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Hemopyrrole and Kryptopyrrole Are Absent from the Urine of Schizophrenics and Normal Persons

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We describe a method for detecting hemopyrrole and kryptopyrrole in urine, with a detection limit of 100 μg/liter (1 part in 10^7). Urine is thoroughly extracted with methylene chloride and the extract is concentrated and examined by gas chromatography and gas chromatography–mass spectrometry. No hemopyrrole or kryptopyrrole could be detected in 52 samples, from 17 controls, 29 schizophrenics, and six persons with acute intermittent porphyria.

Additional Keyphrases: *2,4-dimethyl-3-ethylpyrrole · 2,3-dimethyl-4-ethylpyrrole · synthetic procedures · physico-chemical characterization*

During the past 15 years, the search for metabolic bases of mental disorders has led researchers to examine the biological fluids of various clinical populations. Several groups report finding kryptopyrrole (2,4-dimethyl-3-ethylpyrrole) in the urine of schizophrenics and persons with acute intermittent porphyria (*1–19*). By use of a complex procedure that involves adsorption/desorption from charcoal, extensive chromatography (paper or thin-layer, or both), exposure of sensitive compounds to atmospheric oxygen and acidic conditions, and, finally, spraying with Ehrlich's reagent, kryptopyrrole was assigned as the "mauve factor." It was reported to appear as a five-spot pattern, and to be present in concentrations as great as 9.5 mg/liter (*11*).

Additional reports relating kryptopyrrole to other human physiological conditions make its detection and identification of further interest. In theory, kryptopyrrole can be a by-product of abnormal porphyrin biosynthesis (*10*) and binds with, and thus depletes, pyridoxal phosphate (*13, 18*). Indeed, the presence in urine of a compound presumed to be kryptopyrrole is used as a criterion to the advisability of "mega-vitamin" and pyridoxine treatment (*14, 15, 18*).

Acute intermittent porphyria is marked by gastrointestinal, psychiatric, and neurological symptoms. It would be significant if the enzymically caused porphyric attack could be related to increased kryptopyrrole excretion and psychological disturbance.

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Doubt has recently arisen as to whether or not any kryptopyrrole is actually present in urine. The mauve spot has instead been attributed to a phenothiazine metabolite (*5*), and neither kryptopyrrole nor its isomer, hemopyrrole (2,3-dimethyl-4-ethylpyrrole), was present in the urine of any schizophrenic cases studied (*21*). The authors of the original report now also doubt whether kryptopyrrole is the compound responsible for the "mauve spot" (*20*).

In an attempt to clarify this problem, we have carefully assessed whether hemopyrrole or kryptopyrrole, or both, are present in the urine of schizophrenics or persons with acute intermittent porphyria. Using the purified synthetic compounds in control specimens and gas chromatography and gas chromatography–mass spectrometry for sample analysis, we analyzed urines representative of several human populations.

The considerable quantities of hemopyrrole and kryptopyrrole needed to complete our control experiments necessitated that they be synthesized. Previous syntheses were modified to effect more convenience and higher yields, especially in the case of hemopyrrole.

Materials and Methods

Instrumentation

For the synthetic work, melting points were measured with a "Mel-Temp" apparatus (Laboratory Devices, Cambridge, Mass. 02139) and are uncorrected; ultraviolet visible spectra were taken with a Cary 14 spectrophotometer; ^{1}H NMR spectra were taken on a Varian T-60 (Varian Instrument Division, Palo Alto, Calif. 94351) with internal tetramethylsilane; and mass spectra were taken with Varian Model M-66, MS 12, or CEC 110 spectrometers. Elemental analyses were done by the Analytical Laboratory of this department.

Preparative gas chromatography was done with an Aerograph Autoprep A-700 with glass injector insert, a 152 cm × 4 mm (i.d.) glass column packed with 5% SE-30 on Chromosorb W, 60–80 mesh (acid-washed/dimethylchlorosilane-treated), with He carrier gas at 50 ml/min, reduced to about 30 ml/min during collection; column temp. 110 °C; injector temp. 165 °C. Retention times were 3.5 min for hemopyrrole, 3.4 min for kryptopyrrole.

