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Identification of a pyrovalerone metabolite in the rat by gas chromatography–mass spectrometry and determination of pyrovalerone by gas chromatography–nitrogen–phosphorus detection

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Abstract

Pyrovalerone and its hydroxylated metabolite have been identified by gas chromatography–mass spectrometry in rat urine and plasma. A sensitive gas chromatographic method for the quantitative analysis of pyrovalerone in rat urine and plasma is described. The method also permits the quantitative monitoring of the urinary excretion of the drug and its metabolite. Pyrovalerone and its hydroxylated metabolite are detected up to 18 h after a single oral administration to the rat at a dose of 20 mg/kg.

Keywords: Pyrovalerone

1. Introduction

Pyrovalerone, which is not allowed for athletes according to regulations of the International Olympic Committee, is a member of a group of psycho-stimulants [1]. A number of pharmacological studies of the biochemical and physiological effects of pyrovalerone and their mechanisms of action have been reported in the literature [2–14]. However, there have been few reports of the pharmacokinetics of the drug.

Beckett [15] described a method to determine stimulants including pyrovalerone in urine. In a metabolism study in the human, rabbit and mouse performed by Michaelis et al. [16], it was observed that pyrovalerone was readily metabolized by all three species. No unchanged pyrovalerone has been detected in the urine and only one polar metabolite has been formed as a result of oxidation of the aromatic methyl group to a carboxylic acid [16]. After administration of pyrovalerone to the rat at a dose of 20 mg/kg, we have found that the urine and plasma samples contained unchanged pyrovalerone and a metabolite other than that detected by Michaelis et al. [16]. This new pyrovalerone metabolite in the rat is identified by gas chromatography–mass spectrometry (GC–MS). A method using GC with a nitrogen–phosphorus detector for the determination of pyrovalerone and its metabolite in rat plasma is developed and the urinary excretion of unchagned pyrovalerone is monitored. The simultaneous detection of the drug and its metabolite can help to

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Table 1
Gas chromatographic conditions.

Parameter	Condition
Column	HP fused-silica capillary, cross-linked 5% phenyl methyl silicone (SE-54); 16 m× 0.2 mm I.D., film thickness 0.33 μm
Detector temperature	300°C
Initial temperature	100°C
Program rate	20°C/min
Final temperature	300°C
Carrier gas flow-rate	Helium at 1.5 ml/min
Auxillary gas flow-rate	Helium at 20 ml/min
	Hydrogen at 4.0 ml/min
	Air at 100 ml/min
Split ratio	1:7

establish the abuse of pyrovalerone in suspected doping cases.

2. Experimental

2.1. Reagents

Pyrovalerone [1-(4-methylphenyl)-2-(1-pyrrolidinyl)-1-pentanone] and pentazocine [1,2,3,4,5,6-hexahydro- 6,11 -dimethyl- 3 -(3-methyl- 2 -butenyl)-2,6-methano-3-benzazocine-8-ol], which was chosen as the internal standard, were supplied by Eli Lilly (Indianapolis, IN, USA). Methyl iodide was purchased from Aldrich (Milwaukee, WI, USA) and MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) was from Sigma (St. Louis, MO, USA). The following regents were used: diethyl ether, diethylacetone, methanol, *n*-pentane and 2-propanol (Merck, Darmstadt, Germany).

2.2. Stock solution

Standard solutions in methanol of pyrovalerone (1 mg/ml) and pentazocine (1 mg/ml) were prepared by adding 10 mg of the drugs to 10 ml of methanol. The solutions were stored at 4°C and were stable under these conditions for several weeks. Working solutions of 10 and 1 μg/ml were prepared by sequential dilutions.

2.3. Drug administration and sample collection

Male Wistar rats weighing 250–300 g were used. Pyrovalerone was given orally at a dose of 20 mg/kg as a 5 mg/ml aqueous solution to rats. Urine samples were collected at 0 (blank), 5, 12, 13 and 18 h and blood samples were collected at 0 (blank), 0.23, 0.50, 0.75, 1.00, 1.50, 2.50, 4.50 and 8.00 h. Control urine was collected for 12 h before administration.

