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Separation of Enantiomers of Metolazone by Thin Layer Chromatography Using a Chiral Selector

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Abstract

Enantiomeric separation of (RS)-Metolazone has been achieved by thin-layer chromatography (TLC) using both direct modes. cyclodextrin, containing hydroxylic group, was utilized as the chiral selector. For enantioseparation, cyclodextrin was used as a chiral additive in the stationary phase in a non-covalent mode and there was no chiral additive in the mobile phase; the native enantiomers were isolated and characterized. Cyclodextrin was also added to mobile phase and there was no chiral selector in the stationary phase. The spots were then isolated and characterized. The effect of the composition of the mobile phase, pH, and temperature was studied for the optimization of successful separation conditions. The spots were located in an iodine chamber.

***Keywords**: beta cyclodextrin, Metolazone, Chiral separation, Thin-layer chromatography*

1. Introduction

Metolazone (Metzn, Fig. 1) is a quinazoline derived of the similar diuretics quinethazone. Chemically it is 7-chloro-2-methyl-3-(2-methylphenyl)-4-oxo-1,2-dihydroquinazoline-6-sulfonamide. Metzn is an oral diuretic drug, and is mainly used to cure congestive heart failure and high blood pressure. It is frequently used together with loop diuretics such as furosemide or bumetanide[1]. Metzn indirectly reduces the amount of water reabsorbed in to the bloodstream by the kidney, so that blood volume decreases and urine volume increases. The action of Metzn results from interference with the renal tubular mechanism of electrolyte reabsorption. Metzn behaves primarily to prevent Na reabsorption at the cortical diluting site and to a smaller extent in the proximal convoluted tubule. Na and chloride ions are evacuated in nearly equal amounts. Metzn has been developed and marketed as racemate. Metzn standard brand name in U.S. Zaroxolyn and in Canada it is Mykrox[2].

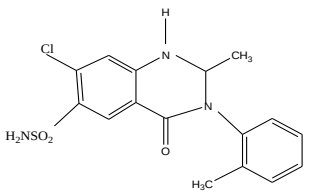

Fig 1. Chemical structure of Metzn

Many chiral pharmaceuticals drugs are still sold as racemate. Recent global advance in new regulatory guidelines for racemate or 'pure' pharmaceutical products necessitate development of rapid, sensitive and reproducible methods for quality control of optical antipodes present in drug substance. Determination of enantiomeric purity, is of special importance in the control of the purity of chiral synthetic materials and chiral pharmaceuticals. Separation of enantiomers has become very important in analytical chemistry, especially in the pharmaceutical and biological fields, because some stereoisomers of racemic drugs have quite different pharmacokinetics and different pharmacological or toxicological effects[3]. The development of stereoselective HPLC method for the determination of enantiomeric drugs has received considerable attention in recent years because of its importance in analysis of quality control of pharmaceutical formulations. Chiral separation techniques mainly include high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE). Chiral HPLC has been recognized as a useful methodology for the separation of chiral drugs than other techniques. A through literature survey revealed that a few HPLC methods were reported for determination of Metzn in human urine. Also in literature, some of HPLC methods were cited for the determination of Metzn in human plasma and pharmaceutical dosage form[1]. Polysaccharide-based stationary phases are quite popular with wide recognition for direct resolution of enantiomers. A simple, rapid and validated method for parting and justification of Metzn enantiomers on amylose based stationary phase Chiralpak AD-H column was developed as a novel, simple and robust chiral HPLC method for enantioseparation of Metzn. The developed method was validated with respective linearity, precision, LOD, LOQ and robustness[4].

