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RESEARCH ARTICLE

Synthesis, Characterization and HPLC Analysis of Structurally Related Compounds of Tetrazepam

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ABSTRACT

Analogues of tetrazepam, a 1,4-benzodiazepine derivative, offer potential improvements in therapeutic profiles, including enhanced efficacy, reduced side effects, and better pharmacokinetics. This study aimed to synthesize tetrazepam and its structurally related compounds, characterize and quantify their impurities, and optimize the synthesis process to improve the impurity profile, ultimately enhancing safety and reducing toxicity in drug therapy. Tetrazepam was synthesized through the reaction of anthranilic acid with sulfuryl chloride, followed by a Grignard reaction, hydrolysis, and cyclization. Structurally related impurities were generated by modifying these steps and characterized using TLC, IR, NMR, and mass spectrometry. An HPLC method with acetonitrile and potassium dihydrogen phosphate effectively separated tetrazepam from its related compounds. The yields of tetrazepam and its related compounds ranged from 68% to 95%. Structural confirmation was achieved using TLC, IR, NMR, and mass spectrometry. Tetrazepam yielded 74.82% with an Rf of 0.34 and Rt of 33.56 minutes. The impurities (IMP I–IV) had yields between 68.18% and 95.23%, with Rf values of 0.40–0.65 and Rt values of 22.52–54.35 minutes, confirming their identities. A novel approach was developed to synthesize tetrazepam and its related impurities, achieving improved purity and reduced toxicity. A new HPLC method was created to separate and identify these impurities, showing enhanced resolution and peak symmetry. This advancement significantly contributed to medicinal chemistry by providing a more refined process for analyzing benzodiazepine derivatives.
Keywords: 1,4-benzodiazepine; Tetrazepam; HPLC; TLC; IR spectroscopy; ^{1}H-NMR

INTRODUCTION

Tetrazepam, 7-Chloro-5-cyclohex-1-en-1-yl-1- methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one, occurs as light yellow or yellow crystalline powder, practically insoluble in water, freely soluble in methylene chloride and soluble in acetonitrile[1]. It is used therapeutically as muscle relaxant[2]. It is an unusual benzodiazepine in its molecular structure as it has cyclohexyl group which has substituted the typical 5-phenylmoietyseen. Identification of tetrazepam is carried out by infrared absorption spectrophotometry[1], also thin layer chromatography, gas chromatography, high performance liquid chromatography, and ultraviolet spectrum was reported as methods of determination[3]. So far very few liquid / gas chromatography procedures have been described for the determination of tetrazepam[4–10].

Benzodiazepines, and 1,4-benzodiazepin-2-ones in particular, form a well-known class of pharmacologically active heterocyclic compounds with sedative, hypnotics, anxiolytic, and anticonvulsant properties[11]. Recent medicinal chemistry investigations have sown that the conformational chirality of the 1,4-benzodiazepine core of these molecules plays a very important role in determining their bioactivity: binding to both human serum albumin HSA and GABAA (gamma-aminobutyric acid type A) receptors is strongly stereo dependent and Favors the (M)-chiral conformation as revealed by HSA induced circular dichroism for fast interconverting species like diazepam[12] and by direct GABAA receptors affinity measurements for single enantiomers of slowly interconverting diazepam derivatives[13]. The study aimed to synthesize tetrazepam and its related compounds, followed by thorough characterization using IR, NMR, and mass spectroscopy. An analytical HPLC method was developed for their identification and quantification. The goal was to improve manufacturing processes, producing a purer drug with fewer impurities and reduced side effects. The research involved isolating impurities through

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column chromatography and preparative TLC and analyzing them using HPLC and spectroscopic methods for accurate identification and quantification.