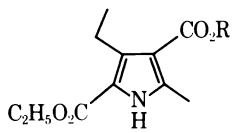
Analytical gas chromatography was done with a Hewlett-Packard 402 Chromatograph with an all-glass

system: 102 cm × 3 mm (i.d.), 3% XE-60 on Chromosorb W, 100–120 mesh (acid-washed/dimethylchlorosilane-treated); He at 60 ml/min; column temp. 100 °C; injector temp. 175 °C, detector temp. 175 °C. Retention times were: for kryptopyrrole, 12.3 min, for hemopyrrole, 14.1 min; at column temp. 125 °C, these times were 5.3 and 5.9 min, respectively.

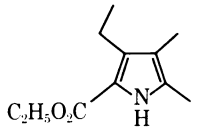
Computerized gas chromatographic/mass spectrometric analyses were done with a double-focusing Du Pont 21-492B Mass Spectrometer interfaced with a Varian 204-1B Gas Chromatograph with a linear temperature programmer. The mass spectral data were acquired and processed with a Du Pont 21-094 data system. The conditions used were as follows: 610 cm × 7.6 mm (i.d.) column, 10% XE-60 on Chromosorb W 100–120 mesh (acid-washed/dimethylchlorosilane-treated); He flow, 6 ml/min; temperature, isothermal at 100 °C for 1 min, then linear temperature increase to 200 °C at 6 °C/min. Mass spectrometry: total effluent, low resolution at 133×10^{-6} Pa (1×10^{-6} Torr) source pressure, 200 °C source temperature at 70 eV; retention times for krypto- and hemopyrrole were 11.0 and 11.5 min, respectively.

Syntheses

2,4-Diethoxycarbonyl-3-ethyl-5-methylpyrrole (**1**). This was synthesized according to Ellis et al. (*22*). Treatment of **1** with 100% sulfuric acid gives the pure β-acid (**2**) in 71% yield. Diborane reduction of **2** introduces the β-methyl group in **3** cleanly (82% yield) and hemopyrrole (**4**) is produced after treatment of **3** with NaOH (2.5 mol/liter) overnight at 165 °C in a sealed tube.



1, R = C_2H_5
2, R = H



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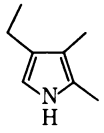

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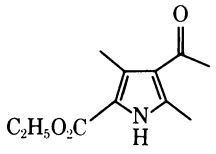

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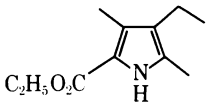

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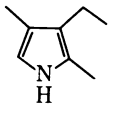

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Kryptopyrrole (**7**). The β-acetylpyrrole (**5**) was prepared (85% yield) according to Fischer and Orth (*23*), except that 1.65 mol of crystalline ethyl 2-oximino-3-oxobutanoate (*24*) per 1.00 mol of ethyl 3-oxobutanoate was used in the Knorr condensation. Reduction to the β-ethylpyrrole (**6**) with diborane (92% yield) was followed by an analogous NaOH sealed-tube reaction as for hemopyrrole to give **7** (80% yield).

2,4-Diethoxycarbonyl-3-ethyl-5-methylpyrrole (**1**) was prepared according to Ellis et al. (*22*). The yield was 54%; mp 116–117 °C.