Need for Enantioseparation: Generally, drug action is the result of pharmacological and pharmacokinetic processes. There is a broad range of examples where the enantiomers of drugs exhibit differences in bioavailability, distribution, metabolic and excretion behaviour and where stereochemical parameters play fundamental significance in their actions and disposition in biological systems. In 1992, the U.S. Food and Drug Administration issued a guideline that only the therapeutically active enantiomer of a chiral drug can be brought into market. In addition, each enantiomer of chiral drug compounds should be studied separately for its pharmacological and metabolic pathways[5,6]. Therefore, manufacturers must develop quantitative assays for enantiomers individually in in vivo samples during drug development to evaluate pharmacokinetics, which will lead to assessment of the potential of interconversion and the absorption, distribution, metabolism and elimination profile of individual enantiomers. If a candidate drug product is racemic with different pharmacokinetic profiles from its enantiomers, manufacturers have to monitor the pharmacological effects of the enantiomers individually so as to measure the properties such as dose linearity, effects of altered metabolic or excretory function and drug-drug interactions. Since a pair of enantiomers have almost identical properties, special chiral techniques are usually required for their separation, quantitation and sometimes identification. Among them, enantioseparation, to separate enantiomers based on their subtle differences in properties, is in a decisive position. Enantioseparation can be applied in simultaneous production of both enantiomers (dual-isomer recovery) or only the target enantiomer (single-isomer recovery). The former is often used in the manufacture of chiral intermediates as both enantiomers are of market outlets. The chiral technology selects one enantiomer, leaving the other behind and both are ultimately recovered by conventional means[3]. The latter is usually applied to the manufacture of end-use chemicals or intermediates when only one enantiomer is commercial. The chiral technology can also select the target enantiomer while deliberately racemizing the other enantiomer and recycling it in the selection process to produce the target enantiomer. The large demand of enantiomerically pure products has stimulated the progress of enantioseparation, which is considered as one of the most important areas of research in both industry and academia during the last decades. Beside pharmaceutics and medicinal sciences, enantioseparation also received more and more attention in geochemistry, geochronology, biochemistry and materials science[3,4].

The production of enantiomerically pure compounds by separation of racemic mixture is known as enantioseparation. The enantiopure drugs are essential because the organic structure is astonishingly chirally selective. More than five hundred medications are chiral however the medicine activity resides with just one compound, termed as eutomer, whereas the opposite compound that is inactive or less potent metabolizes by a distinct means within the body. Therefore, it is necessary to separate chiral drugs into their potent and less potent isomers as they both show different pharmacokinetic and the pharmacological effect[4,6].

Enantioseparation of Metzn

A LC method was established and confirmed for the humble, fast and robust enantiomeric separation of Metzn. a Chiralpak AD-H (amylose based stationary phase) columnwas used for enantiomers resolution of Metzn. The mobile phase consisting of Hexane: 2-propanol: MeOH: AcOH (80:10:10:0.2, v/v) at a flow rate of 1.0 mLmin^{-1}. The resolution between the enantiomers was found to be not less than 3.0 in optimized method. The presence of AcOH in the mobile phase played an important role, in enhancing chromatographic efficiency and resolution between the enantiomers. The established method was largelyconfirmed and evidenced to be robust. The calibration curve for enantiomers showed excellent linearity over the concentration range of 5mgmL^{-1} to 50mgmL^{-1}. The limit of detection and limit of quantification for enantiomers were 0.05 and 0.16 mgmL-1, respectively. The proposed method was found to be suitable and accurate for quantitative determination of enantiomers in bulk drug substance[4].

To determine Metzn in drug substance and pharmaceutical dosage form in the presence of its degradation products two robust and selective stability-indicating chromatographic methods were developed and validated. The HPLC method a Kromasil C18 (250 × 4.6,5 µm) column was used. The mobile phase used was acetonitrile: 0.2% orthophosphoric acid (32:68 v/v) at a flow rate 2 mL/min and detection at 238 nm. HPLC isocratic mode was used for the separation of enantiomers of Metzn. The robustness of the proposed scheme was measured using the Plackett–Burman design, parameters affecting system fitness were established and non-significant breaks for the significant parameters were measured. Nano-SIL-20 UV254 HPTLC plates as adsorbent used in HPTLC method. Developing solvent was ethyl acetate: toluene: AcOH solution (4:4:0.5, v/v/v), and densitometric detection at 238 nm. Metzn was treated to different stress conditions, including alkaline and acid hydrolysis and photolytic and oxidative degradation. LC-MS method was used for characterisation and validation of main degradation products obtained[7].