METHODOLOGY

Chemicals, Reagents, and Analytical Techniques

Chemicals and reagents of AR and LR grade were sourced from various suppliers, including Lancaster, Nice, Sigma, Spectrum, NR Chem, Rolex, S.D.-fine Chem. Ltd, and Merck. The physical properties of the synthesized compounds were assessed by determining their uncorrected melting points in open capillary tubes. Characterization was performed using infrared (IR) spectroscopy, proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, and mass spectrometry. IR spectra were recorded on a Shimadzu 8700S FT-IR spectrophotometer at Lake Chemicals, Bangalore, over the range of 4000-400 cm^{-1} using KBr pellets. ^{1}H-NMR spectra were obtained at 200 MHz with an Amx-200 and at 400 MHz with a Bruker spectrometer, using deuterated chloroform at IISc Bangalore and Bal Pharma, respectively, with chemical shifts reported in ppm relative to Tetramethylsilane (TMS). Mass spectra were acquired using ESI-MS on a Thermo Finnigan LCQ Deca XP Max at IISc, Bangalore.

Synthesis of Tetrazepam

Scheme A

a. Synthesis of 2-Amino-5-chlorobenzoic acid (TNS-A1)

The synthesis of 2-Amino-5-chlorobenzoic acid (TNS-A1) involved cooling a mixture of sulfuryl chloride and DCM, then gradually adding anthranilic acid while maintaining a temperature below 0°C. After distilling off the DCM, the mixture was treated with 8% HCl, heated, and neutralized with NaOH to precipitate the product. The final compound ($CHClNO_2$) had a melting point of 206-210°C, a yield of 67.90%, and an Rf value of 0.37 (TLC: ethyl acetate 2:4).

b. Synthesis of 6-Chloro-2-methyl-4H-3, 1-benzoxazin-4-one (TNS-A2)

6-Chloro-2-methyl-4H-3,1-benzoxazin-4-one (TNS-A2) was synthesized by refluxing TNS-A1 with acetic anhydride for 2 hours. After cooling, the product was filtered, washed with hexane, and dried at 65-70°C. The final compound (C_9HClNO_2) had a melting point of 130-133°C, a yield of 92.99%, and an Rf value of 0.78 (TLC: ethyl acetate 2:4).

c. Synthesis of Bromocyclohexane

Bromocyclohexane was synthesized by heating cyclohexanol with hydrobromic acid and distilling the mixture over 6 hours. The distillate was treated with water, and the crude bromide was purified by washing with concentrated HCl, sodium bicarbonate solution, and water, followed by drying with calcium chloride. The final compound ($CH_{11}Br$) had a boiling point of 166-167°C, with a theoretical yield of 407.5 g and a practical yield of 270 g (66.25%). TLC was not applicable due to the absence of a chromophore.

d. Synthesis of N-[4-chloro-2-(Cyclohexylcarbonyl) phenyl] acetamide (TNS-A3)

N-[4-Chloro-2-(Cyclohexylcarbonyl) phenyl] acetamide was synthesized in two steps. First, a Grignard reagent was prepared by reacting bromocyclohexane with magnesium in THF under a nitrogen atmosphere, refluxing for 3 hours. In the second step, TNS-A2 was dissolved in THF, cooled to -5°C, and the Grignard reagent was added dropwise. The mixture was stirred for 10 hours, then treated with saturated ammonium chloride, and the product was extracted, washed, dried, and distilled. The final compound ($C_{15}H_1ClNO_2$) had a melting point of 180-183°C, a practical yield of 45 g (62.95%), and an Rf value of 0.45 in a 2:2 ethyl acetate:hexane system.

e. Synthesis of (2-Amino-5-chlorophenyl) (Cyclohexyl) methanone (TNS-A4)

(2-Amino-5-chlorophenyl) (Cyclohexyl) methanone (TNS-A4) was synthesized by refluxing TNS-A3 (40 g, 0.143 mol) with 60% H_2SO_4 for 2 hours. The reaction mixture was then added to ice water, neutralized with NaOH, and the resulting precipitate was filtered and dried. The compound ($C_{13}H_1ClNO$) had a melting point of 102-109°C, with a practical yield of 30 g (88.28%) and an Rf value of 0.65 in a 2:2 ethyl acetate:hexane system.

f. Synthesis of 2-Chloro-N-[2-(cyclohexylcarbonyl) phenyl] acetamide (TNS-A5)

2-Chloro-N-[2-(cyclohexylcarbonyl) phenyl] acetamide (TNS-A5) was synthesized by reacting TNS-A4 with chloroacetyl chloride in the presence of sodium carbonate in acetone at 45-55°C. The product was precipitated by adding ice water and adjusting the pH. The compound had a melting point of 155-157°C, a practical yield of 33 g (83.2%), and an Rf value of 0.36 in a 2:2 ethyl acetate:hexane system.

