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SEPARATION AND QUANTITATION OF *cis*- AND *trans*-THIOTHIXENE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A high-performance liquid chromatographic procedure is described for the separation of *cis* and *trans* isomers of thiothixene, a thioxanthene derivative used as an antipsychotic agent. A radial compression module (RCM-100) was used with both silica and cyanopropyl cartridges. A fixed-wavelength UV detector (254 nm) was used in these studies for quantitation. Mesoridazine is used as an internal standard because of its separation characteristics and reproducible quantitation. C_{18} Sep-Pak cartridges are used for biological sample clean-up. Plasma samples from patients treated with thiothixene (Navane) were assayed for *cis* and *trans*-thiothixene. No *trans*-thiothixene was detectable and *cis*-thiothixene concentrations ranged from 0 to 22.5 ng/ml.

INTRODUCTION

Thiothixene (Navane®; N,N-dimethyl-9-[3-(4-methyl-1-piperazinyl)-propylidene]-thioxanthene-2-sulfonamide) is a low-dose antipsychotic agent of the thioxanthene class. It is now recognized that monitoring plasma levels of anti-

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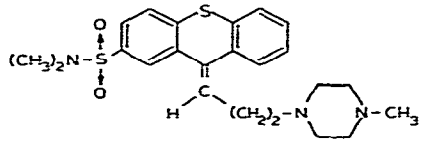
psychotic drugs and their metabolites in some cases aids the evaluation of the clinical response to the drug. A very sensitive method is required in view of the low concentrations found in plasma samples (4—20 ng/ml). Further, thiothixene (TTX) exists in two isomeric forms, *cis* (I) and *trans* (II), the *cis* being



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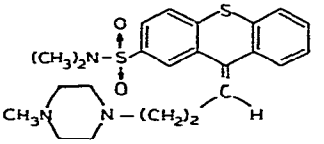

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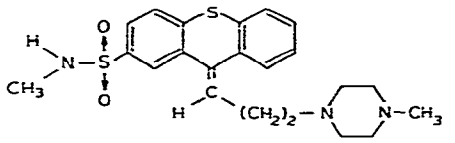
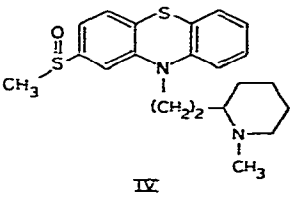


III



IV

the active antipsychotic and the *trans* being inactive. It is believed that differences in clinical response between patients treated with TTX may be due to different concentrations of the *cis* and *trans* forms of the drug produced by isomerization of the *cis* form in vivo. In order to verify this hypothesis the analytical methods should be capable of separating the *cis* and *trans* forms. In addition, N-desmethylthiothixene (III) is a major metabolite [1] and there are no published reports for its quantitation.

Previous methods of analyses used spectrofluorometry [2] and gas chromatography—mass spectrometry (GC—MS) [3, 4]. The spectrofluorometric method is sensitive but not specific since it cannot distinguish between the *cis* and *trans* isomers and is susceptible to interference by metabolites. Hobbs et al. [3] used a GC—MS method with electron impact ionization and selected ion monitoring. C^2H_3-thiothixene was used as internal standard and they monitored the base peak at *m/z* 113 for TTX and *m/z* 116 for the internal standard. This method also did not distinguish the *cis* and *trans* isomers.

Bombardt and Friedel [4] improved the sensitivity of the CG—MS procedure by using methane chemical ionization and monitoring the [M + 1] ion of TTX (*m/z* 444) and the C^2H_3-internal standard (*m/z* 447). They described a GC procedure to separate the *cis* and *trans* isomers and demonstrated that patients show varying capacities to convert the active *cis* isomer to the relatively inactive *trans* form. Drawbacks of this procedure include the expense and expertise needed for the routine assay of clinical samples by GC—MS and the inconsistency of *cis* and *trans* separation by GC due to thermal rearrangement.