For the simultaneous estimation of two diuretic drugs, spironolactone (SPL) and Metzn a new, precise and delicate reversed-phase HPLC method was established. Spherisorb-ODS 2 C18 column having 150 mm 4.6 mm i.d., 5 mm particle size, best chromatographic separation was achieved within 5.0 min. A mobile phase consisting of 0.02 M phosphate buffer and MeOH (30: 70) v/v at pH 3.0 was used. The examination was accomplished at a flow rate of 1 mL min-1 with UV detection at 235 nm. As an internal standard (IS) Xipamide was used. The projected method was straight-lined over the ranges of 0.05–1.0 mg mL^{-1} and 0.5–10.0 mg mL^{-1} with limits of detection (LOD) of 0.009, 0.04 ng mL-1and limits of quantification (LOQ) of 0.03, 0.11 mg mL-1 for Metzn and SPL, respectively. The proposed scheme was effectively applied for the instantaneous analysis of the studied drugs in their laboratory prepared mixtures, single tablets and coformulated tablets. The process was further stretched to the determination of both drugs in spiked human urine. The mean percentage recoveries of Metzn and SPL in spiked human urine were 99.33, 2.37 and 99.72, 3.27, respectively. The anticipated method was also useful for the determination of the studied drugs in the presence of some co-administered or co-formulated drugs without any interference. The proposed and comparison methods revealed no significant difference between the two methods regarding accuracy and precision obtained bystatistical evaluation and comparison of the data[8].

For simultaneous determination of Losartan potassium and Metzn a HPLC method has been described. This process is founded on a HPLC separation of the two drugs on the ThermoHypersil BDS–C_{18} (250 mm × 4.6 mm, 5.0 µm) with isocratic settings. A mobile phase containing acetonitrile: water (60:40) at a flow rate of 0.8 mL/min using UV detection at 237 nm. This method has been useful to a marketed formulation without interference of excipients. The linear regression analysis data for the calibration plots exposed a good linear relationship over the concentration range of 2–12 µg/mL for Losartan potassium and 0.2–1.2 µg/mL for Metzn, respectively. The method was confirmed for accuracy, robustness and recovery. The method is repeatable and selective for the estimation of Losartan potassium and Metzn according to statistical analysis data[9].

A macrocyclic amide receptor based new chiral stationary phase (CSP) was prepared starting from (1R, 2R)-1, 2-diphenylethylenediamine. The CSP was effectivelyfunctional to the resolution of numerous N-(substituted benzoyl)-α-amino amides with practically good separation aspects and resolutions (α = 1.75 ~ 2.97 and RS =

2.89 ~ 6.82 for 16 analytes). The new CSP give good resolution result when applied to 3-substituted 1, 4-benzodiazepin-2-ones and some diuretic chiral drugs including methylchlothiazide, Metzn and bendroflumethiazide[6].

Structure of Cyclodextrin

Cyclodextrins (CD, Fig 2) are a cluster of structurally linked natural products designedthrough bacterial breakdown of cellulose. These cyclic oligosaccharides comprise of (α-1,4)-linked α-D-glucopyranose units having a lipophilic central cavity and a hydrophilic outer surface. As the presence of chair conformation of the glucopyranose units, the cyclodextrins are designed like a truncated cone rather than perfect cylinders. The hydroxyl functions are positioned to the cone exterior with the primary hydroxyl groups of the sugar deposits at the narrow edge of the cone and the secondary hydroxyl groups at the wider edge. The central void is lined by the skeletal carbons and ethereal oxygens of the glucose residues, which gives it a lipophilic character[10].