g. Synthesis of 7-Chloro-5-cyclohexyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-A6)

7-Chloro-5-cyclohexyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-A6) was synthesized by refluxing TNS-A5 with hexamine, ammonium acetate, and ethanol for 6 hours. After distilling off the ethanol, water was added to precipitate the product. The compound had a melting point of 175-178°C, a practical yield of 20 g (75.72%), and an Rf value of 0.42 in a 2:2 ethyl acetate:hexane system.

h. Synthesis of 1, 7-Dichloro-5-cyclohexyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-A7)

1,7-Dichloro-5-cyclohexyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-A7) was synthesized by reacting TNS-A6 with t-butyl hypochlorite in dichloromethane, followed by evaporation under reduced pressure. Crystals formed after dissolving the residue in di-isopropyl ether were separated and dried. The compound had a melting point of 143-144°C, a practical yield of 17 g (83.99%), and

an Rf value of 0.70 in a 2:4 dichloromethane:hexane system.

i. Synthesis of 7-Chloro-5-(1'-chlorocyclohexyl) - 1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-A8)

7-Chloro-5-(1'-chlorocyclohexyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-A8) was synthesized by refluxing TNS-A7 (15 g, 0.048 mol) in 60 mL of ethyl acetate until a precipitate formed and a negative test with sodium iodide in acetone was obtained. The mixture was then cooled, and the solid was filtered, dried, and recrystallized from ethyl acetate. The product had a melting point of 196-197°C, a practical yield of 14 g (93.33%), and an Rf value of 0.73 in a 2:4 dichloromethane:hexane system.

j. Synthesis of 7-chloro-5-(1'-cyclohexenyl) - 1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-A9)

7-Chloro-5-(1'-cyclohexenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-A9) was synthesized by heating TNS-A8 (12 g), lithium carbonate (6 g), lithium bromide (3 g), and DMF (60 mL) to 100°C, then to 110°C for 15 minutes. After cooling, the solvent was evaporated, and the residue was recrystallized from ethyl acetate. The compound had a melting point of 208-210°C, a practical yield of 8 g (61.20%), and an Rf value of 0.69.

k. Synthesis of 7-chloro-5-(1'-cyclohexenyl) -1- methyl - 1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-A10) (Tetrazepam)

7-Chloro-5-(1'-cyclohexenyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-A10), also known as Tetrazepam, was synthesized by stirring TNS-A9 (7 g) with dry DMF (50 mL) and sodium carbonate (4.11 g) for 30 minutes. Methyl iodide (14.33 g) in anhydrous DMF (20 mL) was then added, and the mixture was stirred for 2 hours. After dilution with water and extraction with ethyl acetate, the solvent was evaporated, and the residue was crystallized from ethyl acetate. The compound had a melting point of 139-142°C, a practical yield of 5.5 g (74.82%), and an Rf value of 0.34.

Scheme B

a. Synthesis of 6-Chloro-2H-3, 1-benzoxazine-2, 4(1H) -dione (TNS-B1) (5-Chloro-Isatoic anhydride)

6-Chloro-2H-3,1-benzoxazine-2,4(1H)-dione (TNS-B1) was synthesized by refluxing TNS-A1 with ethyl chloroformate, followed by the addition of chloroacetic chloride and further refluxing. The product (CH_4ClNO_3, 197.5 g/mol) had a melting point of 280-283°C. The theoretical yield was 57.58 g, with a practical yield of 49 g (85.09%). TLC (ethyl acetate 2:4) showed an Rf value of 0.3.

b. Synthesis of 6-chloro-1-methyl-2H-3, 1-benzoxazine-2, 4(1H) -dione (TNS-B2)

6-Chloro-1-methyl-2H-3,1-benzoxazine-2,4(1H)-dione (TNS-B2) was synthesized by reacting TNS-B1 (25 g, 0.126 mol) with anhydrous sodium carbonate and dimethyl formamide. Methyl iodide (34.61 g, 0.243 mol) was added dropwise, and the mixture was stirred for 4 hours at room temperature. The reaction mixture was poured into water, and the product was filtered. The compound (C_9HClNO_3, 211.5 g/mol) had a melting point of 198-200°C. The theoretical yield was 30.38 g, with a practical yield of 25 g (81.08%). TLC (ethyl acetate 2:4) showed an Rf value of 0.42.