The purpose of this investigation was to develop a means of measuring *cis*-TTX in human plasma that is simple, sensitive and specific. We decided to

develop a high-performance liquid chromatographic (HPLC) method for the separation and quantitation of *cis*- and *trans*-TTX and validate the results by the more specific GC—MS method. The only LC methods reported in the literature for TTX were for quantitation in pharmaceutical formulations [5]. Li Wan Po and Irwin [6] reported the separation of *cis—trans* isomers of tricyclic neuroleptics, fluopenthixol, clopenthiol and chlorprothixene but could not separate TTX isomers. In our present work, one aim was to compare different LC columns, solvent phases and detector conditions for the quantitation of *cis*-TTX in plasma samples. In addition, we developed an extraction method and found a suitable internal standard, mesoridazine (MSZ, IV).

MATERIALS AND METHODS

Reagents

Ethyl acetate, hexane, isopropanol, methanol and acetonitrile were distilled in glass, LC-grade (Burdick and Jackson, Muskegon, MI, U.S.A.). Triethylamine was 99% pure (Aldrich, Milwaukee, WI, U.S.A.). Ammonium hydroxide was ACS reagent grade 28%. All water used was deionized reagent quality. All other chemicals were analytical grade.

2 *N* NaOH was diluted from stock 10 *N* NaOH (Fisher Certified, Fisher Scientific, Pittsburgh, PA, U.S.A.). 0.05 *N* NaOH was diluted from 2 *N* NaOH. 0.6 *M* NaH_2PO_4 was prepared by dissolving 8.28 g of NaH_2PO_4 in 100 ml of water. 8.5% H_3PO_4 was prepared by diluting 85% phosphoric acid.

All HPLC mobile phases were filtered through 0.22-μm membrane filters and degassed under vacuum. Mobile phases containing 0.03 *M* aqueous NaH_2PO_4 were adjusted to the desired pH using 8.5% H_3PO_4 or 2 *N* NaOH.

Standards

Cis- and *trans*-TTX and N-desmethyl-TTX were obtained as the free bases (Charles Pfizer, New York, NY, U.S.A.). A 50-mg mesoridazine tablet (Serentil, Sandoz, Hanover, NJ, U.S.A.) was dissolved in 50 ml 0.5 *N* HCl, then the solution was adjusted to pH 9 with 10 *N* NaOH and extracted five times with 20 ml of hexane—isopropanol (9:1). A solution of approximately 100 μg/ml was prepared by diluting 10 ml to 50 ml with methanol.

Separate stock solutions of 1 mg/ml of *cis*- and *trans*-TTX and N-desmethyl-TTX were each made in methanol. These were diluted with methanol to 10 μg/ml.

A solution containing the internal standard, mesoridazine, and "carrier" *cis*-thiothixene was prepared for addition to all standard and unknown plasma samples. This solution was prepared by mixing 0.20 ml of the approximately 100 μg/ml MSZ solution, 1.6 ml of the 10 mg/ml *cis*-TTX solution and 38.2 ml of deionized water. This solution contained 400 ng/ml of *cis*-TTX and approximately 500 ng/ml of MSZ.

Cis-TTX plasma standards of 5, 10 and 20 ng/ml were prepared in drug-free blood bank plasma. Aqueous *cis*-TTX standards of 5, 10, 20, 50 and 100 ng/ml were prepared by dilution of 1 mg/ml methanolic standards with deionized water.

Collection of specimen

Blood was collected into 10-ml green-stoppered Venoject tubes containing sodium heparin (Terumo Medical, Elkton, MD, U.S.A.). The specimens were centrifuged and the plasma layer removed as soon as possible. The plasma was stored at −20°C and protected from light until analyzed.

Special apparatus

C_{18} Sep-Pak cartridges were supplied by Waters Assoc., Milford, MA, U.S.A. Multifit glass syringes (10 ml) were obtained from Beckton-Dickinson, Oxnard, CA, U.S.A.

