations of 1–10 μg/ml were prepared and analysed. The calibration graph of S_V/S_{st} *versus* pemoline concentration obtained was linear in this range. The function $y = 0.594x - 0.067$ with a correlation coefficient of 0.99 was obtained using the least-squares method.

The results obtained demonstrated that trimethylanilinium hydroxide is a suitable alkylating agent and can be used to identify pemoline in biological samples with good reliability. The devised procedure was used for the analysis of biological samples from athletes in doping control during the XXII Olympic Games.

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