c. Synthesis of [5-chloro-2-(methylamino) phenyl] (Cyclohexyl) methanone (TNS-B3)

[5-Chloro-2-(methylamino) phenyl] (cyclohexyl) methanone (TNS-B3) was synthesized from TNS-B2 using a procedure similar to that for TNS-A3. The compound ($C_{14}H_1ClNO$, 251.5 g/mol) had a melting point of 170-172°C. The theoretical yield was 29.72 g, with a practical yield of 23 g (77.38%). TLC (ethyl acetate 2:4) showed an Rf value of 0.34.

d. Synthesis of 2-chloro-N-[4-chloro-2-(cyclohexylcarbonyl) phenyl]-N-methylacetamide (TNSB4)

2-Chloro-N-[4-chloro-2-(cyclohexylcarbonyl) phenyl]-N-methylacetamide (TNS-B4) was synthesized using a procedure similar to that for TNS-A5. The compound ($C_1H_{19}Cl_2NO_2$, 328 g/mol) had a melting point of 167-170°C. The theoretical yield was 26.08 g, with a practical yield of 25 g (95.85%). TLC (ethyl acetate 2:4) showed an Rf value of 0.48.

e. Synthesis of 7-chloro-5-cyclohexyl-1-methyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNSB5)

7-Chloro-5-cyclohexyl-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-B5) was synthesized from TNS-B4 using a procedure similar to that for TNS-A6. The compound ($C_1H_{19}ClN_2O$, 290.5 g/mol) had a melting point of 182-185°C. The theoretical yield was 21.25 g, with a practical yield of 18 g (84.70%). TLC (ethyl acetate 2:2) showed an Rf value of 0.52.

f. Synthesis of 7-chloro-5-(1-chlorocyclohexyl) -1-methyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-B6)

7-Chloro-5-(1-chlorocyclohexyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-B6) was synthesized from TNS-B5 using a procedure similar to that for TNS-A7. The compound ($C_1H_1Cl_2N_2O$, 325 g/mol) had a melting point of 150-153°C. The theoretical yield was 19.01 g, with a practical yield of 17 g (89.42%). TLC (dichloromethane 2:4) showed an Rf value of 0.62.

g. Synthesis of 7-chloro-5-(1'-cyclohexenyl) -1- methyl - 1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-B7)

7-Chloro-5-(1'-cyclohexenyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-B7) was synthesized from TNS-B6 using a procedure similar to that for TNS-A9. The compound ($C_1H_1ClN_2O$, 288.5 g/mol) had a melting point of 141-142°C. The theoretical yield was 14.94 g, with a practical yield of 12 g (80.32%). TLC (dichloromethane 2:4) showed an Rf value of 0.33.

Scheme C

a.Synthesis of (2-Amino-5-chlorophenyl) (cyclohexyl) methanone (TNS-C1)

(2-Amino-5-chlorophenyl) (cyclohexyl) methanone (TNS-C1) was synthesized by reacting 2-amino-5-chlorobenzonitrile with cyclohexyl bromide in dry THF under nitrogen at -40°C, followed by the dropwise addition of n-butyl lithium. The reaction was quenched with 5% HCl and extracted. The product ($C_{13}H_1ClNO$, 237.5 g/mol) had a melting point of 105-110°C. The theoretical yield was 31.14 g, with a practical yield of 20 g (64.22%). TLC (ethyl acetate 2:4) showed an Rf value of 0.6

b. Synthesis of 2-Chloro-N-[2-(cyclohexylcarbonyl) phenyl] acetamide (TNS-C2)

The synthesis of 2-Chloro-N-[2-(cyclohexylcarbonyl) phenyl] acetamide (TNS-C2) followed a procedure similar to that for TNS-A5. The product ($C_{15}H_1Cl_2NO_2$, 314 g/mol) had a melting point of 150-152°C. The theoretical yield was 26.44 g, with a practical yield of 23 g (86.98%). TLC (ethyl acetate 2:2) showed an Rf value of 0.36.