Instrumentation

The HPLC system used was a Waters Assoc. Model 20A consisting of a U6K injection loop, a Model 6000A positive displacement solvent delivery system, a Model 440 254-nm UV detector and a Model RCM-100 radial compression module for chromatography cartridges. A 10 cm × 5 mm (10 μm) Radial-Pak silica cartridge and a 10 cm × 8 mm (10 μm) Radial-Pak Nitrile CN cartridge (both Waters Assoc.) were used. A C_{18} Guard-Pak (Waters Assoc.) pre-column insert was used with the Nitrile CN HPLC cartridge. A stainless-steel 50 cm × 4.6 I.D. pre-column filled with HC Pellosil (Whatman, Clifton, NJ, U.S.A.) high-capacity silica gel bonded to 30—38 μm glass bead was used with the silica HPLC cartridge. An Omni Scribe Model B5117-2 strip chart recorder (Houston Instruments, Austin, TX, U.S.A.) was used to record detector response.

A Model 4000 (Finnigan MAT, Sunnyvale, CA, U.S.A.) GC—MS system was used for identification of thiothixene from the HPLC fractions. A 0.6-m glass column packed with 1.5% SP-2100 was used at 230°C with a helium flow of 35 ml/min. The base peak ion at *m/z* 113 was monitored to identify thiothixene.

C_{18} Sep-Pak extraction

The use of C_{18} Sep-Pak cartridges for biological sample clean-up was reported by Narasimhachari [7]. The Sep-Pak method has now been adopted with some modification for the extraction of TTX from plasma samples.

C_{18} Sep-Pak cartridges were activated prior to use by passing 2 ml of methanol followed by 5 ml of deionized water at a flow-rate of 5 ml/min. Two ml of plasma standards (20 ng/ml), 100 μl of internal standard MSZ and carrier TTX solution were mixed with 2 *N* NaOH (0.5 ml) and passed through separate Sep-Pak cartridges followed by 3 ml of 0.05 *N* NaOH as a wash. The Sep-Pak cartridges were then eluted twice in 4 ml of different organic solvents: ethyl acetate, hexane, hexane—ethyl acetate (1:1), hexane—isopropanol (1:1), hexane—isopropanol (4:1), methanol and isopropanol. All solvents contained 0.1% triethylamine. The eluates were collected in 15-ml centrifuge tubes and centrifuged at 1000 *g*. The lower aqueous layer was removed and discarded. The organic solvents were evaporated at 40°C under nitrogen. Finally, the sides of the tubes were washed with 0.5 ml of extraction solvent, vortexed and dried. The residue in each tube was dissolved in 40 μl of LC mobile phase and 50% of this was injected into the LC system. From the peak height ratio of extracted sample and direct standard injection the extraction efficiency for thiothixene

was calculated for each solvent system. Thirteen control plasma samples containing no thiothixene were analyzed after adding a standard MSZ—TTX mixture using the Sep-Pak method to calculate the extraction efficiency for each compound and to evaluate the reproducibility of peak height ratio of TTX and the internal standard, MSZ.

Cyanopropyl stationary phase

LC system: A 10-μm radial compression module (RCM) silica cartridge was used in the earlier studies. In view of poor stability of silica columns at alkaline pH a cyanopropyl cartridge was evaluated as a reversed phase for the separation of *cis*- and *trans*-thiothixene, desmethylthiothixene and the internal standard. A mobile phase consisting of methanol—acetonitrile—0.03 *M* NaH_2PO_4—triethylamine (400:50:50:1.0) adjusted to pH 7.45 with phosphoric acid gave complete separation of all the four compounds.

Specificity

The LC peak corresponding to the retention time of *cis*-thiothixene was recycled through the chromatographic system five times when one symmetrical peak was always obtained. The fraction corresponding to this peak was collected and evaporated under nitrogen. The residue was dissolved in methanol and used for GC—MS identification.

Duplicate plasma samples were run by GC—MS—selected ion monitoring technique for quantitation of *cis*-TTX. For this purpose 2 ml of plasma sample containing 200 ng of d_3-thiothixene was processed by the C_{18} Sep-Pak procedure, using 10 ml of hexane—isopropanol (9:1) as eluting solvent. The solvent was evaporated under nitrogen, the residue redissolved in 20 μl ehtyl acetate and 2 μl injected into the GC—MS system. The ionizer temperature was 270°C and separator temperature 260°C. Electron ionization at 70 eV and ionizing current 0.45 mA was used. Ions *m/z* 113 and 116 were monitored for sample and internal standard, respectively. It was found necessary to saturate the column at least by three injections containing 1 μg/ml of standard *cis*-TTX solutions before using the system for quantitation.

