animals. In view of the unusual structure, however, this assignment should be considered tentative.

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Keyphrases

Thiazesim metabolism—*in vivo*, *in vitro*
Metabolites, thiazesim—isolated, identified
Liver, rats—*in vitro* metabolism
TLC—separation, identification
Column chromatography—separation
NMR spectroscopy—structure
Mass spectroscopy—structure
UV spectrophotometry—structure

Nonpolar Metabolites of Trifluoperazine in Rats

By C. L. HUANG and K. G. BHANSALI*

Urinary excretion of nonpolar metabolites of trifluoperazine was studied in six albino rats which were administered intraperitoneally with 5 mg. trifluoperazine dihydrochloride daily for a period of 5 days. The 24-hr. urine specimens were collected and analyzed for trifluoperazine sulfoxide and the unchanged drug by means of a spectrophotometric and thin-layer chromatographic techniques. The average urinary excretion of trifluoperazine sulfoxide was found to be 10.77% of the administered dose in the first 24-hr. period. Only a trace or a negligible amount of the unchanged drug was found in urine.

TRIFLUOPERAZINE (TOP) is one of the widely used neuroleptics today. During the past decade TOP has become established as an effective neuroleptic agent. Many clinical reports have appeared attesting to its efficacy in the treatment of certain types of psychotic symptomatology (1–12). TOP, a member of the phenothiazine family, is approximately 10 times more potent than chlorpromazine and is reported to have fewer side reactions. It differs from chlorpromazine by possessing a trifluoromethyl group on position 2 and a piperazinyl function on the propyl side chain. Comparative clinical studies (13–18) have indicated differences in the sedative effect, potency, rapidity of action, and incidence of neurological complications between TOP and other neuroleptics; however, factors responsible for these differences are as yet largely unknown. Despite a large number of clinical investigations reported in the past years, little is known as to its metabolic pathway. Because of the relatively small dosage level, metabolic study of this compound presents a formidable problem especially in man. It would be of interest to study the metabolic pathway of this relatively potent drug and compare it with that of chlorpromazine. Such comparative data might help to explain the question as to why this compound is more potent and has less side effects than chlorpromazine. A survey of the literature revealed that there is little information available regarding the metabolism of TOP. A rapid color test utilizing ferric chloride in mineral acids to detect urinary TOP metabolites was reported (19, 20).

The present investigation is focused on identification and quantitative determination of nonpolar metabolites of TOP in urine of rats. Thin-layer chromatography and paper partition chro-

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matography were utilized to elucidate metabolites in this study.

MATERIALS AND METHODS

Six albino rats weighing 150–200 g. were selected. They were administered intraperitoneally with 5 mg. of TOP dihydrochloride in saline solution daily for a period of 5 days. The animals were placed in individual metabolic cages with free access to food and water. Daily 24-hr. urine specimens were collected and analyzed by means of a Beckman DU spectrophotometer.

On the first day of the experiment, all rats were sedated and remained motionless most of the time but were easily aroused by a light touch on their fur. Beginning the second day, animals showed a lighter degree of sedation and were able to feed and to drink water.

Chromatography of Reference TOP—Reference TOP dihydrochloride was dissolved in normal rat urine. An aliquot of 0.1 ml. was placed on a thin-layer plate and the chromatogram was developed in a solvent system, acetone–methanol (1:1) for a period of 20 min. The developed chromatogram was dried and sprayed with uranyl nitrate reagent in concentrated hydrochloric acid (19). A bluish-purple spot with R_f 0.5 was observed. The sensitivity of this reagent to TOP is 5 mcg. in 10-ml. urine specimen.

Synthesis of TOPO—A reference TOPO was synthesized according to the following procedure. TOP dihydrochloride (1 g.) was dissolved in water and hydrogen peroxide (0.3 ml., 30%) was added. The mixture was left overnight at room temperature. Then the mixture was evaporated to dryness *in vacuo* and the residue dissolved in 2 ml. methanol. The methanol solution was chromatographed on Whatman 3 MM paper by a linear chromatographic technique in a solvent system, ethanol–butanol–water (2:5:2) to a height of approximately 30 cm. Then the chromatogram was processed according to the guiding-strip technique below (21). The chromatogram was dried in the air and about 3-cm. strips from both edges were cut and sprayed with 50% sulfuric acid. Three colored bands, namely, light orange (R_f 0.8), orange (R_f 0.7), and pink (R_f 0.6) were observed. The acid-sprayed paper strips were covered with a thin plastic sheet (Saran wrap) and placed back to the original chromatogram. Using these colored strips as a guide, the chromatogram was marked with a lead pencil and cut into three fractions. The accumulated fraction of each band above was extracted several times with methanol in a Waring blender. The combined methanol extracts were evaporated to dryness and the residue recrystallized from methanol to an amorphous powder. From the physical properties, the second band above was identified to be TOPO (m.p. 173–177°, $\lambda_{max.}$, 235, 277, 305 mμ). The first and the third bands were identified to be unchanged TOP and TOP-sulfone, respectively.