c. Synthesis of 7-Chloro-5-cyclohexyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-C3)

7-Chloro-5-(1'-cyclohexenyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-B7) was synthesized from TNS-B6 using a procedure similar to that for TNS-A9. The compound ($C_1H_1ClN_2O$, 288.5 g/mol) had a melting point of 141-142°C. The theoretical yield was 14.94 g, with a practical yield of 12 g (80.32%). TLC (dichloromethane 2:4) showed an Rf value of 0.33.

d. Synthesis of 1, 7-Dichloro-5-cyclohexyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-C4)

Procedure: The compound, with a molecular formula of $C_{15}H_1Cl_2N_2O$ and a molecular weight of 311 g/mol, had a melting point of 143-144°C. The synthesis yielded a theoretical amount of 11.24 g, with a practical yield of 11 g, resulting in a high percentage yield of 97.86%. Thin-layer chromatography (TLC) using a solvent system of dichloromethane and hexane in a 2:4 ratio produced an Rf value of 0.70.

e. Synthesis of 7-Dichloro-5-(1'-chlorocyclohexyl) - 1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-C5)

7-Dichloro-5-(1'-chlorocyclohexyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-C5) was synthesized following a procedure similar to that for TNS-A8. The compound ($C_{15}H_1Cl_3N_2O$, 311 g/mol) had a melting point of 195-196°C. The theoretical yield was 10 g, with a practical yield of 9 g (90%). TLC (dichloromethane 2:4) showed an Rf value of 0.73.

f. Synthesis of 7-chloro-5-(1'-cyclohexenyl) - 1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-C6)

7-Chloro-5-(1'-cyclohexenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-C6) was synthesized following a procedure similar to that for TNS-A9. The compound ($C_{15}H_{15}ClN_2O$, 274.5 g/mol) had a melting point of 208-209°C. The theoretical yield was 7.06 g, with a practical yield of 5 g (70.82%). TLC (dichloromethane 2:4) showed an Rf value of 0.70.

g. Synthesis of 7-chloro-5-(1'-cyclohexenyl -1- methyl -1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (TNS-C7)

7-Chloro-5-(1'-cyclohexenyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (TNS-C7) was synthesized following a procedure similar to that for TNS-A10. The compound ($C_1H_1ClN_2O$, 288.5 g/mol) had a melting point of 140-142°C. The theoretical yield was 7.35 g, with a practical yield of 6 g (81.63%). TLC (dichloromethane 2:4) showed an Rf value of 0.34.

Synthesis of Structurally Related Compounds of Tetrazepam

a. Synthesis of 7-chloro-5-cyclohexyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (IMP-I)

7-Chloro-5-cyclohexyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (IMP-I) was synthesized following a procedure similar to that for TNS-A6. The compound ($C_{15}H_1ClN_2O$, 276.5 g/mol) had a melting point of 175-178°C. The theoretical yield was 4.40 g, with a practical yield of 3 g (68.18%). TLC (ethyl acetate 2:2) showed an Rf value of 0.42.

b. Synthesis of 7-chloro-5-cyclohexyl-1-methyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (IMP-II)

7-Chloro-5-cyclohexyl-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (IMP-II) was synthesized using a procedure similar to that for TNS-A10. The compound ($C_1H_{19}ClN_2O$, 290.5 g/mol) had a melting point of 149-150°C. The theoretical yield was 5.25 g, with a practical yield of 5 g (95.23%). TLC (ethyl acetate 2:4) showed an Rf value of 0.40.

c. Synthesis of 7-chloro-5-(1-chlorocyclohexyl) -1-methyl-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (IMP-III)

7-Chloro-5-(1-chlorocyclohexyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (IMP-III) was synthesized using a procedure similar to that for TNS-A7. The compound ($C_1H_1Cl_2N_2O$, 325 g/mol) had a melting point of 150-152°C. The theoretical yield was 4.47 g, with a practical yield of 3.5 g (78.29%). TLC (dichloromethane 2:4) showed an Rf value of 0.62.

d. Synthesis of 5-(cyclohex-1-en-1-yl) -1, 3-dihydro-2H-1, 4-benzodiazepin-2-one (IMP-IV)

5-(Cyclohex-1-en-1-yl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (IMP-IV) was synthesized following a procedure similar to that for TNS-A9. The compound ($C_{15}H_1N_2O$, 240 g/mol) had a melting point of 170-172°C. The synthesis resulted in a theoretical yield of 3.47 g, with a practical yield of 3 g (86.45%). TLC (ethyl acetate 2:4) showed an Rf value of 0.65.

