Liquid chromatographic enantioseparation, determination, bioassay and isolation of enantiomers of Ketorolac: A review

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ABSTRACT

Ketorolac (Ket) is a potent non-narcotic analgesic drug (among the nonsteroidal anti-inflammatory drugs). The physiological activity of Ket resides with (S)-(−)-Ket while the drug is marketed and administered as a racemic mixture. Therefore, it is desirable that the pharmacokinetics is measured and quantified for enantiomers individually and not as a total drug. The present paper is focused on relevant literature on LC enantioseparation of (RS)-Ket along with bioassay, pharmacokinetic and clinical studies within the discipline of analytical chemistry. HPLC and Thin layer chromatography (TLC) methods using both direct and indirect approaches are discussed. The methods provide chirality recognition even in the absence of pure enantiomers. Besides, a brief discussion on resolution by crystallization and enzymatic methods is included. The most interesting aspects include establishment of structure and molecular asymmetry of diastereomeric derivatives using LC-MS, proton nuclear magnetic resonance spectrometry, and by drawing conformations in three dimensional views by using certain software. A brief discussion has also been provided on the recovery of native enantiomers by TLC.

KEYWORDS

(RS)-Ketorolac, enantioresolution, bioassay, liquid chromatography, enzymatic methods, isolation of enantiomers

INTRODUCTION

Ketorolac (Ket) is a synthetic pyrrolizine carboxylic acid derivative, and chemically designated as (RS)-5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid (Fig. 1a). It is a potent non-narcotic analgesic drug (among the NSAIDs, i.e., nonsteroidal anti-inflammatory drugs). Ket was patented in 1976 and was approved by USFDA for medical use in 1989 [1] while different formulations of Ket were approved by the FDA in different years (during 1992–2010). Ket was introduced as a safer intravenous alternative to opioid analgesics [2]. It is available as a generic medication. In 2017, it was the 271st most commonly prescribed medication in the United States. Ket is mainly used (as the water-soluble tromethamine salt to treat painful states such as post-operative pain, acute musculoskeletal pain, and dental pain. The total combined (oral, intravenous or intramuscular dosing) duration of use of Ket should not exceed 5 days. Ket can cause peptic ulcers, gastrointestinal bleeding and/or perforation of the stomach or intestines which can be fatal. Though the mechanism of action of Ket, like that of other NSAIDs, is not completely understood but may be by blocking cyclooxygenase 1 and 2 (COX1 and COX2), thereby decreasing production of prostaglandins. There is an increased risk of gastrointestinal bleeding when selective serotonin reuptake inhibitors (SSRIs) are combined with NSAIDs [3]. Nevertheless, it is more effective than other NSAIDs in reducing pain from both inflammatory and non-inflammatory causes. It also has antipyretic properties 20 times more potent than those of aspirin.

Ket possesses one stereogenic center within the pyrrole ring and thus differs from most other NSAIDs, in which the asymmetric carbon is within the 2-arylpropionic acid side-chain.
The physiological activity (the analgesic and cyclooxygenase inhibitor activity, and also the ulcerogenic activity) of Ket resides primarily with \((S)/(R)\)-Ket \[4\], Ket tromethamine salt, m. p., 165–167°C; \(\Delta H^\circ = -176^\circ\) \((c = 1\text{ in methanol})\). The evaluation of COX inhibition activity of the two enantiomers of Ket showed that \((S)\)-Ket exhibited potent COX1 and COX2 enzyme inhibition, whereas \((R)\)-Ket was >100-fold less active on both COX subtypes \[5\] while the drug is marketed and administered as a racemic mixture.

After absorption or injection, the Ket-tromethamine salt dissociates into the anion form of ketorolac at physiological pH \[6\]. The pharmacokinetics of Ket is linear; at the higher recommended doses, there is a proportional increase in the concentrations of free and bound racemate. It has been observed that in humans there was very little or no interconversion of \((R)\)-Ket to \((S)\)-Ket, and interconversion of \((S)\)-Ket to \((R)\)-Ket was minimal (6.5%); the data also demonstrated that the kinetics and interconversion of the enantiomers of ketorolac were different in animals and humans as well as from most other NSAIDs \[4\]. It is thus contended that such (and certain other) differences shown \textit{in vivo} by \((S)\)- and \((R)\)-Ket enantiomers are because of differences in their affinities at receptor sites, affinities for tissue and protein binding sites, and rates of biotransformation and the interaction or binding with the drug receptor, enzyme or, an antibody in a stereoselective manner.

In the field of drug discovery, significant efforts have been made to develop enantiopure Ket with the aim of enhancing beneficial efficacies and/or eliminating side effects. Some of the pioneering papers on enantioselective synthesis of \((S)\)-Ket include (i) considerably efficient and practical five-step route providing \(\approx 45\%\) yield (nearly racemic) from pyrrole \[7\], (ii) direct coupling of pyrroles (at C-2) with carbonyl compounds (using potential toxic reagents) furnishing \((S)\)-Ket up to \(90\%\) ee determined by chiral HPLC and 38% isolated yield over two steps \[8\], and (iii) synthesis of optically enriched \((S)\)-Ket with 89% ee in 82% yield using a Friedel-Crafts type C–C bond forming cyclization of pyrrolic allyl alcohol in the presence of a bimetallic gold(I) salt complex \[9\]. These methods and related literature too suggest that only enantioselective synthesis may not be sufficient to get the single desired enantiomer. The economic viability and technical feasibility of an enantioselective industrial method of synthesis and/or separation/resolution method would remain an important point of consideration. As the number of enantiomeric (still racemates by and large) drugs launched into the market is increasing yearly, the need for fast and efficient enantioseparation methods with minimal costs is becoming more compelling.
Approach to LC enantioseparation

There have been two basic approaches for enantioseparation by chromatographic methods: a direct and an indirect method. Both the approaches have their own advantages and limitations and choice of method would depend upon source and amount of sample available, chemical structure of the analyte and the ease of availability of laboratory facilities. Different reviews and monographs on enantiomeric resolution [10–13] including those on separation of enantiomers of different active pharmaceutical ingredients (APIs) of a variety of racemic drugs [14–16] have discussed various issues related to ‘direct’ and ‘indirect’ (modes of) enantioseparation, therefore, such general aspects are not being discussed in this paper.

Literature reveals that there has been no attempt to review methods of LC enantioseparation of Ket in spite of its pharmaceutical applications in modern clinical therapy and the importance of enantioseparation. The present paper is focused on relevant literature on separation and determination of enantiomers of Ket using liquid chromatography involving enantioselective bioassay and enantioresolution from pharmaceutical formulations. It does not claim to cover all the references on the subject but it covers major, important references with a briefing of methods that lead to sensitive enantioseparation and thus provides a good starting point for chemists in analytical and pharmaceutical areas and in enantioselective organic synthesis, for control of enantiomeric purity. The present paper is in line with the growing demand in pharmaceutical industries for pure chiral products with increasing awareness of use of single enantiomer as the key factor which drives and supports the growth of chiral technology globally.

Various CSPs used for HPLC resolution, bioassay, pharmacokinetic and clinical studies were based on proteins (like α-glycoprotein, and human serum albumin), macrocyclic glycopeptides (MG), and polysaccharides. The alpha-globulin glycoprotein, and peptide moieties from MGs like teicoplanin aglycone, vancomycin, and Ristocetin A are covalently bonded to high purity 5 μm silica while retaining stability and essential components for chiral recognition. The immobilization of α1-acid glycoprotein (α1-AGP) on spherical 5 μm silica micro-particles led to Chiral AGP as the second generation CSP. Polysaccharide-based CSPs have, for example, amylose tris-(3,5-dimethylphenylcarbamate) or amylose (tris-5-chloro-2-methylphenylcarbamate) or cellulose tris(3-chloro-4-methylphenylcarbamate) or certain other such derivatives as the chiral selectors. Discussion on the focal points of this article is systematically and, by and large chronologically, presented with the application of various such CSPs.

HPLC SEPARATION AND DETERMINATION OF ENANTIOMERS IN PLASMA USING CSPS WITH DIFFERENT CHIRAL SELECTORS

Literature reveals a few reports on pharmacokinetic studies establishing enantioselective distribution of Ket enantiomers using chiral HPLC. The characterization of the stereo-specific pharmacokinetics by these methods may be useful in future studies looking at multiple dosing because of the difference in pharmacological properties of the two isomers. Multiple dosing means that each successive dosage(s) are administered before the preceding doses are completely eliminated, and accumulation of the drug would occur within the body yielding a higher plasma drug concentration.

The HPLC conditions and results of enantioseparation of (RS)-Ket for the pharmacokinetic studies using CSPs with different chiral selectors are discussed below.