2.4. Extraction procedure

For each analysis, 0.1 ml of rat plasma and 1.0 ml of rat urine were used. A 10-μl volume of a solution containing 10 μg/ml of pentazocine was added as the internal standard to each sample of plasma or urine in a glass centrifuge tube. A 7-ml volume of *n*-pentane–2-propanol (97:3, v/v) and 100 μl of 5 *M* NaOH were added. The tubes were then stoppered, shaken mechanically for 20 min and centrifuged for 5 min at 750 *g*. The organic layer was transferred to a 15-ml glass centrifuge tube. A 0.5-ml volume of 0.2 *M* HCl was added and the extraction was performed by mixing for 10 min in a mechanical shaker. The solution was centrifuged for 5 min at 750 *g* and the organic layer was aspirated and discarded. The aqueous layer was alkalinized with 5 *M* NaOH to pH 14, reextracted with 7 ml of distilled diethyl ether by shaking for 20 min, and centrifuged for 5 min at 750 *g*. The organic layer was transferred into another tube, evaporated to dryness at reduced pressure, and dissolved in 50 μl of methanol, 3 μl of which was injected into the gas chromatograph.

2.5. Calibration curve and quantitation

A pyrovalerone standard curve was established by adding 1, 5, 25, 50, 100, 250 and 500 ng of pyrovalerone and 100 ng of pentazocine to 0.1 ml of drug-free plasma or 1 ml of control urine. The ratios of the peak areas for pyrovalerone–pentazocine were used to calculate a calibration curve, the slope of which was used in the quantitation of pyrovalerone in the plasma or urine samples.

2.6. Trimethylsilylation

The residues were dissolved in 50 μl of a mixture of CH_3CN–CF_3COOH (60:40, v/v), which contained 200 ppm of methyl orange. MSTFA was added to the solutions until the colour changed from red to yellow. The reaction mixture was heated for 50 min at 80°C and analyzed by GC–MS.

2.7. Methylation

The residues were dissolved in 100 μl of acetone and 50 μl of CH_3I, and 100 mg of K_2CO_3 were added. The reaction mixtures were heated overnight at 60°C and analyzed by GC–MS.

2.8. Gas chromatography

All GC experiments were performed with a Hewlett-Packard (HP) 5890A gas chromatograph equipped with a nitrogen–phosphorus detector (NPD) and connected to an HP 3392A integrator. All injections were made using an HP 7673A auto sampler. GC conditions for the plasma and urine analyses are shown in Table 1.

2.9. Gas chromatography–mass spectrometry

Most of the mass spectra were obtained with a Hewlett-Packard 5890/5970B instrument. The same operating conditions as described above for the GC conditions were also used for GC–MS. Chemical ionization (CI) mass spectra were obtained with a Hewlett-Packard 5890/5988 GC–MS instrument. Methane was used as the chemical ionization reagent gas in the ion source. The electron energy was 230 eV.

3. Results and discussion

3.1. Identification of pyrovalerone and its metabolite in urine

The urinary excretion of metabolites by the rat after oral administration of pyrovalerone is shown in Fig. 1. Unchanged pyrovalerone (peak A) and its metabolite (peak B) were detected in the basic extract of the urine. The metabolite was identified by interpretation of the background-substracted mass spectra of its methylated and trimethylsilylated derivatives. Peak A showed the same retention time as authentic pyrovalerone. The EI and CI mass spectra of the compound corresponding to peak A were identical to those of the authentic pyrovalerone. The EI mass spectrum showed the base peak at m/z 126 and other diagnostic ions at m/z 91, 119, 131 and 202. The CI mass spectrum of peak A gave m/z 246 (M^{+}+1), 274 (M^{+}+C_2H_5) and 286 (M^{+}+C_3H_5) and principal fragment ions at m/z 126 and 202. Peak B had a retention time that was longer than that of pyrovalerone. The EI mass spectrum of the compound corresponding to peak B showed the base peak at m/z 126 and other diagnostic ions at m/z 107, 135, 148 and 218. The CI mass spectrum of peak B gave m/z 262 (M^{+}+1), 290 (M^{+}+C_2H_5), 302 (M^{+}+C_3H_5) and principal fragmentions at m/z 126 and 281. Most of diagnositic ions of peak B had mass 16 a.m.u. higher than the corresponding ions of pyrovalerone. Therefore, the mass spectral data suggest the presence of an additional oxygen atom in the aromatic ring, either as a phenolic hydroxyl group or as an aliphatic hydroxyl group at the benzylic position of pyrovalerone. In general, a phenolic hydroxyl group is easily methylated with methyl iodide but an aliphatic one is not [2]. However, both aliphatic and phenolic hydroxyl groups are trimethylsilylated with MSTFA [2]. The pyrovalerone metabolite was not derivatized with methyl iodide in spite of an overnight reaction, but was derivatized with MSTFA. The chromatographic and mass spectral data of the trimethylsilylated product of the metabolite showed the substitution of a hydroxyl group on the benzylic position of pyrovalerone. The mass spectrum of the trimethylsilyl derivative is shown in Fig. 2. The gas chromatogram of the derivative showed a retention time that

Fig. 1. Gas chromatogram and the electron impact mass spectra of pyrovalerone (peak A) and its metabolite (peak B).