Calculations of unknowns

Plasma samples were obtained from patients on standard antipsychotic TTX therapy and were drawn during initiation of therapy, steady-state and withdrawal of medication.

One hundred fifty plasma TTX samples have been assayed using the standard addition method, the C_{18} Sep-Pak extraction and either silica normal-phase or cyanopropyl reversed-phase LC. UV absorbance at 254 nm was used to detect *cis*-TTX and the internal standard, MSZ. Levels of *cis*-TTX were calculated from a peak height ratio of *cis*-TTX to MSZ versus plasma standard concentration graph.

RESULTS

Sample extraction

Previous to the use of the C_{18} Sep-Pak cartridges, various solvent mixtures

were tried for the extraction of thiothixene. Of the solvents we used, hexane—isopropanol (4:1) was found to be most satisfactory. Solvent extractions produced dirtier chromatograms than did C_{18} Sep-Pak extraction especially when used with a silica stationary phase for chromatography. A three-step solvent extraction reduced interferences but also reduced extraction efficiency, critical for low therapeutic concentrations of thiothixene. The extraction efficiencies for different solvent systems for *cis*-thiothixene from plasma standards are shown in Table I.

TABLE I

EXTRACTION EFFICIENCIES FOR DIFFERENT SOLVENT SYSTEMS FOR *cis*-TTX FROM PLASMA STANDARDS

Solvent		Extraction efficiency (%)
Hexane		37
Hexane—ethyl acetate	(1:1)	11
Hexane—isopropanol	(9:1)	40
Hexane—isopropanol	(8:2)	70
Hexane—isopropanol	(7:3)	62
Hexane—isopropanol	(6:4)	76
Hexane—isopropanol	(5:5)	57

For C_{18} Sep-Pak extraction, ethyl acetate was found to be the best solvent of those we tried. Table II shows the solvent system and the corresponding extraction efficiency. C_{18} Sep-Pak extraction gave consistently cleaner chromatograms than one-step hexane—isopropanol (4:1) solvent extraction and also saves time compared to the solvent extraction method. The Sep-Pak cartridges can be re-used when washed with 5 ml of methanol followed by 5 ml of water.

TABLE II

PERCENT RECOVERY OF *cis*-TTX FOR DIFFERENT SOLVENT SYSTEMS FROM C_{18} SEP-PAK CARTRIDGE

Solvent		Extraction efficiency (%)
Ethyl acetate		62
Hexane—ethyl acetate	(1:1)	40.5
Hexane—isopropanol	(1:1)	55
Hexane—isopropanol	(4:1)	50
Methanol		0
Isopropanol		45

We found variation in extraction efficiency using the Sep-Pak procedure. The extraction of 13 blank plasma samples using the standard addition—internal standard method gave mean extraction efficiencies of 65% and 63% for *cis*-TTX and MSZ, respectively. The standard deviations were 11.2% and 12.2% for *cis*-TTX and MSZ extractions, respectively. However, the peak height ratio of *cis*-TTX and MSZ was 0.331 with a standard deviation of 0.008. Thus, the presence of internal standard is necessary to correct for the variation in the extraction.

Chromatography

We investigated the separation of *cis*- and *trans*-thiothixene on 10-μm silica columns (μPorasil, Waters Assoc.) and silica cartridge (5 mm I.D.). Fig. 1 shows typical chromatograms of a plasma based standard and a patient plasma standard. We found that the silica column was not stable with the hexane—isopropanol—methanol—ammonium hydroxide (400:400:200:1) mobile phase that we used. Silica is soluble in low-molecular-weight alcohols at alkaline pH. Column instability prompted us to try reversed-phase columns. C_{18} Reversed-phase chromatography of these compounds yielded broad peaks. We found that the cyanopropyl reversed-phase columns gave good chromatography of thiothixene and mesoridazine.