Human Serum Albumin (HSA) or (S)-tert-Leucine

One of the initial reports, for an enantioselective assay and determination of the enantiomers of Ket and its metabolite (p-hydroxy-ketorolac, p-OH-Ket) in human plasma and urine [17] was from Diaz-Perez et al., in 1994. The second major metabolic route of Ket in humans accounts for 12% of the administered dose in the form of p-OH-Ket [18]. The HPLC analysis showed [17] that the enantioselective distribution of Ket enantiomers in plasma and urine was [(R)-(+-)Ket: (S)-(--)Ket = 3.89 ± 0.93] and [(R)-(++)Ket: (S)-(--)Ket = 1.26 ± 0.09], respectively, i.e., the concentrations of (R)-Ket were greater than (S)-Ket in both the plasma and urine. Diaz-Perez et al., [17] utilized a coupled achiral-chiral HPLC system. The initial separation of Ket from p-hydroxy-Ket and matrix interferences was made on a C18-stationary phase and the enantioselective separation of the two solutes was accomplished on chiral analytical column of HSA-CSP, and the two columns were attached in sequence and the assay was carried out without the necessity of column-switching technique. The mobile phase was sodium dihydrogen phosphate-disodium hydrogen phosphate (0.05 M, pH 6.9), modified with 13% (v/v) acetonitrile with a flow rate of 1.0 mL min⁻¹ and detection at 313 nm. Under these chromatographic conditions, the enantiomers of Ket and p-OH-Ket were resolved on HSA based column after independent injections of rac-Ket and rac-p-OH-Ket and the enantioselective resolution factor (R) was 8.0 and 2.73, respectively. The limit of detection was 50 ng mL⁻¹ and 25 ng mL⁻¹, respectively, for each enantiomer of Ket and p-OH-Ket. The method was validated for use in pharmacokinetic and metabolic studies to quantitate the enantiomers of p-OH-Ket and Ket in plasma and urine from patients undergoing cataract surgery and dosed with rac-Ket.

Vakily et al., in 1995 reported [19] a direct HPLC assay to delineate pharmacokinetics of Ket in both humans and rats. After administration of racemic Ket, the pharmacokinetics studies revealed that plasma concentrations of (R)-Ket were significantly higher than those of (S)-Ket. The (S)/(R) ratio in human plasma was found to be 0.26 while it was 0.45 in rat plasma. This was likely due to more extensive plasma protein binding of (S)-Ket than the (R)-Ket. Vakily et al., [19] adopted an approach similar to the one reported by Diaz-Perez et al., [17] in terms of using a 12.5 cm Partisil ODS 3 column followed by a 5 cm chiral tert-leucine column.

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[chiral phase consisted of (S)-tert-leucine and 3,5-dinitroaniline or (S)-1-(a-naphthyl)ethylamine urea linkage, electron acceptor designed for the separation of carboxylic acids, and amino acid derivatives, from Phenomenex]; the baseline separation of Ket enantiomers was achieved using a mixture of methanol-ethyl acetate-isopropanol (50:50:2, v/v) in 0.5 mM ammonium acetate (pH around 4.75) as the mobile phase at a flow rate of 0.8 mL min\(^{-1}\). The sensitivity of the assay was 25 ng mL\(^{-1}\) which was much lower in comparison to the value reported by Diaz-Perez et al., [17]. No chiral conversion was noted when individual pure enantiomers were incubated in various buffer solutions with pH ranging from 1 to 11 using this direct approach. However, complete racemization of (S)-Ket was observed by addition of 100 μL of 1 M NaOH to 100 μL of (S)-Ket (containing 1 μg per mL) [19]. The reason for racemization may be attributed to the reaction conditions to which Ket was exposed.

### Ristocetin A

Lynn et al., in 2007 reported [20] plasma concentrations of the (R)(+)- and (S)(-)-enantiomers of Ket from a randomized study of Ket pharmacokinetics by administering racemic Ket in infants aged 6–12 months and toddlers aged 12–18 months, postoperatively. It was contended that the infants of 6–18 months rapidly cleared the active (S)(-)-Ket isomer (elimination half-life of 50 min), while the (R)(+)-isomer clearance was slower. Under identical experimental conditions, the pharmacokinetic parameter values compared favorably in infants aged 2–6 mo [21] showing elimination half-lives of 40 min for the (S)(-)-Ket which means that the stereo-specific pharmacokinetics of Ket in infants aged 2–6 months showed more rapid elimination of the analgesic (S)(-)-isomer. However, (S)(-)-Ket clearance was observed to be 4–5 times faster than the (R)(+)-isomer. Lynn et al., [20] used Ristocetin A based chiral column for separation and determination of concentrations of the (R)(+)- and (S)(-)-enantiomers of Ket in plasma by HPLC with UV detection at 313 nm. Baseline separation of the enantiomers was achieved using an Astec Chirobiotic R chiral column with a binary gradient mobile phase of 0.07 g ammonium formate and 24 μL glacial acetic acid per liter methanol and increasing to 100% methanol for 17.5 min at a flow rate of 1.0 mL min\(^{-1}\). The retention times of (R)(+)- and (S)(-)-Ket were 6.6, and 7.2 min, respectively. The lower limit of detection was 0.01 μg mL\(^{-1}\).

### α1-Acid glycoprotein

Chiral-AGP columns (first developed by immobilization of orosomucoid onto silica microparticles by Hermansson in 1983) [22], were relatively more extensively used in comparison to the CSPs containing human serum albumin, or (S)-tert-leucine, or Ristocetin A, a glycopeptide antibiotic [23] as the chiral selectors, during the same period. α1-Acid glycoprotein (AGP) or orosomucoid based CSPs are expensive to the extent that they are out of reach of majority of small laboratories and Institutes and have a short life with limited applications and do suffer with one or more types of limitations as they require (a) storage in a solution of isopropanol-water (2:8, v/v) at room temperature or at 1–4 °C when not in use, and (b) washing after each use with water for 30 min followed by washing with isopropanol-water (2.8, v/v) for 30 min; mobile phase with greater than 25% isopropanol is not recommended as it may cause protein precipitation. Besides, the separation power of AGP columns is reported to decrease with an increasing number of loads for enantioseparation. The HPLC conditions and results of enantioseparation of (RS)-Ket for the pharmacokinetic studies establishing enantioselective distribution of Ket enantiomers using chiral-AGP columns are discussed below. In most of the cases experiments have been carried out using plasma samples taken from healthy volunteers administered with (RS)-Ket or the plasma samples were spiked with the racemic Ket or its enantiomers, therefore, these may not deal with enantioselective distribution of Ket enantiomers in plasma or other clinical samples. Nevertheless, depending upon the nature of sample and facilities available any one of these methods may be successful for pharmacokinetic studies establishing enantioselective distribution of Ket enantiomers.

In the year 1994, Mills et al. [24] administered 90 mg of racemic Ket trometamol intravenously over 10 s to an adult merino female sheep and collected the arterial blood samples during the next 90 min. The samples of Ket were prepared, by a very cumbersome sample preparation approach, for injecting onto the HPLC column from the plasma; it required acidification of plasma (reducing the pH to 1.5) followed by extraction with 0.4 mL of 10% pentan-2-ol in hexane and further extraction into 0.15 mL of base (20 mM NaOH, pH to 7–8) and centrifugation at each step. The authors did not report enantioselective distribution of Ket enantiomers in plasma. However, the resolution factor (R\(_s\)) was 3.7 and the chromatogram of the plasma sample revealed that the level of (R)(+)-Ket was much higher than the level of (S)(-)-Ket. The HPLC conditions reported by Mills et al., [24] for resolution of [(R)(+)-] and [(S)(-)-] for pharmacokinetic studies in plasma were application of α1-acid glycoprotein column and 4% isopropanol in sodium dihydrogen phosphate buffer (0.1 M, pH 5.5) as the mobile phase, at a flow rate of 0.9 mL min\(^{-1}\) and detection at 325 nm. The metabolite p-OH-Ket was well separated from the parent compound, but was not resolved enantiomerically.

Later, in the same year 1994, Jones and Bjorksten [25] determined the concentration of Ket enantiomers in human plasma of a patient undergoing major abdominal surgery who was given an intravenous infusion of racemic Ket for three days. The blood samples were taken for three days following surgery. The method did not address stereospecifity and enantimetric interconversion during the assay procedure. However, the levels of (R)(+)-Ket were found seven to ten times higher than the levels of (S)(-)-Ket during the infusion, as shown in the results reported by Mills et al., [24]. Plasma samples containing Ket were acidified and extracted into diethyl ether. The ethereal extract was evaporated to dryness and the residue reconstituted in
mobile phase before injecting (a 10 μL aliquot) onto the chiral column. Jones and Bjorksten [25] used chiral-AGP column. The plasma sample after a simple work up was injected onto the column. Potassium dihydrogen phosphate buffer (20 mM, pH 7) - iso-propanol (99.5 : 0.5, v/v) was used as mobile phase with detection at 320 nm; the LOD was 5 ng mL⁻¹ for each enantiomer and the enantioselective resolution factor (Rₛ) was 2.5. Variable injection volumes were used to quantify Ket enantiomers though less than 5 nmol was normally recommended as injected amount for the Chiral AGP column. The method required column washing after each use with water for 30 min followed by 25% iso-propanol to maintain column performance.

Tsina et al. [26] in 1996 (at Syntex, U.S.A.) reported HPLC determination of (R)- and (S)-Ket in clinical samples of human plasma by using an AGP column after extracting Ket enantiomers from plasma by using C₁₈ solid-phase extraction (SPE) column and then injecting the reconstituted extract onto the chiral column. Mobile phase of iso-propanol-phosphate buffer (0.05 M; pH 5.5) at a ratio of 5:95 (v/v) and a flow rate of 0.4 mL min⁻¹ with UV detection at 317 nm provided the quantification limit of 20 ng mL⁻¹ for (R)- or (S)-Ket using a maximum volume of 1.0 mL of plasma for analysis. The method was specific for each enantiomer and that less than 1.8% of one enantiomer was measured as the other enantiomer.