HPLC Analysis

The HPLC method for identifying and quantifying tetrazepam-related compounds utilized a 0.25 m x 4.6 mm column packed with octadecylsilyl silica gel (5 μm). The mobile phases were- Phase A (40% acetonitrile, 60% 3.4 g/L potassium dihydrogen phosphate solution) and Phase B (pure acetonitrile). Detection was at 229 nm, with a flow rate of 1.5 ml/min and an injection volume of 20 μl. The system included a Shimadzu 10 ATVP pump, Rhedyn injector, 10 AVD UV-visible detector, and CLASS-VP software. A tetrazepam standard solution (5 mg/10 ml) was prepared in acetonitrile.

Preparation of Individual solution of related compounds of Tetrazepam I, II, III, and IV:

Five milligrams each of the related compounds of tetrazepam (I, II, III, and IV) were accurately weighed and dissolved in approximately 5 ml of acetonitrile in separate 10 ml volumetric flasks. The flasks were sonicated for 5 minutes, and the volume was then adjusted to 10 ml with acetonitrile. Each sample was injected separately (20 μl), and the retention times were recorded for each compound. A standard sample solution was also injected to verify the retention times (Tables 1 and 2).

Table 1: HPLC data for IMP-I, II, III and IV

Peak #	Name	Retention Time	Area	Area %
1	IMP-III	22.859	2018949	19.666
2	IMP-IV	41.251	14569321	29.821
3	IMP-I	50.049	45633968	34.514
4	IMP-II	54.314	7454489	15.999
Total	–	–	69676727	100.00

Table 2: HPLC data for IMP-I, II, III, and tetrazepam

Peak #	Name	Retention Time	Area	Area%
1	IMP-III	22.520	16707389	0.294
2	Tetrazepam	33.562	358668905	96.668
3	IMP-IV	41.332	55073114	1.328
4	IMP-I	50.125	53812390	1.652
5	IMP-II	54.356	12061121	0.058
Total	–	–	496322919	100.00

RESULTS AND DISCUSSION

The results for tetrazepam and its structurally related compounds are summarized in Table 3. The synthesized compounds were identified and characterized using multiple analytical techniques, including thin-layer chromatography (TLC) with solvent systems of ethyl acetate (2:4) and dichloromethane (2:4), infrared (IR) spectroscopy on a SHIMADZU FTIR 8400S spectrometer with the KBr pellet method, proton nuclear magnetic resonance (^{1}H-NMR) analysis on a Bruker Spectrospin-200 NMR spectrophotometer, and mass spectrometry on a SHIMADZU spectrometer 210 (Figures 1 and 2).

Table 3: Physical and Chemical Characteristics of Synthesized Tetrazepam-Related Compounds

Sr. No.	Sample code	Molecular formula	Molecular weight	Yield	Rf value	Rt value
1	Tetrazepam	$C_{16}H_{17}ClN_2O$	288.5	74.82%	0.34	33.56
2	IMP I	$C_{15}H_{17}ClN_2O$	276.5	68.18%	0.42	50.12
3	IMP II	$C_{16}H_{19}ClN_2O$	290.5	95.23%	0.40	54.35
4	IMP III	$C_{16}H_{18}Cl_2N_2O$	325	78.29%	0.62	22.52
5	IMP IV	$C_{15}H_{16}N_2O$	240	86.45%	0.65	41.33