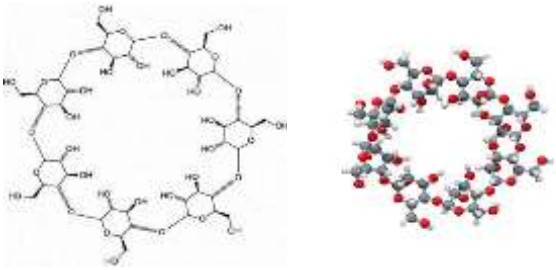

Fig 2. Structure of cyclodextrins

The natural CDs, in particular β-CD, are of restricted aqueous solubility meaning that complexes resultant from interaction of lipophiles with these cyclodextrin can be of limited solubility resulting in precipitation of solid cyclodextrin complexes from water and other aqueous systems. In general, the aqueous solubility of the natural CDs is considerably lower than that of similar acyclic saccharides. This happened due to relatively strong intermolecular hydrogen bonding in the crystal state. Exchange of any of the hydrogen bond forming hydroxyl groups, even by lipophilic methoxy functions, results in affected enhancement in their aqueous solubility[11].

In HPLC as well as capillary electrophoresis CDs have been used as chiral selectors and signify the most commonly used type of chiral selectors for a wide application range. Maximum CDs have enough solubility in the mobile phases and low UV absorbance. Additionally, several CD derivatives (native, methylated, and hydroxypropylated derivatives) are comparatively cheap. The structure of β-CD is shown in Fig. 2. The molecules have the shape of a truncated cone[12]. The primary C-6 hydroxyl groups are positioned at the narrower rim while the secondary C-2 and C-3 hydroxyl groups are on the wider rim. CDs have several chiral centers (five in every glucose unit). The creation of inclusion host–guest complexes is assumed to be a key interface in the chiral recognition by CDs. In this case, hydrophobic groups of analyte are involved into hydrophobic cavity of CD. Secondary interactions between analyte and the hydroxyl groups on the rims can also support to the chiral acknowledgement[13].

Solubilities

Metzn is soluble in ethanol, DMF and DMSO; it is very slightly soluble in water.The equilibrium solubilities of Metzn in aqueous buffer solutions over the pH range of 1 through 10. It can be seen that the aqueous solubility of the compound is 0.1 mg/mL or less at pH values of 8 or below. The rapid increase in solubility beginning at about pH 8 is the result of the formation of the more soluble ionized species.

The separations using CDs as chiral selectors can be carried out in the polar organic mode, the normal-phase mode, and the reversed-phase mode[14].

2. MATERIAL AND METHODS

Materials

Metzn tablets (2.5 mg) were from Selco Enterprises Private Limited, Mumbai, India. Silica gel G with 13% calcium sulphate as binder, having led, chloride and iron impurities up to 0.02%, with pH 7.0 in a 10% aqueous suspension was obtained from Merck (Mumbai, India). Other chemicals and reagents, of analytical grade, were obtained from Merck (Mumbai, India) and BDH (Mumbai, India). Some of the equipment used were, a direct Q (Millipore, France) water purifier dispensing system for supplying purified water, the terminal 740 (Indolab) pH meter, previously calibrated, for pH buffer adjustments, and SYSTONIC (Panchkula, Haryana, India) spectrophotometer (single beam, spectral bandwidth-2nm,10mm matched quartz cells) for recording UV-VIS spectra in MeOH. Mobile phases and other solutions were submitted to ultrasonication with the help of an ultrasonic Elma Transsonic bath (model T700H, Tovatech, NJ, USA). Compact Quartz Polarimeter from Friends (Ambala, Haryana, India) measuring range of optical rotation: +/- 180, Division Value: 1 degree, least count: 0.05 degree or less, Monochromatic light source: 580-590 nm, Stabilization time (approx.): 5 minutes.