In 1998, Campanero et al. [27] quantified [(R)-(+-)] and [(S)-(+-)-Ket] in human plasma for pharmacokinetics studies. Plasma samples were collected prior to dosing and at different time intervals in 24 h after dosing 30 mg of racemic Ket trometamol intravenously over 60 s to ten healthy volunteers. The final extraction of the plasma sample was made with methyl-t-butyl ether which was evaporated to dryness and the residue reconstituted in mobile phase was chromatographed on a chiral-acid glycoprotein column (Astec) using 8.5% iso-propanol in phosphate buffer (0.09 M Na₂HPO₄·H₂O, 0.01 M Na₂HPO₄, 0.002 M dimethylcylamine, pH 5.5) at a flow-rate of 0.5 mL min⁻¹. The LOD was 2 ng mL⁻¹ for both enantiomers (using a volume of 1.0 mL of plasma for analysis) and no racemization was observed. After a few analyses, the column was required to be washed consecutively with 120 mL each, of water, 25% iso-propanol and water at a flow rate of 0.2 mL min⁻¹ before re-equilibration with mobile phase. However, the method is simpler in terms of sample preparation, and extraction procedure. Quality control samples and the working standard solutions containing rac-Ket in plasma and water, at three different concentrations, did not show changes in [(R)-(+-)-] and [(S)-(+-)-Ket] concentrations, after one month of storage at 4 °C. Overall, the method could be rated better than those reported by Mills et al., [24] and Jones and Bjorksten [25]. The method developed by Jones and Bjorksten [25] had comparable sensitivity to this method but had quantification limits and run time twice. The methods were specific for each enantiomer but were not suitable for pharmaceutical industries as per prescribed guidelines (of less than 1% of the enantiomeric impurity) since less than 1.64% of one enantiomer was measured as the other enantiomer.

A LC-MS/MS method was developed by Patri et al., in 2011 [28] for simultaneous determination of [(R)-(+-)] and [(S)-(+-)-Ket] in human plasma samples of a bioequivalence study. The study involved twenty-two healthy male volunteers of 18–45 years of age who were given 10 mg of Ket trometamol mouth dissolving tablet (of Ranbaxy Laboratories Ltd) (test) under fasting condition and the same was compared with the 10 mg tablet of Roche Products Ltd (as reference drug). Venous blood samples were collected at different time intervals in 24.00 h after dosing and plasma was separated by centrifugation for analysis. The mean Cmax of (R)-(+-)-Ket was found nearly 2.4 times higher in comparison to that of Cmax for (S)-(+-)-Ket for both the reference drug and test products. For development of HPLC separation conditions plasma samples were spiked with solutions of individual standard enantiomers of Ket. The method was mainly developed for SPE for quantification of Ket in human plasma. Internal standard [(S)-(+-)-Ket] dilution (50 μL of 2,500.00 ng mL⁻¹) was added to 300 μL plasma sample followed by addition of 100 μL of 5% formic acid solution (v/v). After SPE and further work up, samples were injected into the LC/MS/MS for analysis. Chiral-AGP column and a mobile phase of ammonium formate buffer (10 mM, pH 4.70 ± 0.05)-acetoniitrile (85:15, v/v) and 70:30, v/v in a gradient time program [28] was successful to quantify the enantiomers. Quantification was achieved using a positive electrospray ionization (ESI+) interface under multiple reaction monitoring (MRM) condition. The LOQ was 9.37 ng mL⁻¹ and 6.09 ng mL⁻¹ for [(R)-(+-)] and [(S)-(+-)-Ket], respectively. The Chirobiotic V2 column with a mobile phase of ammonium formate buffer (10 mM, pH 4.00–5.50)-acetoniitrile (70:30, v/v) was not successful for baseline chromatographic resolution. The SPE approach [28] had certain advantages over liquid-liquid extraction (LLE) reported by Ing-Lorenzini et al [29] (for use on polysaccharide based column). The SPE approach resulted into (i) almost 100% recoveries, (ii) less time for extraction with cleaner extracts, (iii) selective removal of matrix components, and (iv) less consumption of solvent without emulsion formation.

To quantify the impact of pregnancy on Ket disposition, samples of plasma and urine were collected at delivery and in postpartum of women by Kulo et al., in 2014 [30] since Ket is frequently used as analgesic in this population. The results showed that [(S)-(+-)-Ket] was cleared faster compared to [(R)-(+-)-Ket] in women at delivery compared to the same women 4–5 months postpartum. SPE columns were used before injecting the reconstituted extract sample (volume varied between 10 and 60 μL) onto the chiral column. Direct resolution and quantification of enantiomers of Ket was achieved in such samples of plasma and urine using chiral AGP column and a mixture of isopropl alcohol and 50 mM potassium dihydrogen phosphate buffer pH 5.5 (5:95, v/v for plasma and 4:96, v/v for urine) as the mobile phase at a flow rate of 1.0 and 0.5 mL min⁻¹, respectively with UV detection at 313 nm [30]. The LLOQ for [(R)-(+-)] and [(S)-(+-)-Ket] was 0.025 mg L⁻¹. No interferences with endogenous compounds from pooled
human drug-free plasma and drug-free urine were detected using the extraction and chromatographic conditions. To illustrate the applicability of the method (\(R\))-(\(+\))-Ket and (\(S\))-(\(\neg\))-Ket were quantified in 80 plasma samples and in 16 urine samples collected in eight women at delivery and in 4-5 months postpartum. Previously reported method [28] was relatively time consuming, expensive and complicated. The present method provided identical procedures for determination of racemic Ket and its enantiomers in both plasma and urine [30].

The chiral AGP column with mobile phase of 0.1 M sodium phosphate buffer (pH 4.5)–isopropanol (98:2, v/v) at a flow rate of 1 mL min\(^{-1}\) and detection at 322 nm in RP mode was found successful in baseline resolution (as shown in Fig. 2) of enantiomers of Ket (\(R_k = 2.3\)) with LOD value of 5 ng mL\(^{-1}\) for each enantiomer [31]. A Chiralpak AGP column (having \(\alpha_1\)-acid glycoprotein as the chiral selector immobilized on spherical 5 \(\mu\)m silica particles) was found successful in resolving enantiomers of Ket [32] with 10 mmol L\(^{-1}\) ammonium acetate (pH 5.5)-isopropyl alcohol (97:3, v/v) as the mobile phase at a flow rate of 1.0 mL min\(^{-1}\) and detection at 324 nm. The resolution (\(R_s\)) was 2.8. The methods [31, 32] simply present the application of AGP column for analytical separation of (\(RS\))-Ket into enantiomers.

**Effect of pH, buffer and organic modifier on enantioselectivity using AGP.** The characteristic behavior of the property and the concerns over stability of the \(\alpha_1\)-acid glycoprotein column are visible in the above cases [24–27, 30–32] for resolution of (\(RS\))-Ket, (i) the column was used in the reversed-phase mode, (ii) the enantioselectivity and the retention was regulated by the pH of the mobile phase (ranging from pH 4.5–7.0), concentration and nature of the buffer (phosphate buffer, or ammonium formate/acetate buffer), and also with the nature and concentration of the organic modifier, and (c) separation of enantiomers required high percentage of aqueous component (70–98%) in the mobile phase in the form of a buffer. The LOD was the lowest in the method reported by Mills et al [24]. The method reported by Tsina et al [26] (in 1996) for direct approach, provided a higher LOD (in comparison to other methods using AGP column) and, overall appeared to be cumbersome in terms of sample preparation. However, the studies were successful to investigate the kinetics of each enantiomer and the \(in vivo\) interconversion of the enantiomers in humans. Using \(\alpha_1\)-acid glycoprotein (AGP) columns with MS detection (via the frequently used ion sources, electrospray and atmospheric pressure chemical ionization) the nonvolatile phosphate buffers are incompatible owing to the complexity of ionization processes and highly aqueous mobile phases considerably reduce the sensitivity of the MS detection. Therefore, the selection of eluent composition requires particular attention because a solvent which is optimal for analyte ionization may not provide acceptable retention and resolution in LC [33].

**Derivatized polysaccharides**

The chiral selectors in the polysaccharide-based CSPs are immobilized (covalently bonded) or coated on silica gel support. Such columns are by no means less expensive than the AGP or any other type of chiral columns. Since the coating swells and/or dissolves in chloroform, dichloromethane, acetone, ethyl acetate and tetrahydrofuran the enantioselective capacity of these coated CSPs gets destroyed so the use of such organic modifiers is prohibited. However, the immobilized CSPs are relatively robust and can be used with a variety of solvents. However, the *guide to users* provided by the manufacturers of cellulose or amylose based CSPs clearly advise that one can use only MeCN, EtOH or MeOH as the mobile phase, use of alcohols causes back pressure, phosphate buffer for pH >8 should not be used as the basic conditions damage the silica gel matrix and high percentage of organic modifier may precipitate the buffering salt leading to irreversible clogging of column. In LC-MS approach, in particular, such organic mobile phases are used which avoid the risk of explosion inside the ion source and that help improve ionization of drugs [34].