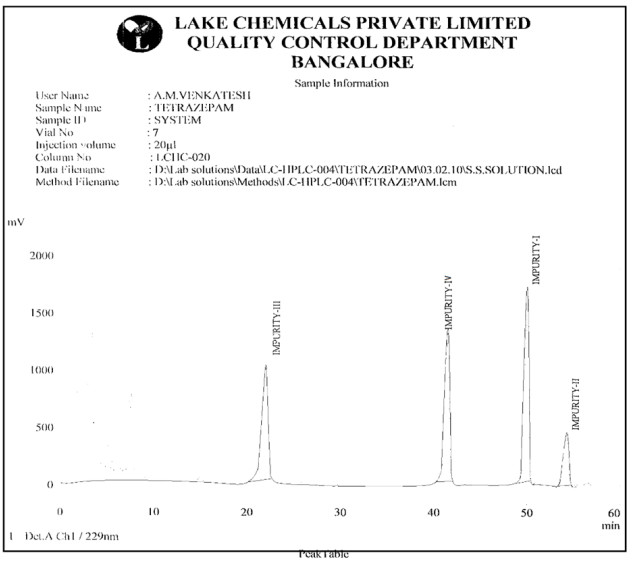

Fig. 1: HPLC chromatogram of the IMP Mixture

The study of the impurity profile of bulk drugs, such as tetrazepam, is a critical area of research in the bulk drug industry. Identifying various impurities present in the bulk drug helps in producing a pure drug with minimal impurities and reduced side effects. During bulk drug production, several opportunities for impurity generation arise. Characterizing and identifying both identified and unidentified impurities in a bulk drug substance is essential, known as the impurity profile. It is necessary to identify and quantify impurities present in excess of 0.1% using sufficiently selective methods. Therefore, this study aimed to synthesize, characterize, and identify the impurities formed during the manufacture of tetrazepam.

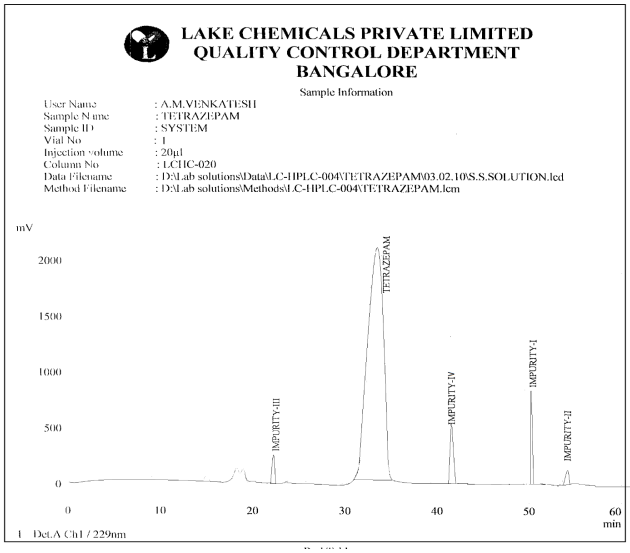

Fig. 2: HPLC Chromatograms of the synthesized Tetrazepam related compounds

Synthesis and Characterization of Tetrazepam and Related Compounds

Tetrazepam and its analogs were synthesized using Grignard reactions, acetylation, hydrolysis, bond migration, oxidation, dehydrogenation, dehydrohalogenation, cyclization, and recrystallization. Characterization was performed via thin-layer chromatography (TLC) with ethyl acetate/dichloromethane (2:4), infrared (IR) spectroscopy using the KBr pellet method, and nuclear magnetic resonance (NMR) spectroscopy on a Bruker Spectrospin-200 NMR spectrophotometer with $CDCl_3$ at Bal Pharma and IISc, Bangalore. High-performance liquid chromatography (HPLC) was employed to separate tetrazepam-related impurities using a mobile phase of 40% acetonitrile and 60% 3.4 g/L potassium dihydrogen phosphate solution, achieving effective separation with high resolution and peak symmetry.

CONCLUSION

The study successfully synthesized, characterized, and analyzed tetrazepam and its structurally related compounds. Using optimized chemical reactions and thorough characterization techniques such as TLC, IR, NMR, and mass spectrometry, the structural identities and purity of the compounds were confirmed. An HPLC method was developed to effectively separate and quantify tetrazepam and its impurities, achieving high resolution and peak symmetry. This research contributes significantly to the understanding of tetrazepam's chemical and physical properties, ensuring improved safety and reduced toxicity in drug therapy by identifying and quantifying potential impurities.