Identification of TOP and TOPO in Urine—A urine specimen (50 ml.) from rats administered with TOP dihydrochloride was adjusted to pH 10, and extracted three times with equal volume of methylene dichloride. The combined methylene dichloride extracts were filtered through a layer of anhydrous sodium sulfate and the solvent was removed *in vacuo*. The residue was dissolved in 1 ml. methanol and an aliquot of 0.1 ml. was placed on a thin-layer plate (Chromagram, Eastman). The chromatogram was developed in a solvent system, acetone–methanol (1:1) for 20 min. to a height of about 15 cm. The developed chromatogram was dried in the air and sprayed with uranyl nitrate reagent in concentrated hydrochloric acid. Two spots, with a bluish-purple color (R_f 0.5) and a pink color (R_f 0.4) were observed. They were identified to be the unchanged TOP and TOPO, respectively, from the color reaction, R_f value and cochromatogram with reference TOP and TOPO. The ultraviolet absorption spectra of TOP and TOPO isolated from rat urine are shown in Fig. 1.

Construction of Standard Curves of TOP and TOPO—A series of dilutions of TOP and TOPO in normal rat urine was prepared. An aliquot (10 ml.) of each dilution was adjusted to pH 10 and extracted three times with a 10-ml. portion of methylene dichloride. The combined methylene dichloride extracts were dried over anhydrous sodium sulfate and evaporated to dryness under a reduced pressure. The residue was taken up in 1 ml. methanol and the methanol extract was applied linearly on 10 × 20 cm. thin-layer plate (Chromagram, Eastman). The chromatogram was developed in a solvent system, acetone–methanol (1:1) for 20 min. to a height of about 15 cm. The chromatogram was air dried and scanned under ultraviolet lamps (Mineralight, UVS-11 and UVL-21) to locate the spots. The silica gel support of the chromatogram including the spot was scraped into a container and extracted with methanol (3.5 ml.). The methanol extract was centrifuged and the supernatant liquid was transferred into a cell. The absorbance was recorded in a Beckman DU spectrophotometer at 262 mμ for TOP and 277 mμ for TOPO. The absorbances of both TOP and TOPO followed Beer's law.

Quantitative Determination of TOP and TOPO in Urine—An aliquot (10 ml.) of a 24-hr. urine specimen was measured and analyzed for TOP and

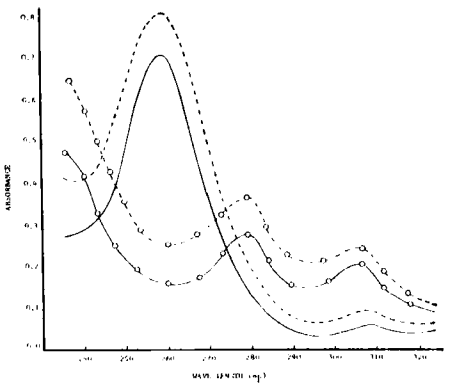

Fig. 1—Ultraviolet absorption spectra of trifluoperazine (TOP) and trifluoperazine sulfoxide (TOPO). Key: ——, reference TOP dehydrochloride; --- TOP isolated from rat urine; O—O, reference TOPO dihydrochloride; O--O, TOPO isolated from rat urine

TABLE I—URINARY EXCRETION OF TRIFLUOPERAZINE SULFOXIDE IN RATS[a]

Rat	Day 1	Day 2	Day 3	Day 4	Day 5
1	0.400	0.206	0.354	0.224	0.213
2	0.832	1.520	0.395	0.266	died
3	0.486	0.304	0.186	0.196	0.187
4	0.364	0.292	0.236	0.248	0.292
5	0.353	0.318	0.218	died	—
6	0.306	0.246	0.227	0.208	0.200
Av. mg. excretion	0.457	0.419	0.269	0.228	0.223
Av. % excretion of the administered dose	10.77	9.89	6.35	5.37	5.25

[a] Five milligrams trifluoperazine dihydrochloride (equivalent to 4.24-mg. base) was administered intraperitoneally for a period of 5 days. Absorbance was recorded in a Beckman DU spectrophotometer at 277 mμ.

TOPO according to the procedure described above for the construction of standard curves. The concentrations (mcg./ml.) were calculated with the factor obtained from the standard curves. The recovery data of the urinary TOPO are shown in Table I.

RESULTS

Six albino rats injected intraperitoneally with 5 mg. (4.24-mg. base) of TOP dihydrochloride were maintained for at least 5 days. Two rats died, one on the fourth day and the other on the fifth day. Average urinary excretion of TOPO on the first day and the second day was found to be 10.77 and 9.89% of the administered dose, respectively. Thereafter, the average daily excretion was almost constant being between 6.35 to 5.25% (22).