To overcome the limitations of AGP columns in LC-MS approach, Ing-Lorenzini et al. in 2009, [29] chose a polysaccharide-based chiral column of amylose tris-(3,5-dimethylphenylcarbamate) coated on 5 \(\mu\)m silica-gel (ChiralPak® AD-RH) and a two dimensional LC-MS approach for determination of Ket enantiomers in human plasma (with simultaneous determination of paracetamol). The pharmacokinetic study in healthy human volunteers who had received an intravenous single dose of both paracetamol (1 g) and racemic ketorolac (20 mg) showed that the decline in the plasma drug concentration for (\(S\))-(\(\neg\))-Ket was 3.6 times higher than that of (\(R\))-(\(+\))-Ket. The first-dimension (non-stereoselective) separation was achieved on a RP C18 column by using a gradient solvent system consisting of 0.1% \(aq\) formic acid and acetonitrile (MeCN). The effluent between 8.9 and 9.9 min, corresponding to phenacetin (internal standard) and racemic ketorolac peaks, was transferred to a polysaccharide-based chiral column of amylose tris-(3,5-
dimethylphenylcarbamate) coated on 5 μm silica-gel (ChiralPak® AD-RH), by using a six-port switching valve. The enantiomers of Ket were subsequently separated on this chiral column using an isocratic mobile phase composed of MeCN - 0.1% formic acid (50:50; v/v) at a flow rate of 0.15 mL min⁻¹. The total run-time was less than 18 min. The effluents from both columns were directed into an ion trap mass spectrometer by using a tee equipped with an electrospray ionisation source (ESI) working in positive ion mode (ketorolac m/z 256). The LOQ, estimated according to the S/N ratio was 0.05 ng mL⁻¹ for each ketorolac enantiomer. Overall the method required an expensive experimental set-up and suffered from a major limitation of involvement of tedious and time consuming extraction procedure using liquid liquid extraction (LLE). The SPE approach adopted by Patri et al., [28] had certain advantages over liquid-liquid extraction (LLE), though Patri et al., used Chiral-AGP column for enantioseparation which has its own disadvantages in comparison to polysaccharide-based chiral columns.

In 2012 Dossau et al., evaluated [35] the resolving power of a few polysaccharide-based CSPs (namely, Sepapak-2 containing cellulose tris(3-chloro-4-methylphenylcarbamate), Sepapak-4 having cellulose tris(4-chloro-3-methylphenylcarbamate) and Sepapak-5 containing cellulose tris(3,5-di-chloro-phenylcarbamate), adsorbed on aminopropylsilanized silica of particle size 5 μm) toward resolution of a series of chiral acidic drugs including (RS)-Ket using trifluoroacetic acid (TFA), formic acid (FA), and acetic acid (HOAc) and basic butylyamine (BuA), or diethylamine (DEA) or triethylamine (TEA) as additives in the mobile phase [35]. Baseline resolution of enantiomers of Ket (Rₜ = 2.4) was achieved on Sepapak-2 column with acetonitrile as the main solvent of the mobile phase (at a flow-rate of 1.0 mL min⁻¹, and detection at 240 nm) and found acetic acid (0.2%) as the most useful acidic additive since they found it better than TFA and FA to lead to enantioseparation of Ket and other chiral acidic drugs. Using acetonitrile as the main mobile phase containing 0.2% acetic acid and one of the basic additives (0.05% of butylyamine, or diethylamine or triethylamine) also provided complete enantioresolution of Ket (with Rₜ = 2.2 or 2.3).

For a paediatric pharmacokinetic study Mohammed et al., in 2013 [36] developed a method to measure the concentration of [(R)-(+-)] and [(S)-(−)] enantiomers of Ket using micro volumes of blood from five children aged 11–15 years undergoing elective surgery who were administered intravenous (i.v.) rac-Ket 0.5 mg kg⁻¹ (maximum 10 mg). Ket enantiomers were extracted (15 min post 10 mg dose of i.v. Ket) from plasma by liquid–liquid extraction. The effects of patient covariates such as age and body size on the pharmacokinetic parameters were not assessed. Enantiomers were separated (shown in Fig. 3) by HPLC using ChiralPak® AD-RH column, containing amyllose tris-(3,5-dimethylphenylcarbamate) coated on 5 μm silica-gel using the mobile phase composed of acetonitrile-0.1% aq formic acid (50:50; v/v), delivered in isocratic mode at a flow rate of 0.2 mL min⁻¹. Detection was made by a TSQ quantum triple quadrupole mass spectrometer with an electrospray ionization source operating in a positive ion mode. The mass transition for ketorolac was: m/z 256→105. The LOQ were 0.15 ng and 0.31 ng on column for [(R)-(+-)] and [(S)-(−)-Ket], respectively. There were no interfering peaks from endogenous plasma components, and there was a clear resolution of [(R)-(+-)] and [(S)-(−)-Ket] and separation from the internal standard. In terms of resolution of enantiomers of Ket and LOQ the method is simpler and more sensitive than that reported by Ing-Lorenzini et al [29].

The median clearances of [(R)-(+-)] and [(S)-(−)-Ket] observed in this study were lower than the values reported previously by Lynn et al. [20] who used Ristocetin A based chiral column for infants and toddlers.

Guaiifenesin (Gua, having expectorant properties) is known to enhance the bioavailability of coadministered Ket, therefore, dosage adjustment of the latter is important to avoid the severe side effects that may arise with increased Ket levels upon ingestion of a normal dose. In 2014, Maher et al., [37] collected the plasma sample of a healthy female volunteer who was administered orally 100 mg Gua and 10

Fig. 3. HPLC separation using ChiralPak® AD-RH column, containing amyllose tris-(3,5-dimethylphenylcarbamate) coated on 5 μm silica-gel, mobile phase: acetonitrile-0.1% aq formic acid (50:50; v/v), flow rate 0.2 mL min⁻¹, run time 6 min, detection by a TSQ quantum triple quadrupole mass spectrometer, the mass transition for Ket was: m/z 256→105. The peak at 3.78 min corresponds to [(R)-Ket] and the peak at 4.81 min corresponds to (S)-enantiomer.[36] Adapted from [36], Mohammed, B. S.; Engelhardt, T.; Cameron, G. A.; Hawwa, A. F. Bio- pharm. Drug. Dispos. 2013, 34, 377–386.
mg Ket; venous blood was collected after 1.5 h of drug administration. Plasma was extracted by LLE method and the clean-up was based on protein precipitation by methanol followed by direct extraction of drug residue in ethanol. The optimized conditions for HPLC enantioresolution of (RS)-Ket along with (RS)-Gua from plasma samples included use of a polysaccharide based column (amylose-2), having amylose \((3,5\text{-dimethylphenylcarbamate)})\) under the normal phase isocratic mode, using the mobile phase composed of hexane-isopropanol-TFA (85:15:0.05, \(v/v/v\)) at a flow rate of 2.0 mL min\(^{-1}\) with detection at 305 nm. The LOD values were 0.019 and 0.023 \(\mu g\) mL\(^{-1}\) for \((R)-(+)\) and \((S)-(+)\)-Ket, while the values for \((S)-(+)\) and \((R)-(+)\)-Gua were 0.016 and 0.018 \(\mu g\) mL\(^{-1}\), respectively. Though the method was successful for simultaneous quantitative analysis of the two drugs in trace amounts and to monitor plasma levels of Ket in clinical samples the sample size was too small and should be further testified to recommend for routine work.

In 2016, two polysaccharide-based CSPs, namely, Lux Amylose-2 (LA-2) and Lux Cellulose-2 (LC-2) containing amylose \((3,5\text{-chloro-2-methylphenylcarbamate)}\) and cellulose \((3\text{-chloro-4-methylphenylcarbamate)}\), respectively, were found successful for analytical and semi-preparative HPLC enantioresolution of (RS)-Ket in spiked plasma sample [38]. Enantioresolution of (RS)-Ket in spiked plasma sample, aqueous ammonium formate buffer (20 mM, pH 3.8)-MeCN (70:30, \(v/v\)) was successful under RP conditions using photo diode array detection at 320 nm on both the columns and better resolved on amylose based column. A mixture of \(n\text{-hexane-isopropanol-formic acid (85:15:0.1, v/v/v)}}\) and \(n\text{-hexane-ethanol-formic acid (85:15:0.1, v/v/v)}}\) with a flow rate of 1 mL min\(^{-1}\) was successful for semi preparative enantioresolution using Lux Amylose-2 and Lux Cellulose-2 column under normal phase (NP) elution mode, respectively. The peaks of interest were adequately resolved and were free from interfering endogenous plasma-derived signals under acidic conditions (pH 3.8). A comparison of the separation performance of two CSPs in terms of retention and separation of enantiomers showed that the best resolution was observed on cellulose based CSP using EtOH while using iso-propyl alcohol (15%) and amylose based CSP highest retention was obtained. Formic acid (pKa 3.75) was used as preferred acidic additive, for improving Rs and peak shapes of ionizable acidic analytes [39] over TFA [40]. For complete analysis [38] it required around 7.9 min at room temperature (25 °C) which was lesser as compared to ~20 min, 12 min, and 10 min required for direct determination of Ket by HPLC using polysaccharide-based CSP (amylose-2) under NP mode [37] using AGP chiral column, and ChiralPak AD-RH column [38], respectively. The LOD and LOQ values were 5 ng mL\(^{-1}\) and 15.5 ng mL\(^{-1}\) for each enantiomer of (RS)-Ket, respectively. The LOQ was lesser [38] in comparison to the values of 0.02 \(\mu g\) mL\(^{-1}\) and 0.043 \(\mu g\) mL\(^{-1}\) reported, respectively, by Ing-Lorenzini et al. [29] and Maher et al. [37] using polysaccharide-based CSPs. Earlier, it was observed that reversal of elution order of enantiomers of Ket (and several other chiral carboxylic acid drugs) was a function of the nature of polysaccharide-based chiral columns and of the use of polar organic mobile phase in HPLC [41].