Extraction, isolation and purification of active API

Commercial 20 tablets of Metzn were taken to extract about 50 mg of the API. All tablets were crushed/grinded to fine powder and was subjected to extraction in MeOH by sonication for 15 min. The solutions were centrifuged at 3,000 rpm for 10 min; the residue was further extracted with MeOH and centrifuged. The combined supernatant was concentrated in vacuum and left to cool until crystals appeared. The mother liquor was decanted and the crystals were dried. The samples were further purified by recrystallization with MeOH and were used as standard analytes[4,6].

Preparation of Standard Solutions

Stock standard solutions with concentrations of 1 mg mL^{-1} were prepared using MeOH as the solvent. Working standard solutions were freshly prepared by dilution with MeOH to obtain solutions having concentrations of 0.1 mg mL^{-1}.

Procedures

Development of chromatograms

The analysis was performed on pre-coated 20 cm × 20 cm silica gel 60 F_{254} aluminium sheets Solution of Metzn was spotted (10µL) using Hamilton syringe, on thin-layer plates 2 cm above the margin. Chromatograms were developed using different solvents such as, acetonitrile, MeOH, ethyl acetate, toluene, and glacial AcOH, in various systematic compositions (binary, ternary and quaternary) to achieve the enantioseparation. Chromatograms were developed in cleaned, dried and paper-lined rectangular glass chambers pre-equilibrated with the mobile phase at different temperatures (17±2, 25±2, and 32±2 °C) for 15 min under the controlled conditions. The chromatograms so developed were dried in air for 10 to 15 min. The spots were located as dark brown spots in an iodine chamber.

Isolation of API from TLC plates

The Metzn was also spotted on Homemade TLC plates (10 × 10 cm with 1.5 mm thickness) prepared by spreading slurry of silica gel G (25 g) in distilled water (50 mL), with a Stahl-type applicator and drying the plates at room temperature and then activating them for 8-10 h at 60 ±2 °C. In one of the methods, the slurry was prepared by dissolving requisite amount of b- CD in the slurry[11]. Spots corresponding to each enantiomer (separated from the racemic mixture) were marked on the chromatograms and iodine was allowed to evaporate. Silica gel of each of the spots was scraped (from nearly 10 chromatograms); the combined silica gel for each spot was extracted by sonication with MeOH (5 mL, five times). The combined extracts for each of the analytes were filtered through Whatman filter paper grade number-1 and the residues were further treated thrice with the same solvent mixture and filtered. The combined extracts (for each case) were then dried and characterized.

Method Optimization

The effect of the concentration of impregnating reagents with silica-gel and in the mobile phase was investigated. It was observed that the best resolution was at 0.5% of the impregnating reagent. As the

concentration was decreased to 0.4% and 0.3%, the resolution became poorer in all the solvent combinations. An increase in the concentration of the impregnating reagent to 0.6% also resulted in poor resolution.

The chromatographic conditions were also optimized by spotting the drug on TLC plates and developing different solvent systems in order to achieve the best separation. Initially, a combination of Toluene-ethyl acetate-MeOH- glacial AcOH- β- CD in different ratios was tried. After trying several combination ratios, best separation was observed with Toluene-ethyl acetate-MeOH-glacial AcOH – 5mM CD (6:4:1:0.1:1:0). The average R_F values of enantiomers of Metzn using both the methods is tabulated in Table 1.
Chromatographic plates showing the separation of enantiomers of Metzn is shown in Fig 3