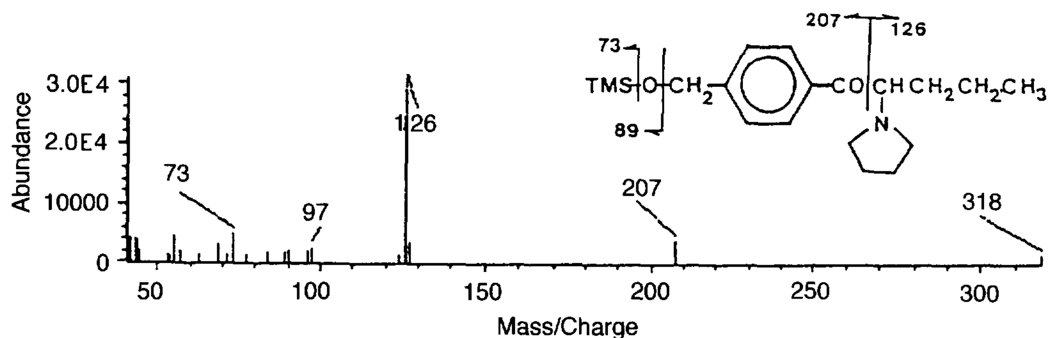

Fig. 2. Mass spectrum of the trimethylsilyl derivative of the metabolite.

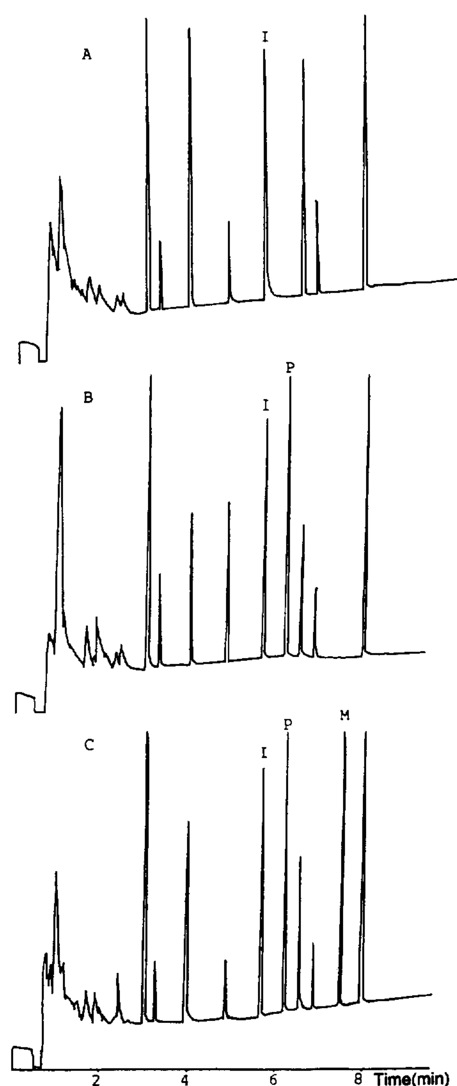

Fig. 3. Chromatograms of the rat plasma extracts. (A) Blank plasma; (B) plasma to which 250 ng/ml of pyrovalerone were added; (C) a plasma sample after an oral dose of 20 mg/kg; I, pentazocine; P, pyrovalerone; M, metabolite of pyrovalerone.

was 1.5 min longer than that of the underivatized metabolite. The mass spectrum showed the characteristic fragment at m/z 318 (M^+-15), due to the loss of a methyl group from the molecular ion and m/z 73 from the trimethylsilyl group. Also, m/z 207 and 126 were formed from fragmentation at the same position as that in the case of pyrovalerone.

3.2. Linearity, precision, detection limit and recovery

The gas chromatogram obtained from a plasma sample to which a known amount of pyrovalerone was added is shown in Fig. 3, together with those obtained from blank plasma and plasma obtained after oral administration. No interfering peaks from endogenous substances were present near the peaks of pyrovalerone and pentazocine.