He et al. [42] in 2018, investigated the separation of Ket enantiomers under the polar organic normal phase mode using Chiralcel polysaccharide-based columns containing amylose \((3,5\text{-dimethylphenylcarbamate)}\) (AD-H), amylose \((3\text{-methylbenzylcarbamate)}\) (AS-H), cellulose \((3,5\text{-dimethylphenylcarbamate)}\) (OD-H), and cellulose \((4\text{-methylbenzoate)}\), CTMB (OJ-H) as chiral selectors coated on 5 \(\mu m\) silica-gel. CTMB-coated packing materials (with 10, 20, 30 and 40% of CTMB) were prepared according to the literature reports [43]. The packing material thus obtained was suspended in methanol and packed in stainless-steel tubes (250 mm x 4.6 mm i.d.) at 400–500 kg/m\(^2\) by a slurry method. Excellent separation was achieved on Chiralcel OJ-H column by using methanol-formic acid (100:0.1, \(v/v\)) as the mobile phase at a flow rate of 1.0 mL min\(^{-1}\) and detection at 254 nm. High values of \(\alpha=2.43\) and \(R_s=9.04\) were obtained. The method so developed was especially attractive for preparative-scale due to the high enantioselectivity and the high solubility of Ket in methanol (100 mg mL\(^{-1}\)) and resolution of 96 mg of Ket was accomplished in 65 min on the said column with 40% CTMB coating and the ee for the first and second enantiomer was 99.8% and 99.6%, respectively. Under the normal phase conditions (mobile phase consisting of hexane-isopropanol-formic acid (85:15:0.1, \(v/v/v\)), enantiomers of Ket were well separated on the OD-H column containing cellulose \((3,5\text{-dimethylphenylcarbamate)}\).

Effect of acid additive in mobile phase using polysaccharide-based CSPs. It is interesting to take note of the reports that Ket has pKa 3.54 and its enantioresolution was observed on amylose based CSP [containing \((3\text{-5-chloro-2-methylphenylcarbamate)}\)] with the mobile phase having TFA [37] (pKa 0.59) while enantioresolution was observed on cellulose based CSP containing \([(3\text{-3-chloro-4-methylphenylcarbamate)}\]), with mobile phase containing formic acid [38] (pKa 3.75) or acetic acid [35] (pKa 4.75). Since Ket is an acidic compound and its dissociation in the mobile phase is hard to avoid, therefore, an acid (TFA or acetic or formic acid) is added to suppress the dissociation of Ket. The type of acid, in general, has no profound effect on the enantioselectivity, it slightly changes the retention time. Since pKa value of formic acid is close to that of Ket it remains the acid of choice for addition to mobile phase. TFA being a strong acid acts as an efficient protonating (and ion-
pairing) agent and is known to bind intimately to polysaccharide based CSPs [44].

**Enantiorecognition on polysaccharide-based CSPs.** It is known that amylose has an α-1,4-glycosidic linkage and cellulose has β-1,4-glycosidic linkage. This difference in linkage causes a difference between the polysaccharide backbones, with multiple chiral centers on a d-glucose unit, in the CSPs of the two types and an effect on enantioselectivity of these CSPs. The cellulose phenylcarbamates have a 3/2 left-handed helical conformation and form a lyotropic liquid crystalline phase at a high concentration in solution which may be less influenced by the immobilization. On the other hand, the corresponding amylose derivatives do not form a lyotropic liquid crystalline phase, therefore, the polymer chains are relatively more flexible, and their conformation may be more sensitively changed depending on the immobilization position [45].

The bimodal (i.e. NP and RP) elution characteristics [46] of the CSPs containing both an alkyl (–CH₃) and halogen (–Cl) substituent on the phenyl group of phenylcarbamate moiety proved [38] to be advantageous over other CSPs based on derivatives of cellulose because (i) the CSPs based on chloromethyl-phenylcarbamate derivatives of cellulose or amylose provide higher fraction of free N–H and >C = O groups (i.e., free carbamate fragments), (ii) with the introduction of halogen substituent, the fraction of free carbamate groups increases while the fraction of carbamate groups involved in intramolecular H-bonding increases with the introduction of an alkyl substituent onto the phenyl moiety [47, 48], (iii) the chloromethyl-phenyl carbamate derivative of cellulose is insoluble in both the hydrocarbon-alcohol and polar eluents in contrast to dichloro-phenyl carbamate derivatives of cellulose in such solvent mixtures, and (iv) the presence of Cl atom in the backbone of the said CSP easily polarizes the racemic mixtures and leads to preferential interaction with one enantiomer. The interactions due to the presence of phenyl ring, carbonyl group (>C = O) and polar groups, (for example, –COOH), favoring π–π interactions, dipole–dipole interaction and strong hydrogen bonding between the chiral compound and the CSP generate transient diastereomeric pair resulting in the observed baseline separations.

**Teicoplanin**

In 2016, Ismail et al. [49] used a new bonding chemistry and prepared an ultra-high performance teicoplanin based CSP starting from 1.9 μm narrow particle size distribution (NPSD) fully porous Titan silica particles, as base material, as was used by Armstrong and coworkers [50]. The overall objective of the report was to study the behavior of the new CSP for its potential use in fast analysis with short columns and its wide elution mode (reversed phase and polar organic) versatility rather than studying stereo-specific pharmacokinetics or enantioreolution of (RS)-Ket in particular. However, baseline enantioseparation of (RS)-Ket was achieved on a 2 cm column of this CSP, in 1 min at a flow rate of 2.0 mL min⁻¹ using the mobile phase (i) methanol-water (85:15) + 20 mM ammonium acetate in reversed phase (Rₛ = 2.78), and (ii) acetonitrile-methanol (60:40) + 0.055% acetic acid + 0.03% triethylamine in polar organic mode (Rₛ = 2.57) with detection at 254 nm. Looking into the structure of teicoplanin (a semi-rigid polypeptidic basket surrounded by sugars) the deprotonated carboxylic group of acidic Ket was considered (by molecular modeling) to form several H-bonds with the aglycone walls making a suitable fit in the basket and resulting into resolution. To the best of author’s knowledge there is no report for the pharmacokinetic studies establishing enantioselective distribution of Ket enantiomers using this method.

**DIRECT ENANTIOSEPARATION BY TLC**

Thin layer chromatography (TLC) is an economically viable yet a consistent analytical technique in various different application fields like fast screening and quality control of pharmaceuticals, phytochemicals, and products of enantioselective synthesis and detection of counterfeit drugs for regulatory purposes. It is a simple, convenient, inexpensive, and rapid separation method requiring little instrumentation. Starek et al. [51] reviewed TLC methods and different TLC techniques, along with patent developments, which were developed and used for determination of NSAIDs (including Ket) in bulk drugs, formulations and biological fluids for the period from 1990 to 2008. Del Bubba et al. [52] reviewed chiral separations by TLC using commercial and non-commercial cellulose and cellulose-derivative plates (including commercial Chiralplates®) and stationary phases obtained by the impregnation of achiral plates with appropriate chiral selectors. Sajewicz, and Kowalska [53] stated that "Among the most challenging applications of TLC, one can name the enantioreolution of the racemic and scaled mixtures and a statement that the chiral TLC in this particular respect outperforms the instrumentally more advanced chromatographic techniques is far from being an exaggeration."

**Ligand exchange reagents as ‘chiral additive in achiral stationary phase’**

Incorporating a ‘chiral additive in achiral stationary phase’ (CAASP) of the TLC plate, in a non-covalent manner, prior to applying the samples on the plates and development of chromatogram is one of the techniques of impregnation [54]. The chiral additive may be a ligand exchange reagent or an enantiomerically pure compound. The inert character of the adsorbent is changed to a turning point where it results into greater improvements for different analytical purposes. The basic principle of chiral ligand exchange chromatography (CLEC) is the reversible coordination of chelating analyte species from the mobile phase into the coordination sphere of a metal ion that is immobilized by complexation with a chelating chiral selector and the formation of mixed ternary metal-ion/chiral selector/analyte is considered to be responsible for chiral discrimination [55, 56].
In 2019, Malik and Bhushan [57] achieved resolution of (RS)-Ket by TLC using chiral ligand exchange reagents (LER) as 'chiral additive in achiral stationary phase' and the chromatograms were developed using mobile phase having no chiral additive. Three enantiomerically pure amino acids (AA) [namely, L-tryptophan (Trp), L-histidine (His) and L-phenylalanine (Phe)] were used as chiral ligands to prepare LERs. The LERs were first prepared [57] by simple reaction of Cu(II) as a bivalent complexing ion with each of the three AAs in appropriate molar ratio of L-AA-Cu(II) as 2:1 and the $\lambda_{\text{max}}$ was recorded and compared in UV region for Cu(II) solution. Each of these LERs was mixed with the slurry of silica gel while preparing the TLC plates. Thus LER served as 'chiral additive in achiral stationary phase' and the chromatograms were developed using mobile phase having no chiral additive. Different solvent combinations were found successful for each case. The solvent systems and the resolution values ($R_s$) are given in Table 1. The spots were located with iodine vapors. The detection was successful up to 0.6 $\mu$g mL$^{-1}$ for Ket. Actual photographs of chromatograms showing TLC resolution of (RS)-Ket by use of Cu(II)-L-amino acids complex, respectively, of (i) L-Trp, (ii) L-His and (iii) L-Phe as ‘chiral additive in silica gel slurry’ are shown in Fig. 4. Thermodynamic enantioselectivity of the system, i.e. the difference in the stability of the above two diastereomeric ternary complexes is considered to be responsible for the observed enantioresolution. The stability of the diastereomeric complexes formed in CLEC is higher than the stability of the diastereomeric adducts formed by other chiral selectors [58]. The method TLC method of enantioseparation via ligand exchanged diastereomeric complexes was successful in isolation of native enantiomers via preparative TLC [57] in a very simple way. 