REFERENCES

1. British Pharmacopeia;vol. 1. 2010. Available from: https://books.google.co.in/books/about/British_Pharmacopoeia_2010.html?id=O-Q9PgAACAAJ&redir_esc=y.
2. Perez-Guerrero C, Herrera MD, Marhuenda E. Relaxant effect of tetrazepam on rat uterine smooth muscle: role of calcium movement. *Journal of Pharmacy and Pharmacology*. 1996;48(11):1169–1173. Available from: https://doi.org/10.1111/j.2042-7158.1996.tb03915.x.
3. Moffat AC, Clarke EGC. Clarkís Analysis of Drug and Poison. Pharmaceutical Press. 2004. Available from: https://books.google.co.in/books/about/Clarke_s_Analysis_of_Drugs_and_Poisons.html?id=_ElrAAAAMAAJ&redir_esc=y.
4. Borrey D, Meyer E, Lambert W, Van Calenbergh S, Van Peteghem C, De Leenheer AP. Sensitive gas chromatographic-mass spectrometric screening of acetylated benzodiazepines. *Journal of Chromatography A*. 2001;910(1):105–118. Available from: https://doi.org/10.1016/S0021-9673(00)01177-8.
5. Pirnay S, Ricordel I, Libong D, Bouchonnet S. Sensitive method for the detection of 22 benzodiazepines by gas chromatography-ion trap tandem mass spectrometry. *Journal of Chromatography A*. 2002;954(1-2):235–245. Available from: https://doi.org/10.1016/s0021-9673(02)00190-5.
6. Maurer H, Pfleger K. Determination of 1, 4-and 1, 5-benzodiazepines in urine using a computerized gas chromatographic-mass spectrometric technique. *Journal of Chromatography B: Biomedical Sciences and Applications*. 1981;222(3):409–419. Available from: https://doi.org/10.1016/S0378-4347(00)84141-9.
7. Quintela O, Cruz A, de Castro A, Concheiro M, Lopez-Rivadulla M. Liquid chromatography-electrospray ionisation mass spectrometry for the determination of nine selected benzodiazepines in human plasma and oral fluid. *Journal of Chromatography B*. 2005;825(1):63–71. Available from: https://doi.org/10.1016/j.jchromb.2004.12.038.
8. Capella-Peiró ME, Bose D, Martinavarro-Domínguez A, Gil-Agustí M, Esteve-Romero J. Direct injection micellar liquid chromatographic determination of benzodiazepines in serum. *Journal of Chromatography B*. 2002;780(2):241–249. Available from: https://doi.org/10.1016/s1570-0232(02)00523-8.
9. Spangenberg B, Seigel A, Kempf J, Weinmann W. Forensic drug analysis by means of diode-array HPTLC using RF and UV library search. *JPC-Journal of Planar Chromatography-Modern TLC*. 2005;18(105):336–343. Available from: https://doi.org/10.1556/JPC.18.2005.5.1.
10. Laloup M, Fernandez MDMR, De Boeck G, Wood M, Maes V, Samyn N. Validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous determination of 26 benzodiazepines and metabolites, zolpidem and zopiclone, in blood, urine, and hair. *Journal of analytical toxicology*. 2005;29(7):616–626. Available from: https://doi.org/10.1093/jat/29.7.616.
11. Olkkola KT, Ahonen J. Midazolam and other benzodiazepines. *Handbook of Experimental Pharmacology*. 2008;182:335–360. Available from: https://doi.org/10.1007/978-3-540-74806-9_16.
12. Fitos I, Visy J, Zsila F, Mády G, Simonyi M. Conformation selectivity in the binding of diazepam and analogues to α1-acid glycoprotein. *Bioorganic & medicinal chemistry*. 2007;15(14):4857–4862. Available from: https://doi.org/10.1016/j.bmc.2007.04.060.
13. Lee S, Kamide T, Tabata H, Takahashi H, Shiro M, Natsugari H. Axial chirality and affinity at the GABAA receptor of pyrimido. *Bioorganic & medicinal chemistry*. 2008;16(21):9519–9523. Available from: https://doi.org/10.1016/j.bmc.2008.09.037.