4-Carboxy-2-ethoxycarbonyl-3-ethyl-5-methylpyrrole (**2**). To 292 ml of concd. H_2SO_4 was added 68.4 ml of H_2SO_4/15% SO_3, with stirring. After the temperature fell to 30 °C, 178 g (0.70 mol) of **1** was added during 5 min, so that the temperature remained below 40 °C. The flask was transferred to an oil bath at 40 °C for 19 min, and the resulting solution was poured onto 550 ml of crushed ice, left overnight, and then filtered. The crude solid was washed with diethyl ether and air-dried to give 150 g (95% yield) of the pyrrole acid, which still contained some starting diester detectable by thin-layer chromatography (ethyl acetate/benzene, 1/9 by vol; R_F of product 0–0.24, of starting material, 0.47). Starting diester was removed by digestion with boiling methanol to leave 112 g (71%) of pure pyrrole ester acid (**2**): mp 245–255 °C; NMR (DMSO-d_6) δ 11.70 (s, br, 2H), 4.26 (q, J = 7, 2H), 3.03 (q, J = 7, 2H), 2.43 (s, 3H), 1.30 (t, J = 7), 1.05 (t, J = 7, 6H); mass spectrum *m/e* (%BP): 226 (7), 2-5 (54, M^+), 210 (10), 196 (31), 182 (20), 180 (18), 179 (21), 178 (100), 164 (41), 162 (23), 161 (12), 160 (65), 124 (45), 133 (26).

2-Ethoxycarbonylhemopyrrole (**3**). To 300 ml of diglyme was added 20.9 g (0.55 mol) of $NaBH_4$ and the mixture was stirred and finally cooled in an ice bath. Slowly, during 165 min, 85 g (0.6 mol) of methyl iodide was added, then 45 g (0.2 mol) of **2** was added in one portion and the mixture was stirred, with ice cooling. After 120 min, methanol was added to the reaction mixture until no more hydrogen was evolved, the solvent was evaporated, and the residue was partitioned between diethyl ether and water, and the aqueous phase washed with two additional portions of ether. The ether washes were combined, washed once with water, dehydrated over $MgSO_4$, filtered, and evaporated. The residue, on crystallization from ethanol/water, gave a 32 g (92%) yield of 2-ethoxycarbonylhemopyrrole: mp 97–98 °C; λ_{max}^{EtOH} (A_{rel}) 284 (1.00), sh 246 (01313); NMR (C^2HCl_3) δ 9.00 (s, br, 1H), 4.31 (q, J = 7, 2H), 2.75 (q, J = 7, 2H), 2.19 (s, 3H), 1.96 (s, 3H), 1.33 (t, J = 7), 1.12 (t, J = 7, 6H total).

Hemopyrrole (**4**). Into a 250-ml tube that was being flushed with nitrogen, we placed 19.5 g (0.1 mol) of **3**, 200 ml of deoxygenated water, and 20 g of sodium hydroxide. The tube was sealed, placed at 165 °C in an oven for 13 h, cooled, and opened in a darkened room. Under a nitrogen atmosphere throughout, the contents were extracted twice with 40-ml portions of ether, and the ether was dehydrated over Na_2SO_4, filtered, and evaporated. The residue, on distillation, gave 8.7 g (71%) of pure hemopyrrole with a bp of 92 °C at 1.6 kPa (12 mmHg). On gas chromatography on a 305 cm × 6 mm (i.d.) column of 5% OV-17 (column temp. 200 °C, helium flow 40 ml/min), the retention time for hemopyrrole was 187 s, for 2-ethoxycarbonylhemopyrrole 548 s. NMR (CCl_4): δ 7.00 (s, br, 1 H); 6.09 (d, J = 3, 1H), 2.33 (q, J = 7, 2H), 2.02 (s, 3H), 1.87 (s, 3H), 1.12 (t, J = 7, 3H); mass spectrum *m/e* (%BP) 124 (3), 123 (37), 122 (9), 108 (100), 107 (7), 100 (8), 94 (7), 77 (4), 39 (11).