Obtaining native enantiomers from the ligand exchanged complexes may not always be required; the experiments so described are clearly an evidence for the formation of such diastereomeric ligand exchanged complexes and could be an approach for obtaining native enantiomers. The method is self-sustained and can be realized even in a small laboratory. Overall, it is less expensive and does not require any spray reagent as spots are visible with iodine vapors. The overall method was a bit cumbersome and lengthy in comparison to the other method of mixing the chiral selector, as such, with the slurry of the silica gel, as mentioned below.

### Table 1. The solvent systems and resolution ($R_s$) values for successful TLC resolution of (RS)-Ket by two approaches

| L-amino acid$^a$ | Solvent system | Ratio (v/v) | $R_s$ |
|------------------|----------------|-------------|-------|
| **Using LER (the Cu(II)-L-amino acid$^b$ complex) as ‘chiral additive in silica gel slurry’** | | | |
| L-Trp | Methanol-acetonitrile-dichloromethane-water | 3:6:1:0.5 | 2.2 |
| L-His | Methanol-acetonitrile-ethyl acetate-water | 2:4:3:1 | 2.0 |
| L-Phe | Methanol-acetonitrile-dichloromethane | 2:3:3 | 1.4 |
| **Using L-amino acids as chiral additive in silica gel slurry** | | | |
| L-Trp | Acetonitrile-methanol-water-chloroform | 4.5:2.5:1:2 | 4.21 |
| L-Val | Acetonitrile-methanol-water-dichloromethane | 6:2:1:1 | 3.89 |
| L-Met | Acetonitrile-methanol-water-chloroform | 5:3:1:1 | 3.86 |
| L-His | Acetonitrile-methanol-water-chloroform | 5:2.0:5:2.5 | 4.03 |

$^a$L-amino acids as Cu(II)-complexes used as LERs, Solvent front, 8.0 cm; Development time, 15–20 min; temperature, 28 °C (RT).  
$^b$L-amino acid mixed in the silica gel slurry for making TLC plates, Solvent front, 12.0 cm; development time, 25–30 min, temperature, 24 °C (RT). Detection, by iodine vapors in both cases.
and the anion of the enantiomer molecule of Ket in the (RS)-Ket sample, resulted in the formation of the transient diastereomers of the type: [(L)-Trp + (R)-Ket]/C0 and [(L)-Trp + (S)-Ket]/C0 without resorting to any covalent linkage [60–62] and along with the three point interaction proposed by Dalgliesh [63]. As per literature “Quantitative analysis in TLC is performed by using in situ densitometry, directly scanning the plate after chromatographic elution in the visible or UV wavelength regions, usually with the reflectance mode” [52] but the method mentioned above is successful in quantitative determination of the enantiomers (with detection up to 0.4 µg mL⁻¹) for each enantiomer of (RS)-Ket and their isolation in pure native forms by using the simple preparative TLC mode with the homemade plates and thus without resorting to expensive densitometric equipment.

**Recovery of native enantiomers**

The above two approaches of TLC enantioresolution involve mixing (impregnation) of the chiral selector, i.e., LER [57] in one case and the L-AA [59] as such in the other case, with the silica gel slurry used for making the TLC plates. In both the cases native enantiomers of Ket have been isolated. The only drawback in both the cases is that such a slurry method of impregnation cannot be applied with commercial precoated TLC plates. However, it is easier to isolate the native enantiomers by the latter approach [59] as the diastereomeric derivatives are ionic in nature and do not require hydrolysis of the ligand exchanged Cu(II)–l-amino acid diastereomeric complex.

The ligand exchanged diastereomeric complexes corresponding to the enantiomers, e.g., [L-AA-(R)-Ket-Cu] and [L-AA-(S)-Ket-Cu], were isolated via preparative TLC [57]. The two spots of ligand exchanged complexes were marked and the silica gel was scrapped from the plates. It was extracted with 90% ethanol by sonication. A drop of conc HCl was added to the alcoholic solution(s) containing the diastereomeric complex(es) and each of these solutions was then irradiated under microwave (80% at 800 W) for 1 min. The resulting solutions were lyophilized and each residue was extracted with dichloromethane. Since the complex broke down into constituent units and only the [(R)-(+)·]- or [(S)-(−)-Ket] went into solution since copper ion and l-amino acid were insoluble in dichloromethane. The native enantiomers dissociated from the diastereomeric complexes were, thus, obtained. The polarimetry measurements confirmed the elution order (the (+)-enantiomer eluted before the (−)-isomer) and also showed that the two isomers were in the ratio of 1:1.

In the second approach of using l-amino acids as ‘chiral additives’ in the slurry of silica gel for direct TLC enantioseparation of (RS)-Ket, the spots corresponding to the two enantiomers were marked and the iodine was allowed to evaporate off, silica gel for each spot was scrapped and was extracted with methanol. Only [(R)-(+)·]- or [(S)-(−)-Ket], from silica gel of each of the two cut spots, passed into the solution since the amino acid (say L-Trp) present with the analyte was insoluble in methanol. The enantiomers of Ket, so separated and isolated, were characterized after further simple work up and purification process and their specific rotation and other physical properties were verified.

**HPLC ENANTIOSEPARATION BY DERIVATIZATION APPROACH**

Most of the time there occurs an un-noticed error leading to misinterpretations of the enantiomeric ratio (er) value of the product of enantioselective synthesis reaction when the same is evaluated by chiral HPLC after the product is purified by

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*Fig. 4. Photographs of actual chromatograms showing TLC resolution of (RS)-Ket by use of Cu(II)–l-amino acids complex, respectively, of (i) L-Trp (ii) L-His and (iii) L-Phe as 'chiral additive in silica gel slurry'. Location of spots by iodine vapors. Solvent front, 8.0 cm; development time, 15–20 min; temperature, 28 °C (RT); location of spots by iodine vapors. [57] [adapted from Malik, P.; Bhushan, R. Ligand Exchange Thin Layer Chromatographic Enantioresolution of (RS)-Ketorolac and (RS)-Etodolac and Recovery of Native Enantiomers. J. Chromatogr. Sci. 2019, 57, 511–517]*
employing TLC or open column chromatography because there may be a change in enantiomeric ratio (ee) value during this purification process by achiral chromatography and majority of synthetic organic chemists are not aware [64]. Straightaway loading of sample onto chiral column (i.e., without appropriate purification by a simple mode of liquid chromatography) may not be feasible. Moreover, high cost and certain limitations posed by chiral columns are other deterrents. Therefore, an indirect approach using suitable chiral derivatizing reagent (CDR) should be helpful and useful to confirm correct ee or enantiomeric excess (ee). A comparison of the stereoselective data of a number of racemic drugs obtained from indirect chiral method (via diastereomer formation) with the similar data generated by the application of a direct chiral method showed that the indirect method is still continued as a viable and important tool for gathering stereoselective data of racemic drugs used in medical practice as well for those racemic drugs still in discovery and developmental stages [65].

The indirect approach using CDRs continues to gain importance in pharmaceutical and biomedical analysis even when a large number of chiral columns have become available. Some of the advantages include easy optimization of chromatographic conditions, excellent detection of the resulting diastereomeric derivatives due to chromophoric or fluorophoric properties of the CDR, and the prospects of using low cost achiral columns in comparison to chiral columns. However, the main disadvantages of the indirect method are (i) the native enantiomer loses its identity, and (ii) the potential different reactivity (both thermodynamic and kinetic) of the two enantiomers towards the chiral selector. On the other hand, in chiral analytical HPLC also the native enantiomer is not recovered and the preparative mode is extremely expensive. In spite of availability of different types of detectors (in HPLC) UV remains the method of choice for simplicity and cost considerations for detection under indirect approach.

Enantioseparation as diastereomeric esters and amides

As early as 1986, HPLC separation of (RS)-Ket was reported using indirect approach as its diastereomeric esters that were prepared by its reaction with (−)-α-phenyl ethyl alcohol (in trifluoro acetic anhydride/triethyl amine, benzene at 0–5 °C); these were separated using Lichrosorb column (9 mm × 50 cm, 10 μm particle size) and hexane-ethyl acetate (96:4, v/v) as mobile phase. Cleavage of each diastereomERICally pure ester with 45% TFA gave (+)- and (−)-Ket, both of them possessed rotations 20° lower than the enantiomerically pure Ket obtained by the classical procedure; partial racemization was observed during transesterification [66].