In contrast to the relatively high rate of sulfoxide excretion, the unchanged TOP in urine was found to be negligible or only trace which indicated almost complete metabolism of this drug. Preliminary information indicated that the intraperitoneally administered TOP was also excreted in the feces (23–25).

The mother liquor of the urine specimens after the methylene dichloride extraction showed a strong purple color reaction with 50% sulfuric acid. This suggests the presence of polar metabolites such as hydroxides and/or glucuronides of TOP which are not extractable in methylene dichloride.

Thin-layer chromatography coupled with an ultraviolet spectrophotometric technique offers a simple yet sensitive method for a quantitative assay of urinary metabolites of TOP. The sensitivity of this method is 0.7 mcg. in 1-ml. urine specimen. Normal urine components such as urea, creatinine and bile acids did not interfere with the assay of these TOP metabolites because none of these compounds have UV absorption peaks or R_f values in the area where the TOP metabolites are found.

CONCLUSION

TOP dihydrochloride (5 mg.) was injected intraperitoneally to six albino rats daily for a period of 5 days. Twenty-four hour urinary excretion of the nonpolar metabolites, namely, TOPO and the unchanged TOP, were determined spectrophotometrically in a Beckman DU spectrophotometer following linear chromatography on a thin-layer plate.

TOPO was found to be the major nonpolar metabolite in the urine with an average excretion of 10.77 and 9.89% in the first 2 days, respectively. Only a trace or a negligible amount of the unchanged trifluoperazine was found in the urine specimens indicative of almost complete metabolism of this drug in rats at this dosage level (5 mg./rat).

Preliminary study on other metabolites indicated that there is a considerable amount of polar metabolites present in urine after being extracted with methylene dichloride. They are speculated to be hydroxides and/or glucuronides of TOP.

An attempt was also made to isolate the nonpolar metabolites by paper chromatographic technique. However, it did not prove to be a satisfactory one for a spectrophotometric determination of TOP and TOPO as interference of the chromatographic paper in the methanol extract was noticed.

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Keyphrases

Trifluoperazine metabolites—nonpolar
Urinary excretion, rats—trifluoperazine metabolites
TLC—separation, identity
Paper chromatography—separation, identity
UV spectrophotometry—analysis

Further Applications of Pentachlorophenyl Active Esters in Lengthening Peptide Chains from C- and N-Terminal Amino Acids Using Dicyclohexylamine C-Protection

By A. KAPOOR, E. J. DAVIS, and MARY J. GRAETZER

In order to eliminate the problems associated with the use of alkali during peptide synthesis, a systematic investigation was carried out to lengthen peptide chains by coupling N-protected pentachlorophenyl active esters of amino acids and peptides with amino acids and peptides, C-protected by dicyclohexylamine. There was an appreciable increase in yields when N-protected pentachlorophenyl active esters of amino acids were coupled with di- and tripeptides instead of single amino acid units C-protected by dicyclohexylamine. From this, it was concluded that peptide chains would be lengthened more profitably from C-terminal instead of N-terminal amino acid residues when the synthesis of peptides is carried out, using pentachlorophenyl active esters in combination with dicyclohexylamine C-protection. In addition to affording relatively better yields, this approach would further limit the degree of racemization as the active ester component used would always be a monomer.

THE SYSTEMATIC SEARCH for suitable "activated" esters, for the synthesis of peptides *via* the aminolysis of esters started with the historic paper of Wieland and Bernhard, when they reported the synthesis of peptides *via* the phenyl thiolesters (1). For more than a decade various active esters have been used extensively in the synthesis of peptides and polypeptides with known sequence of amino acids (2–5). The pentachlorophenyl active esters, which were first reported in the literature in 1961 (6), afford an excellent method for lengthening the peptide chain. The pentachlorophenyl active esters have the following advantages: (*a*) they are one of the most active esters (7), (*b*) they are generally higher melting compounds than other active esters, which leads to their easy crystallization and purification (8), (*c*) they are conveniently prepared without any significant racemization by *N*,*N*′-dicyclohexylcarbodiimide (DCC) method (9), (*d*) they are stable to controlled hydrogenation conditions and make an excellent combination with *N*-carbobenzoxy and *tert*-butyl protecting groups when the incorporation of trifunctional amino acids in the peptides is desired (8). Previously, the peptide chain was lengthened by coupling pentachlorophenyl active esters of *N*-carbobenzoxy amino acids or peptides with C-methyl protected amino acids or peptides. C-methyl protection at each activation stage and at the end of the synthesis was removed by saponification (8, 10–12). Alkali treatment of peptides is usually associated with a number of problems, *e.g.*, racemization (13), transpeptidation (8, 14), *etc.* In addition, removal of C-methyl protection by alkali becomes more difficult as the number of amino acids increases in

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