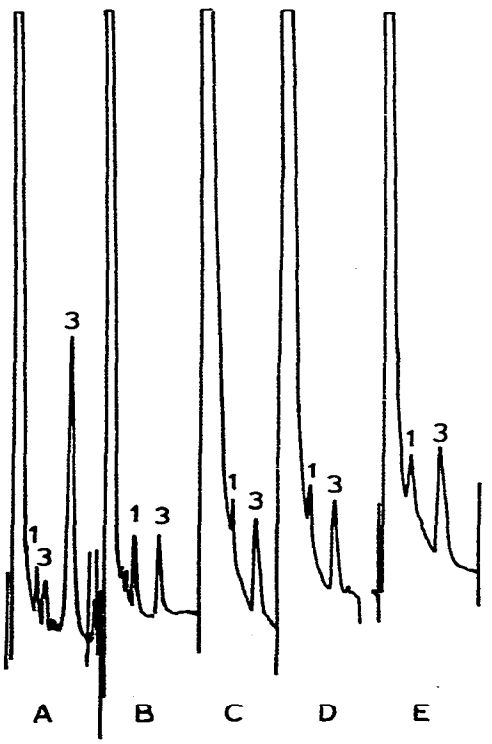


Fig. 1. HPLC separation on RCM silica cartridge of (1) *cis*-TTX (I), (2) *trans*-TTX (II), (3) MSZ (IV) internal standard. (A) Pure standards; (B) plasma standard 20 ng/ml extracted after standard addition; (C and D) drug-free blank plasma extracted after standard addition; (E) patient plasma extracted after standard addition (plasma concentration 4 ng/ml).

We found two satisfactory mobile phase systems for use with the 10-μm cyanopropyl reversed-phase radial compression cartridge. The first, methanol—acetonitrile—0.03 *M* NaH_2PO_4—triethylamine (650:100:250:1.0) pH 3.7, gave us chromatograms similar to the silica phase. There was a large solvent front so that this system is compatible with C_{18} Sep-Pak extraction but not with one-step solvent extraction. The second mobile phase of methanol—acetonitrile—0.03 *M* NaH_2PO_4—triethylamine (400:50:50:1.0) pH 7.45 gave much better

separation of *cis*-TTX, *trans*-TTX, N-desmethyl-TTX and MSZ. The compounds of interest were better separated from the "solvent front" so that C_{18} Sep-Pak or solvent extraction could be used. Fig. 2 shows chromatograms of extracted plasma standard and extracted patient plasma samples. A standard curve of peak height ratio of *cis*-TTX to MSZ versus standard *cis*-TTX concentration was used to calculate unknown *cis*-TTX concentrations in patient plasma samples.

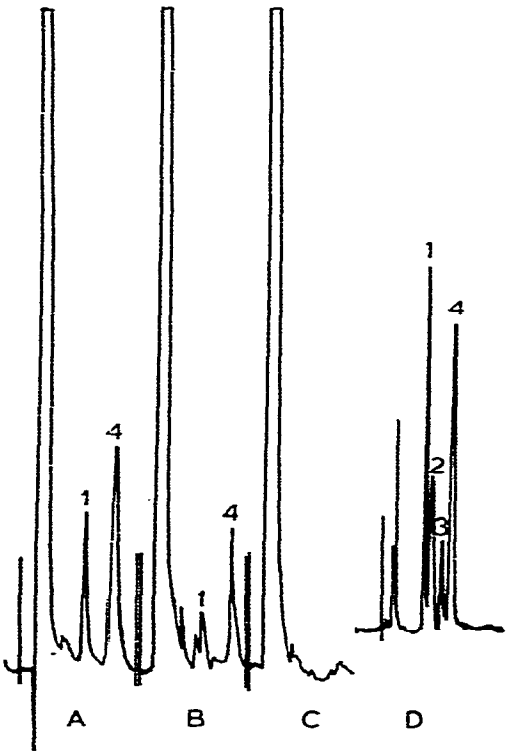


Fig. 2. HPLC separation on RCM-CN-Nitrile cartridge of (1) *cis*-TTX (I), (2) *trans*-TTX (II), (3) nor-TTX (III) and (4) MSZ (IV). Extraction by Sep-Pak method. (A) Plasma standard 20 ng/ml; (B) patient plasma (3.5 ng/ml); (C) drug-free plasma blank; (D) pure standards.