In another approach, diastereomeric amides of Ket were separated by analytical HPLC for quantification of its (R)- and (S)-enantiomers when present together in human plasma [67]. Derivatization was carried out with thionyl chloride/(S)-1-phenyl ethyl amine and the diastereomeric derivatives were separated under RP HPLC conditions in 12 min with 50% acetonitrile and 0.1% triethylamine in aqueous 20 mM sodium acetate buffer (final pH 5.5) at a flow rate of 2.0 mL min⁻¹ and detection at 254 nm. The LOQ for each enantiomer of Ket was 50 ng mL⁻¹ (signal-to-noise ratio >10). The peaks of interest were well resolved and were free of interfering endogenous plasma-derived signals. The derivatization procedure did not lead to detectable racemization of either enantiomer of Ket. The concentration of the enantiomer in minor amount was less than 3.0% when plasma samples spiked with either pure (R)- or (S)-Ket were assayed, stereoselectively. This analytical method for Ket enantiomers was then applied to a pharmacokinetic study in humans upon intramuscular administration of racemic drug and it was observed that the plasma concentrations of the pharmacologically active (S)-Ket were significantly less (five-fold difference) than the corresponding concentrations of (R)-enantiomer at all time points examined [67]. The results in terms of plasma levels of the two enantiomers of Ket were comparable to those reported by Jones and Bjorksten [30] using a direct approach of enantioseparation with AGP column. Using the same HPLC conditions, the concentrations of (S)-Ket in ex vivo plasma samples (expressed in terms of the enantiomer free acids) were measured following a single therapeutic intramuscular dose of the racemic Ket to four young healthy volunteers, it was shown that the time-averaged plasma concentration of the pharmacologically active (S)-Ket was approximately half that of its optical antipode [68], i.e., the disposition of Ket was markedly enantioselective. Prior to synthesis of diastereomeric derivatives, racemic Ket free acid was extracted from Ket tromethamine solution for injection (Toradol® Syntex, Sydney, Australia) followed by separation and isolation of the individual (R)- and (S)-enantiomers by the fractional crystallization of the diastereomeric cinchonidine salt as reported by Gusman et al., [66] The optical purity of each of the (R)- and (S)-Ket so obtained was estimated to be approximately 98% as determined by HPLC using derivatization method. The drawbacks of the method include the use of thionyl chloride which has been discouraged in chemical methodology since a long time, relatively high LOQ, and involvement of a step of fractional crystallization of the diastereomeric cinchonidine salt.

The diastereomeric amide derivatives were also separated by normal phase HPLC on a Microsorb silica (5 μm) column, using a mobile phase of ethyl acetate-hexane (40:60, v/v) at a flow rate of 1.0 mL min⁻¹ and UV detection at 317 nm [26]. The derivatization involved activation of carbonyl group of Ket by 1-hydroxybenzotriazole containing 1% pyridine followed by reaction with (R)-(−)-(1-naphthyl) ethylamine (of enantiomeric purity ≥99%) in presence of N,N′-dicyclohexylcarbodiimide (DCC) at room temperature with a reaction time of 2.5 h when equal amounts of the diastereomeric amides of Ket were formed; there was no report of kinetic resolution since the analyte and the reagent were in the ratio of 1:2. The LOQ for each enantiomer was 20 ng mL⁻¹ plasma. The data indicated that the method was specific for each enantiomer and that less than 3% of one enantiomer was measured as the other enantiomer.
The method was applied to the analysis of plasma from patients who had been administered Ket tromethamine (intramuscularly, or intravenously, or orally) and from healthy subjects treated either with racemic Ket tromethamine or with individual enantiomers of the drug. In the same paper Tsina et al., [26] also reported direct enantioseparation of (RS)-Ket by using chiral APG column (as discussed in Section “Recovery of native enantiomers”); interestingly (i) the quantification limits for (R)- and (S)-Ket were identical by both the methods, and (ii) the column used for direct method was more expensive, less durable and required special care though the method was slightly faster.

Semipreparative HPLC enantioseparation as diastereomeric anhydrides

Taking into account the literature (as cited above), Malik and Bhushan reported [69] a sensitive semipreparative HPLC method of enantioseparation of (RS)-Ket as its diastereomeric anhydrides. The CDR was synthesized by the reaction of (S)-(+)naproxen (Npx) with N-hydroxybenzotriazole (OH-Btz) in THF by adding DCC. So it was Npx ester (Npx-OBtz). The pair of diastereomeric derivatives was synthesized by the reaction of (RS)-Ket (dissolved in 0.1 M NaHCO₃) with the Npx ester (the CDR dissolved in acetonitrile) in presence of triethylamine under microwave irradiation (MWI) for 150 s at 80% power (800 W). The optimized conditions of synthesis (1:2 M ratio of analyte: CDR) were applied for preparative level synthesis of diastereomeric derivatives. Formation of the diastereomeric derivatives of the type, (i) [(R)-Ket-(S)-Npx] and (ii) [(S)-Ket-(S)-Npx] were considered to have been formed (as shown in Fig. 1(b) and (c), respectively). Mobile phase comprising of methanol-triethylamine phosphate buffer (pH 3.5) at a flow-rate of 1.0 mL min⁻¹ using C₁₈ column and detection at 320 nm was successful for base line separation (Rs = 6.1) of diastereomeric pair (shown in Fig. 5). A flow rate of 5 mL min⁻¹ was found successful for separation and isolation of derivatives of (RS)-Ket at preparative scale using the same analytical RP C₁₈ column. An equimolar mixture of the diastereomeric derivatives, [(R)-Ket-(S)-Npx] and [(S)-Ket-(S)-Npx], separated and isolated through HPLC, in a semi preparative mode, was run independently under the same optimized HPLC separation conditions of diastereomeric derivatives for verification. The LOD for the diastereomeric derivatives of (R)- and (S)-Ket were 3.69 and 3.02 ng mL⁻¹, respectively. The structure and configuration of the isolated diastereomeric derivatives, along with spatial orientation of groups with respect to the anhydride bond (Fig. 1(b) and (c)), were determined [69] by recording the ¹H NMR spectra and were verified by constructing the molecular models of the chemical structures. By observing the shielding and deshielding chemical shift values (in the ¹H NMR spectra) conformations were drawn in 3D view by using software ‘Chem3D Pro 12.0’. By these structures, all the δ-values could be explained well. The reported [69] chromatographic separation of diastereomeric derivatives constitutes a noteworthy separation strategy but requires the use of a relatively large amount of solvent.

Recovery of pure enantiomers.

The derivatives so obtained [69] by reaction with Npx ester (the CDR) were anhydride in nature and, therefore, were easily hydrolyzed using methanol/H₂SO₄ under MW for 6 min; the hydrolysates were freeze dried and the residue was extracted with water when only (R)- and (S)-Ket went into solution since (S)-Npx was insoluble in water. Each extract was further lyophilized and the residue was dissolved in small amount of methanol followed by crystallization of each of the native enantiomers. The native enantiomers (and (S)-(+)Npx) so isolated and purified were characterized [69] by recording specific rotation and by spectroscopic techniques like ¹H NMR, HRMS, and IR. The pure enantiomers were correlated with the chemical shift values (in the ¹HNMR spectra) conformations and were verified by constructing the molecular models of the chemical structures. By observing the shielding and deshielding chemical shift values (in the ¹HNMR spectra) conformations were drawn in 3D view by using software ‘Chem3D Pro 12.0’.

Fig. 5. Actual chromatogram showing separation of diastereomeric derivatives of (RS)-Ket on LiChrospher C₁₈ (250 mm × 4.6 mm, I.D., 5 µm particle size) column, mobile phase: methanol-triethylamine phosphate buffer (pH 3.5), binary gradient (75–25%), flow-rate: 1.0 mL min⁻¹; detection, 320 nm; injection volume 10 µL; the peak at 2.27 min corresponds to (R,S)- and the peak at 3.79 min corresponds to (S,S)-diastereomeric derivative.[69] The x-axis shows time in min and y-axis shows absorbance in mAU, [adapted from Malik, P.; Bhushan, R., New Journal of Chemistry, 41 (2017) 13681–13691]. There is no need to obtain permission to reuse own figures, diagrams, etc. that were originally published in a Royal Society of Chemistry publication [http://www.rsc.org/journals-books-databases/journal-authors-reviewers/licences-copyright-permissions/#reuse-permission-requests]
The CDR supports the future usefulness of the reagent and, overall, the method may be considered as self-sustained and complete in totality. The confirmation of molecular asymmetry of diastereomeric derivatives followed by recovery of native enantiomers from the covalently linked diastereomeric derivatives and verification of the configuration of the enantiomers make the method [69] ahead of the literature reports in the area of LC enantioseparation of (RS)-Ket (discussed in this paper) where only the application of CDR remained limited to synthesis, separation and detection of diastereomeric derivatives. Some of the indirect methods that required derivatization were tedious and time-consuming due to the application of abnormal conditions (heat, pH, etc.) [19], and required either long analysis time or were less sensitive [26].

DIRECT RESOLUTION BY CRYSTALLIZATION AND ENZYMATIC METHODS

Though the scope and focus of the present paper is on separation and determination of enantiomers of Ket using liquid chromatography it was considered worthwhile to include the few interesting reports on direct resolution by crystallization and enzymatic methods.

In order to determine the absolute configuration of enantiomers of Ket, direct resolution was effected by crystallization [66] of its cinchonidine salt from ethyl acetate which gave easily purifiable, less soluble salt of the (−)-Ket. Recrystallization of the more soluble salt did not yield the optically pure form of (+)-Ket. Therefore, the mother liquor was evaporated and the crude material was decomposed and the partially resolved (+)-Ket was converted into the diastereomeric cinchonine salt. The latter was obtained in pure form after two crystallizations from ethyl acetate. Decomposition of the above salts with dilute sulphuric acid gave pure (−)- and (+)-Ket. The absolute configuration was determined by a single crystal X-ray analysis of the amide of (+)-Ket prepared by reaction with (+)-(R)-1-(1-naphthyl) ethylamine. Since the absolute configuration of the stereogenic centre in the amine was known the (+)-Ket was deduced to be (R)- by internal reference [70]. Thus, (−)-Ket was concluded to have the (S)- absolute configuration.