3,5-Dimethyl-2-ethoxycarbonyl-4-ethylpyrrole (**6**). To 35.7 g (0.94 mol) of $NaBH_4$ dissolved in 1 liter of diglyme was added 87.6 g (0.42 mol) of **5** and the solution was cooled to 5 °C in an ice/salt bath. Methyl iodide

(133.8 g, 0.94 mol) was added during 3 h, while keeping the temperature at 10 °C. The mixture was then warmed to room temperature, stirred for 4 h, and then cooled below 15 °C as 550 ml of cold 0.6 mol/liter HCl was added. The aqueous solution was extracted twice with diethyl ether, which was washed with saturated aqueous NaCl, dehydrated over $MgSO_4$, and evaporated. The residue, on crystallization from ethanol/water, yielded 75 g (92%) of product: mp 87.5–88 °C. mass spectrum *m/e* (%BP) 196 (14), 195 (100) [M^+], 181 (10), 180 (79), 149 (25), 134 (11), 133 (98); NMR (C^2HCl_3) δ 4.30 (q, 2); *ca.* 2.40 (q), 2.25 (s), 2.18 (s, 8H total); 1.33 (t), 1.07 9t, 6H total).

Kryptopyrrole (**7**) (*25*). In a 250-ml tube that had been flushed with nitrogen we combined 19.5 g (0.1 mol) of **6**, deoxygenated water (200 ml), and sodium hydroxide (20 g). The tube was sealed, placed in an oven at 165 °C for 14 h, cooled, and opened in a darkened room. Under a nitrogen atmosphere, the contents were twice extracted with diethyl ether (100 ml, 75 ml), and the ether was dehydrated over Na_2SO_4 and evaporated. The residue, on distillation [bp 97–98 °C at 2.8 kPa (21 mmHg)], gave kryptopyrrole (9.89 g, 80%): NMR (C^2HCl_3) δ 7.07 (s, br, 1H), 6.08 (d, J =2, 1H), 2.25 (q, J = 7.5, 2H), 2.15 (s, 3H), 1.85 (s, 3H), 0.93 (t, J = 7.5, 3H).

Sampling Procedure

Urine samples were collected in 140-ml polypropylene specimen bottles and frozen immediately in solid CO_2 ("J Ward" samples) or placed in a freezer and frozen within 30 min and then transferred to solid CO_2 within 2 h ("ITRS" samples). Use of an ethanol/solid CO_2 slush shortens freezing time to 15 or 20 min. The samples were then sequentially defrosted by immersion in a 27 °C water bath, and extracted two at a time. Each sample was washed three times with 50 ml of CH_2Cl_2, suction filtering after each washing to break the emulsion. Filtering varied with the amount of particulate matter in each washing. Generally, no. 202 coarse paper (Reeve Angel; Whatman Inc., Clifton, N.J. 07014) was used for filtering the first washing and Whatman no. 1 paper for the next two washes. These washes were combined and refiltered through Whatman no. 1 paper, after first discarding the aqueous layer. This was repeated with use of Whatman no. 2 paper until the emulsion was dispersed. The filtrate was again separated and the organic layer was placed in a dry 1-liter round-bottom flask and rotary-evaporated to 100 μl. This extract was used for gas-chromatographic injection, and was evaporated further under nitrogen if the presence of a suspect peak determined the need for gas chromatography–mass spectrometry.

The above procedure was developed as a result of control studies with use of known amounts of pure, synthetic hemo- and kryptopyrrole collected by preparative gas chromatography. Just before use, each portion of the compounds was subjected to gas chromatography–mass spectrometry to verify its identity. The following observations were made regarding the integrity of hemopyrrole or kryptopyrrole on handling:

A. A standard solution of kryptopyrrole in CH_2Cl_2, 50 mg/liter or 1 g/liter, can be stored under nitrogen at −10 °C for a few weeks. Hemopyrrole is somewhat more sensitive, but losses of either pyrrole can be minimized by sealing the volumetric flask with Parafilm.

B. Urine that has been fortified to 1 mg/liter with a standard solution of hemopyrrole and kryptopyrrole (1 g/liter, in CH_2Cl_2), frozen at −10 °C (freezer compartment) and extracted 22 h later shows only 10% recovery as compared to that from urine fortified to the same concentration and extracted immediately. When 1.0 mg of hemopyrrole or kryptopyrrole per liter of urine is left in the refrigerator for 24 h at 4 °C and then extracted, none is detectable.