Standard curves of the drug over a range of 5 to 500 ng/ml were linear. The best fit for pyrovalerone using the internal standard resulted in the equation $y=0.0095x+0.0184$ ($r=0.9998$), where x is the analyte concentration and y is the peak-area ratio. The lower limit of detection in plasma was estimated to be 5 ng/ml in 0.1 ml of plasma. The coefficient of variation of the method at several concentrations is given in Table 2, together with the recovery of pyrovalerone in Table 3. The method was sufficiently precise and sensitive for quantitation of pyrovalerone in biological samples.

Table 3
Recoveries of pyrovalerone added to 100 μl of rat plasma ($n=6$)

Concentration of standard (ng/ml)	Recovery (mean±S.D.) (%)	Coefficient of variation (%)
50	77.9±5.9	12.9
250	85.5±1.2	3.7
2500	86.1±3.5	4.0

Table 2
Precision and accuracy of determination of pyrovalerone in 100 μl of rat plasma ($n=6$)

Concentration added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	Coefficient of variation (%)
50	51± 6	11.8
250	248± 4	1.6
2500	2499±10	4.0

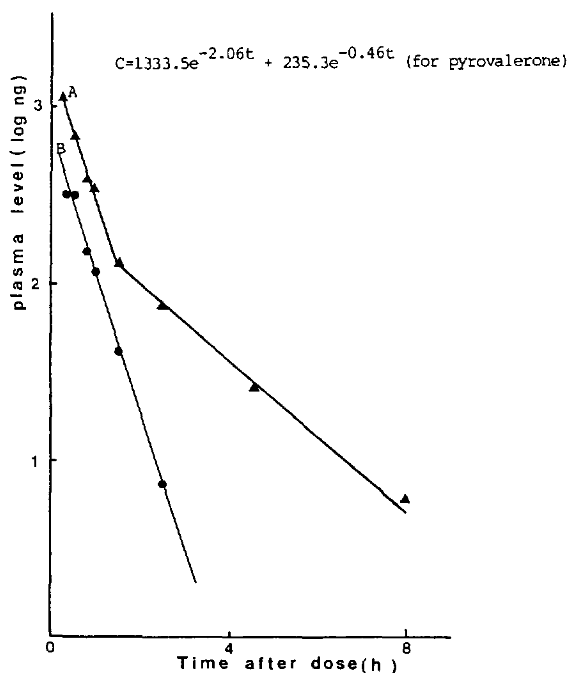

Fig. 4. Plasma levels of pyrovalerone (A) and its metabolite (B) from rats after an oral dose of 20 mg/kg (n=3).

3.3. Elimination of pyrovalerone and its metabolite

This analytical method was used for the pharmacokinetic study of pyrovalerone. The plasma concentration–time curves over 8 h are presented in Fig. 4. The absorption of pyrovalerone was very rapid and the calculated first half-life of pyrovalerone in plasma was 0.34 h and the second half-life was 1.50 h. Also, the calculated half-life of the hydroxylated metabolite in plasma was 0.39 h.

Fig. 5 shows the excretion of pyrovalerone in rat urine. The total amount of pyrovalerone excreted in the urine over 20 h was 0.2% (n=3).

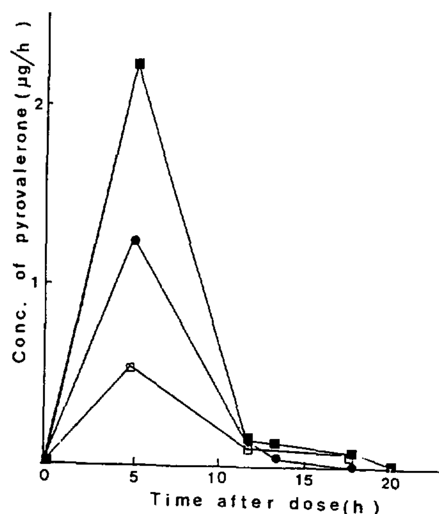

Fig. 5. Urinary excretion of pyrovalerone after an oral dose of 20 mg/kg (n=3).

4. Conclusion

Unchanged pyrovalerone in the rat plasma and urine was identified by comparing the mass spectra and the gas chromatographic behaviour with the corresponding data of the authentic standards. The total amount of pyrovalerone excreted in urine over 20 h was 0.2%. Furthermore, the failure of the pyrovalerone metabolite to react with methyl iodide and the mass spectrum from the trimethylsilylated metabolite suggested the presence of an hydroxyl group at the benzylic position of pyrovalerone.

This procedure has been found to be both sensitive and specific for the analysis of pyrovalerone and its hydroxylated metabolite.

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