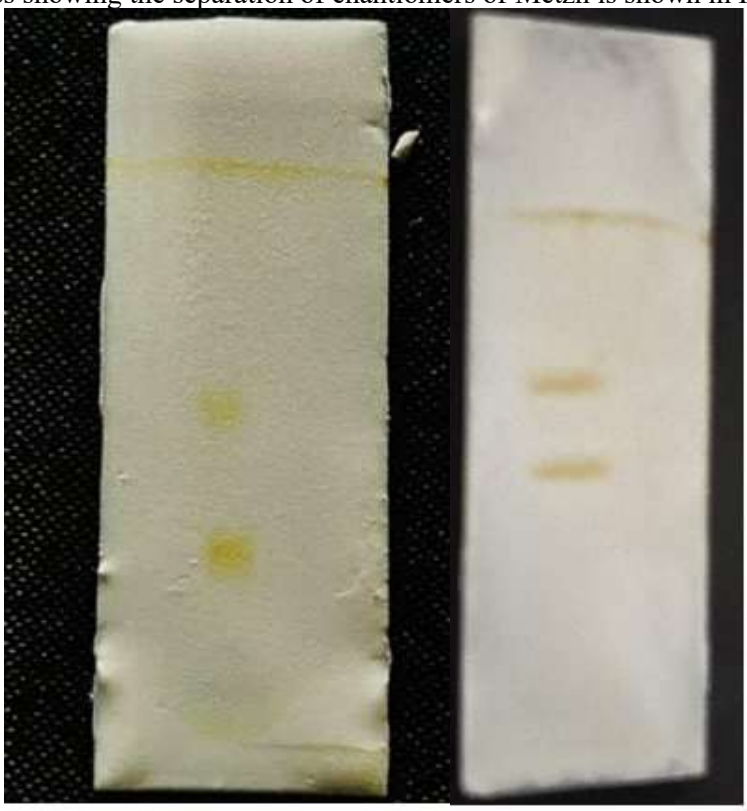

Fig. 3 Photographs of TLC plates, showing separation of enantiomers. a) TLC plates prepared by added chiral selector to the slurry. b) the separation of enantiomers where chiral selector was added to the MP. Chromatographic conditions are mentioned in Table 1.

Table 1. Chromatographic data of separation of Metzn enantiomers on TLC plate

Method	Mobile phase	R_f (lower spot)	R_f (upper spot)	Resolution (Rs)
Adding chiral selector to the slurry	ethyl acetate-toluene-MeOH-glacial AcOH (4:6:1:0.1)	26	44	2.24
Adding chiral selector to the mobile phase	ethyl acetate- Toluene-MeOH-glacial AcOH: CD (4:6:1:0.1: 1.0)	28	49	2.31

3. Results and Discussion

CD and their derivatives have been extensively used as chiral selectors for HPLC chiral separation due to their natural chirality and ability to form inclusion complex with molecules via hydrophobic cavity. It was reported that the combination of hydrophobic interactions and steric effects from the substituents present on the cavity entrance are believed to be responsible for the observed enantioselectivity in reversed-phases HPLC. The chiral recognition of CD CSPs under reverse-phase conditions is thought to be driven by the inclusion complexation between the hydrophobic moiety of analyte and the relatively non-polar interior of the CD cavity. Therefore, the dimension of CD-cavity is likely to have substantial effects on the enantioseparation ability of CD-bonded CSPs under reversed-phase conditions[13,14]. Under normal-phase conditions, however, the CD-cavity is more

likely to be occupied by the non-polar molecules of the mobile phase and the chiral recognition is mainly attributed to the interaction and hydrogen bonding between sites provided by the aromatic and carbonyl substituents on the derivatized CD. Similar recognition mechanism may be applied to explain the enantioseparation of Metzn on β-CD. Their bicyclic moiety containing the chiral center was apt to be "tight-included" into the cavity of β -CD, which resulted in their separation on β-CD[15].

4. Conclusion

The method reported herein provides a simple, rapid, and effective approach in the planar mode for separation of enantiomers of Metzn, which can be realized even in a small laboratory. The method may be applied for successful resolution of a variety of pharmaceuticals and other organic racemic mixture.

Conflict of Interest

Authors declare no conflict of interest

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