C. Before urine samples were to be collected, a 200-ml sample of standard urine at 37 °C was divided into two equal portions. To one half was added 50 μg of hemopyrrole or kryptopyrrole as a standard solution (CH_2Cl_2), and both halves were frozen on solid CO_2. It was shown that up to 60% of the hemopyrrole or kryptopyrrole could be maintained for as long as 24 h if the sample was stored under nitrogen and on solid CO_2. The controls were extracted last, to ensure that the hemopyrrole or kryptopyrrole had survived over the time it took to do the extractions of that day.

D. The lowest concentration of hemopyrrole or kryptopyrrole-fortified urine that we attempted to analyze was 0.1 mg/liter after the specimen had been frozen (−10 °C) for 12 h. The gas-chromatographic trace of the concentrated urine extract shows that the detection limit might be improved by at least a factor of two to three, but we did not pursue this in view of the reported amounts of hemopyrrole of kryptopyrrole in urine (*11*) and our results.

Results

In a previous report (*21*), we presented a method for unequivocal identification of hemopyrrole or kryptopyrrole in urine in concentrations as small as 1 mg/liter (1 part/10^6). That method was applied to 20 normal persons and 16 schizophrenics, and no hemopyrrole or kryptopyrrole was found in their urine.

We now have greatly simplified that method, and increased its sensitivity 10-fold, down to 100 μg/liter (1 part/10^7) with complete certainty and to 30 μg/liter with reasonable confidence, and applied it to a larger population (17 normals, 29 schizophrenics, and six persons with acute intermittent porphyria). Again, we could find neither kryptopyrrole nor hemopyrrole in any of the urines. Because these compounds evidently are absent from urine of schizophrenics, tests for their presence cannot be used diagnostically.

Discussion

This conclusion is in direct conflict with several reports in the literature, and an evaluation of, and possible explanations for, the differences may be of value. Primarily, the reason for the difference lies in the analytical

method. Our method completely eliminates any artifacts, because we are directly isolating and analyzing for kryptopyrrole and hemopyrrole rather than for any derived compounds. The chromatographic methods used by others (e.g. *6–9, 11–14*) are ambiguous, because alkylpyrroles clearly are unstable under the conditions of the analysis, and five spots are obtained from a presumed single compound. A low detection limit (~4 μg/liter) has been claimed (*19*) for this technique, but obviously quantification must remain equivocal. Considering the great instability of polyalkylpyrroles to light, oxygen, and oxygenases (*26–28*), persistence of these compounds in the urine at such low concentrations seems unlikely (*20*). On other occasions, mass spectral analysis has been used (*6,7*) but has consisted merely of assigning fragment ions. Since no prior purification was used, these fragment ions tell us nothing about purity or identity, and certainly convey no quantitative information.

Thus we question both the qualitative and quantitative aspects of these methods. Based on the work presented here and an evaluation of the previous methods, we conclude that no evidence exists for the occurrence of hemopyrrole or kryptopyrrole in urine of either normal persons or schizophrenics.

We are greatly indebted to Dr. Paul Philp and Mr. Steven Brown (Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory) for their comprehensive gas chromatographic–mass spectrometric analysis of our samples. We thank Dr. Ira Glick and the entire staff of the Inpatient Treatment and Research Service (ITRS) at the Langley Porter Neuropsychiatric Institute, San Francisco, for collecting samples. The staffs of I- and J-Wards at Contra Costa Community Hospital also greatly facilitated our collection. We express our appreciation to Dr. D. Montgomery Bissel of UCSF for his assistance in obtaining porphyritic urine. Lastly, our gratitude to all the students, schizophrenics, and acute intermittent porphyritics who enthusiastically donated specimens for our experiments.

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