In 1987, Fulling and Sih [71] reported one of the earliest examples to exploit racemization of carboxylic acid derivatives in order to achieve a dynamic kinetic resolution. Ket was prepared by hydrolysis of the corresponding ester. Whilst most lipases afforded the undesired enantiomer preferentially, a protease from Streptomyces griseus afforded very high enantiospecificity for the required (S)-enantiomer (E = > 100). The substrate was particularly prone to racemization since the intermediate enolate was well stabilized by resonance effects, although a pH 9.7 buffer was required to achieve a useful dynamic resolution reaction because the S. griseus protease is known to be stable at alkaline pH. Thus Ket was formed with complete conversion and with 76% enantiomeric excess (ee). The 12% of the (R)-Ket formed was the result of both racemization ($k_{racs}$) of (S)-Ket and competing nonenzymic hydrolysis.

Kim et al., [72] reported for the first time the resolution of Ket via esterification using Candida antarctica lipase B (CALB, commercially available as Novozym 435). Ket being a carboxylic acid reacted with various alcohols (the ethanol, 1-butanol, 1-octanol, and 1-tetradecanol). The alcohol and ground molecular sieves (4 Å) were added to a solution of Ket in acetonitrile followed by addition of the enzyme CALB (1 mass equiv.) and the mixture was shaken in an incubator at 33.8 °C. A sample drawn from the reactor was dissolved in methanol and was analyzed by HPLC using chiralpak AD column (containing amylose tris(3,5-dimethylphenylcarbamate) coated on 3 μm silica gel), and n-hexane-isopropanol-TFA (90:10:0.1, v/v) as the mobile phase at a flow rate of 0.8 mL min−1 with detection at 310 nm. (RS)-Ket reacted with n-octanol in chlorinated solvents such as dichloro methane and 1,2-dichloro methane and each enantiomer was resolved in up to 99% ee. The conversion rate was the highest in 1,2-dichloroethane (as the pure (S)-Ket was resolved in >99% ee) because the high boiling point (83 °C) of the solvent controlled the reaction temperature to increase the reaction rate without the enzyme destabilization. To get pure (S)-Ket, the temperature was acceptable from 34 to 60 °C (for reaction time ranging from 60 to 24 h) using CALB in the solvent 1,2-dichloroethane. For the enzymic hydrolysis of butyl ester of Ket with different enzymes, butanol and p-toluene sulfonic acid were added to a solution of Ket dissolved in benzene, and the mixture was refluxed for 3 h followed by extraction with ether and the crude Ket butyl ester was purified by column chromatography (n-hexane-ethyl acetate, 10:1). The product was dissolved in phosphate buffer (pH 7.0) and the enzyme (0.2–2 mass equiv.) was added to the solution and an aliquot was analyzed by chiral HPLC, as above. As reported previously [71] the protease, Streptomyces griseus, hydrolyzed Ket butyl ester in favor of pure (S)-Ket, while C. antarctica lipase B favored the (R)-Ket. It is well known that microwave irradiation is an approach for process intensification in a variety of reactions as a green process development. Microwave irradiation results in an instantaneous localized superheating which is achieved due to dipole rotation or ionic conduction. With the objective of improving reaction rate in chiral drug resolution, Shinde and Yadav [73] reported microwave assisted CALB catalyzed resolution of (RS)-Ket. The reaction mixture (consisting of (RS)-Ket, n-octanal, lipase and the solvent) was irradiated under constant microwave irradiation (30–40 W). It was found that the reaction rate improved up to 1.5-fold in a period of 3 h compared with that under conventional heating; Novozym 435 effectively catalyzed the enantioselective esterification showing excellent conversion (50%) and ee > 99% for (S)-Ket. Kim et al., [72] had obtained pure (S)-Ket in ee > 93% (with 58% Ket conversion) by several hours of conventional heating, using n-octanal. A synergism between enzyme catalysis and microwave irradiation was argued considering that (RS)-Ket might be the good microwave absorbing material and its dipole might have reoriented...
quickly under microwave irradiation, making the functional group more active at the interface of (RS)-Ket and n-octanol, therefore, the microwave absorbing character of the feed was contributing to the faster reaction rate and the improvement in the reaction rate was not purely thermal. It was further postulated that there occurred conformational changes in enzyme, making it more active, which facilitated the substrate to approach active sites of enzyme more easily under microwave irradiation. These interpretations are not supported by further experimental evidences though a theoretical model, of limited application, based on classical mechanism of esterification by lipases was postulated.

Under conventional heating, for lipase catalyzed enantioselective resolution, the reaction mixture (consisting of (RS)-Ket, n-octanol, lipase and the solvent) was agitated at 50 °C for 15 min at a speed of 300 rpm; an increase in conversion from 30 to 50% was found when speed of agitation was increased from 100 to 300 rpm and the ee and E obtained were above 99% and 200, respectively [73]. An increase in conversion rate by increasing the rate of agitation, to some extent, is in line with the approach of mechanochemical methods later reported by Pérez-Venegas et al., [74] However, the requirement of solvents was much higher in the method of agitation and conventional heating [73].

In both the situations of microwave irradiation and conventional heating, aliquots drawn from the reaction mixture were analyzed by HPLC to determine the conversion and ee using Chiralpak IA column (containing amylose tris(3,5-dimethylphenylcarbamate) immobilized on 3 μm silica gel) n-hexane-isopropyl alcohol-trifluoroacetic acid (90:10:0.1, v/v) as the mobile phase at a flow rate of 1.0 mL min⁻¹ and detection at 310 nm [73].

Liquid-assisted grinding (LAG) is grinding together individual components, either neat or in the presence of a small amount of a liquid phase, i.e., the amounts of liquid involved in the experiments are typically very small so that the solubilities of individual components are not decisive and the limitations imposed by the solubilities of the components are circumvented. The mechanochemical methods, for example, cogrinding, milling, and kneading, represent viable “green” routes [75].

Pérez-Venegas et al., [74] developed a mechanoenzymatic kinetic resolution protocol for the isolation of both enantiomers of Ket with high enantiopurity using CALB under the liquid assisted grinding (LAG) technique which employed minimal amounts of solvent to carry out chemical transformations in a ball mill. (S)-enantiomer of Ket was conveniently isolated (ee > 83%) by means of two alternative mechanoenzymatic strategies that involved either the kinetic resolution of a racemic mixture of free Ket or the enantioselective hydrolysis of racemic Ket alkyl esters. Both strategies proceeded with an equally high conversion (e = 46%) and with remarkable enantio-discrimination (E >> 500). The resolution by means of enantioselective hydrolysis of (RS)-Ket ester proceeded between 50% and 200% faster than similar strategies reported in solution [71], evidencing the efficiency of the mechanoenzymatic technique. Both enzymatic resolution procedures conducted to the enantiopure form of (R)-Ket (ee > 99%), and highly enantioenriched (S)-Ket (ee ca. 83%) [74]. Thus the activation of immobilized CALB by mechanical force employing a minimal amount of solvent constitutes a promising green strategy in the pharmaceutical field.

CONCLUDING REMARKS

Literature presented herein suggests that stereoselective pharmacokinetic, and pharmacodynamics of (RS)-Ket has been investigated mostly by chiral HPLC which at the same time provided methods for separation of (RS)-Ket into its enantiomers by direct approach. Derivatizing the racemate with a suitable CDR followed by separation of the diastereomeric derivatives using C₁₈ RP HPLC has also been successful for enantioseparation. TLC provided direct enantioseparation by using either enantiomerically pure l-amino acid or a ligand exchange reagent comprising Cu(II)-l-amino acid complex as the ‘chiral additive in achiral stationary phase’. Thus, both direct and indirect LC approaches are used and they have their own advantages/limitations. The success of enantiomeric separation is related to the availability of improved columns or the development of new CDRs or application of different enantioselectively pure molecules as chiral additives in TLC separation. However, these remain within the confines of determination or control of enantiomeric purity in very small amounts but not for separations at large scale, in real terms. Therefore, the modern industry still depends on the methods originated by Pasteur, enzymatic resolution (very selective and generally resulting into loss of one of the two enantiomers), and separation through (fractional crystallization) formation of ionic diastereomeric pair for resolution of racemates of chiral drugs and for obtaining enantioselectively pure materials.

To establish absolute configuration of diastereomeric derivatives becomes desirable in order to establish the success of enantioseparation method, by indirect approach, to be used during enantioselective synthesis or for control of enantiomeric purity of chiral drugs in industry and analytical laboratories (particularly associated with regulatory agencies) because most of the time the pure enantiomer of the analyte is not available (to prepare corresponding diastereomeric derivative for comparison of various chromatographic parameters). A look at the development of enantioseparation methods suggests interesting trends regarding internal standard selection, mobile phase contents and composition, mass spectrometric detection, determination of elution order, analytical manipulations without reference standards and enantioseparation involving multiple analytes.

Both HPLC and TLC methods discussed herein provide an insight and a choice to select a method to separate, isolate and quantify the enantiomers of Ket and to investigate their pharmacokinetics as markedly different species and not as a total drug. TLC results can often be transferred to HPLC or vice versa with some adjustment in eluting solvent.
conditions. The presentation evaluates the field’s status and is in support of the increasing awareness of use of single enantiomer and growing demand in pharmaceutical industries for single enantiomeric products.

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