The linear regression equation of the line gave a slope of 0.019, intercept of 0.324 and r of 0.998. The coefficient of variation of the peak height ratio of 21 extracted plasma standards used to prepare this standard curve was 4.8%. Plasma blanks were run under identical conditions and no interference from any endogenous compounds were observed (Fig. 2). Extracts of plasma samples containing d_3-thiothixene were run on HPLC, the LC peaks for the *cis* form were collected, and after evaporation were quantitated by GC—MS—SIM. A complete mass spectrum of the LC peak was also obtained on GC—MS and also by direct solid probe inlet (Fig. 3). The spectrum did not show any detectable contamination.

Retention data for *cis*- and *trans*-TTX, desmethyl-TTX and internal standard using a cyanopropyl reversed-phase column are presented in Table III.

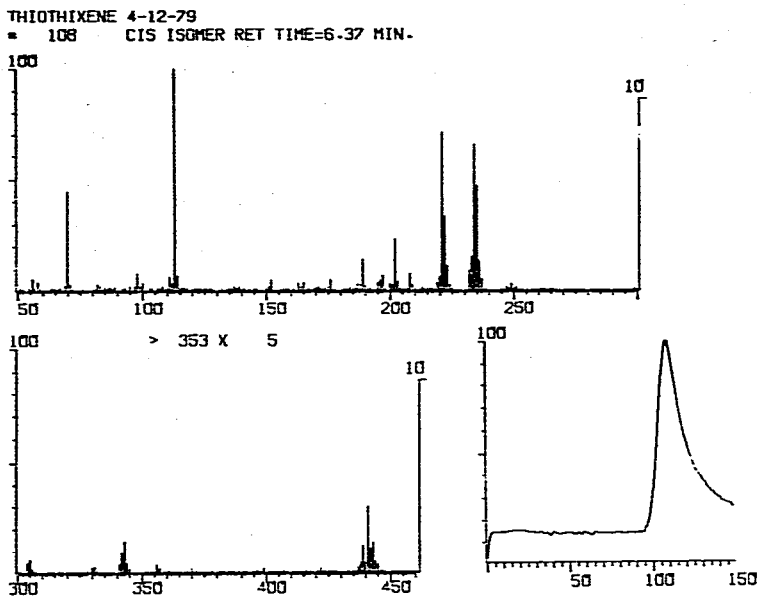


Fig. 3. Mass spectrum and total ion chromatogram (lower right) of HPLC fraction containing *cis*-TTX.

TABLE III

RETENTION DATA ON CN NITRILE RADIAL COMPRESSION CARTRIDGE (8 mm I.D.) WITH MOBILE PHASE OF METHANOL—ACETONITRILE—0.03 *M* AQUEOUS NaH_2PO_4—TRIETHYLAMINE (400:50:50:1) pH 7.45; FLOW-RATE 1.5 ml/min

Compound	Retention time (min)	*k'*
cis-TTX	7.0	3.7
trans-TTX	8.1	4.4
N-Desmethyl-TTX	9.6	5.4
Mesoridazine	11.0	6.3

Plasma cis-thiothixene concentrations

The 150 plasma samples assayed for *cis*-TTX had levels ranging from 0 to 22.5 ng/ml. The mean of all levels was 4.3 ng/ml. No *trans*-TTX was noted in these plasma samples.

DISCUSSION

We report the determination of *cis*-thiothixene in human plasma specimens by HPLC. Mesoridazine is used as internal standard and the addition of a constant amount of *cis*-TTX as a "carrier" increases the sensitivity of the assay. *Cis*-TTX and MSZ can be extracted from alkalinized plasma by either solvent extraction using hexane—isopropanol (4:1) or by the C_{18} Sep-Pak method. We

have found the C_{18} Sep-Pak extraction to reduce interferences and the time required to prepare the specimen for LC. We are routinely using the Sep-Pak procedure and cyanopropyl reversed-phase chromatography to measure *cis*-TTX levels during clinical trials with patients diagnosed as schizophrenic or borderline schizophrenic.

Bombardt and Friedel [4] reported finding varying proportions of *trans*-TTX by GC—MS in plasma of patients receiving *cis*-TTX. In contrast, we have not found *trans*-TTX in the plasma samples we have analyzed. This HPLC procedure clearly separates the isomers whereas GC gives poor separation. During the course of this investigation we found that standard solutions of *cis*-TTX showed contamination by *trans*-TTX after 1 or 2 weeks, the height of the *trans* peak increasing with time. We therefore studied the effect of UV irradiation on solutions of pure *cis*-TTX, in methanol or ethyl acetate. Results of such irradiation by a 15-W UV lamp (TLC scanner) for 15 min is shown in Fig. 4. From these results it is our view that the presence of *trans*-TTX in plasma has to be interpreted as probably artifactual unless adequate precautions are taken in the storage and handling of the samples.

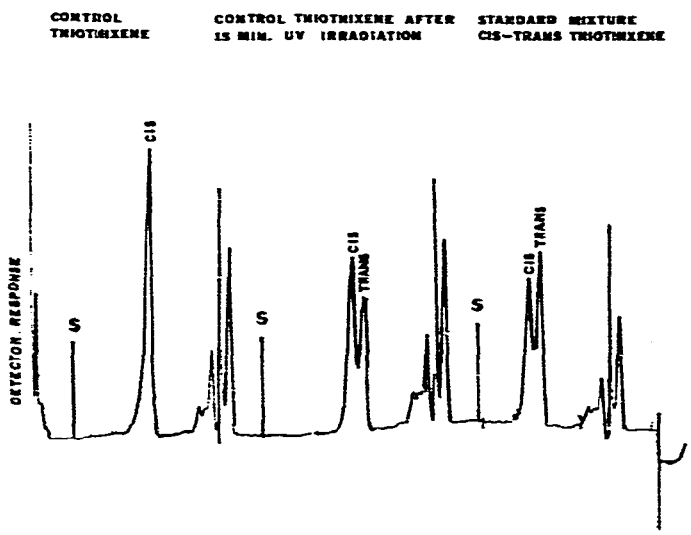


Fig. 4. The effect of UV irradiation on *cis*-TTX. (Left) standard *cis*-TTX; (center) *cis*-TTX after 15 min irradiation; (right) standard *cis—trans* mixture.

The sensitivity of the HPLC assay can be improved by measuring UV absorbance at 229 nm, the UV absorbance maximum for *cis*-TTX. Absorbance is roughly double at 229 nm compared to 254 nm. The use of a variable-wavelength detector at 229 nm would allow the measurement of thiothixene without the standard addition procedure. We have carried out experiments on detection limits for *cis*-TTX using the Beckman Model 150 fixed-wavelength UV detector using a cadmium lamp (229 nm) (Beckman Instruments, Berkeley, CA, U.S.A.). In our future studies we intend using this more sensitive wavelength for TTX quantitation. Hobbs et al. [3] used a GC—MS procedure to

measure *cis*-TTX in plasma of patients taking TTX. They reported a range of peak TTX concentrations of 10.0—22.5 ng/ml which correspond to a dose range of 15—60 mg per day which adequately controlled the symptoms of fifteen chronic schizophrenic patients. The plasma levels we determined were drawn at various times after drug administration. The range of *cis*-TTX plasma concentrations we found was 0—22.5 ng/ml which compares favorably with the reported concentrations [3].

N-Desmethyl-TTX was reported as a metabolite of TTX by Hobbs [1]. N-Desmethyl metabolites of phenothiazines and tricyclic antidepressants have been found to be active agents similar to their parent compounds. We have been able to separate and detect N-desmethyl-TTX on the cyanopropyl reversed-phase column. Simultaneous measurement of *cis*-TTX and N-desmethyl-TTX is possible and may yield additional information in the study of plasma levels and clinical response.

The HPLC procedure described in this paper coupled withthe Sep-Pak method for sample clean-up and the use of a closely related internal standard will be highly useful for routine monitoring of plasma *cis*-TTX levels. This simple, rapid analytical procedure can therefore be used for pharmacokinetic studies, studies in the metabolic differences between responders and non-responders and also for quickly checking compliance among patients. We have recently extended this study for monitoring saliva levels of TTX and found the HPLC method suitable for measuring saliva levels.

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