

Novel Coated Cellulose Carbamate Silica Based Phase to Enhance Selectivity of Compounds of Pharmaceutical Interest

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Abstract

Modern liquid chromatographic (LC) analyses of targets in complex matrices, even with mass spectroscopic (MS) detection, more commonly are also requiring an orthogonal method to ensure peak purity and resolution of all sample impurities. This is especially true when stereoisomer identity and separation are critical criteria. To help address this gap in orthogonality, a novel coated cellulose carbamate stationary phase was developed using a re-optimized coating density, a type B 500-angstrom silica as its base entity, and a secondary amine to facilitate the coating to the base silica. The chiral phase can be used in reverse phase chromatography but is designed to work with mixed polar eluents in both polar organic and normal phase chromatography. It is also selective for achiral applications as well. A critical difference is the stability of this cellulose carbamate stationary phase at higher alcohol concentrations and the prediction capability of the elution order of analytes. This stationary phase showed greater chromatographic selectivity by requiring less alcohol modifier for elution of chiral compounds, when compared to other cellulose carbamate columns and by the use of acetonitrile (ACN) as a modifier. The Cogent EE phase is also rugged and can easily switch between different pH's without loss of resolution or memory effects. Finally this phase was used to separate optical isomers of gossypol and tramadol, and worked well in a case study where diastereomers were isolated from a previously unknown impurity.

Keywords: Chiral chromatography; Cellulose carbamate stationary phases; Gossypol, Tramadol

Introduction

In order to truly test if chromatographic separations have separated all impurities from the parent and show that the assay value of a compound that one gets from the analysis is the true value, the analytical chemist often uses another technique to test if the same value is achieved. For instance quantitative nuclear magnetic resonance (qNMR) is a popular, non-chromatographic technique to determine potency, which can then be compared to the value determined chromatographically [1]. These determinations of potency would be considered orthogonal or completely independent of each other. In this paper the term orthogonal represents different types of chromatography which are independent of each other. Changing from a C8 to C18 column is not considered an orthogonal change since the principles of reverse phase chromatography are the same for both, they differ only by slight selectivity differences. Normal phase chromatography is considered orthogonal to reverse phase chromatography, since the elution order and effect of mobile phase polarity are completely different between the two techniques.

Many recent schemes have been proposed to define the selectivity of different LC phases [2-11]. However, even if the defining criteria for selectivity varies, these schemes all show that the majority of LC phases fall into well defined arenas of restricted selectivity and present as tight groups of selectivity in reverse, normal and polar organic phase chromatography. Each stationary phase has only limited options for orthogonal applications to aid resolution.

The Cogent Type C phases [11-13] and other mixed modal columns (*i.e.* a combination of silica phase and a weak anion exchanger) introduced novel orthogonal solutions to the market. These phases are

able to function in different types of LC modes such as aqueous reverse, aqueous normal phase, hydrophilic interaction chromatography (HILIC), and can often work under traditional normal phase as well.

In searching for other ways to achieve orthogonality, researchers have also tried functionalized cellulose (or amylose) carbamate chiral phases to achieve non-chiral or isomer resolutions [14,15]. Typically however, chiral stationary phases are challenged in their ability to resolve impurity peaks, due to the attached functional groups or larger particle sizes (typically 5 to 10 μm). Impurities remain undetected in chiral separations because they either did not elute from the stationary phase or were unresolved from enantiomer peaks. Second limitation of the carbamate stationary phases is their tendency to retain achiral analytes in a manner that does not relate to their polarity or other readily definable chromatographic criteria when working in normal phase mode. To ensure the suitability of separation, one often uses an additional method to separate the impurities and confirm the purity of the compound.

The carbamate stationary phase presented here is designed for use in the method development of both chiral and achiral compounds.

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Received June 13, 2011; **Accepted** August 30, 2011; **Published** September 02, 2011

Citation: Brown L, Webster GK, Kott L, Rao NKR, Luu TA, et al. (2011) Novel Coated Cellulose Carbamate Silica Based Phase to Enhance Selectivity of Compounds of Pharmaceutical Interest. Pharm Anal Acta 2:134. doi:10.4172/2153-2435.1000134

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Because of their expense, chiral phases are not the phase of first choice for achiral development. However, the authors have seen them used on occasion when traditional reversed phase chemistries fail to yield the required separations. Examples of non-chiral impurity and degradation studies, plus isomer and excellent performance in chiral applications will be shown.

Coated cellulose derivatives, used in columns for chiral separations, pioneered by Okamoto and co workers [16] remain largely unchanged several decades after their inception. Since this work, several coated and bonded versions of these stationary phases have been commercially released. Yet, these phases still follow the elution characteristics, as the original coated cellulose chiral phases. The inherent shortcomings of these chiral phases, however, is that analytes of radically different polarity still elute in a narrow band using standard chiral LC solvent systems either neat, or modified with acids or bases (data shown below). Traditional chiral mobile phases and columns often appear limited to only resolving a single racemic pair of enantiomers. This imposes potential disadvantages for compounds with multiple chiral centers. The goal for traditional cellulose chiral phases *should* be to effectively resolve (i) several chiral centers which are comprised of meso and threo, erythrio analogues, (ii) metabolites and their degradation products from accelerated stability studies, and (iii) difficult to resolve achiral mixtures. The current limited “standard” elution condition of traditional carbamate stationary phases is not a good option for selectivity studies of complex mixtures (achiral or chiral) of compounds.

Described here is an original coated cellulose carbamate stationary phase (Cogent EE) that meets the criteria of an elution order dictated by the molecular polarity in normal phase as opposed to traditional carbamate stationary phases. The Cogent EE column illustrates robustness to higher alcohol concentrations and reduced memory effects as compared to traditional carbamate phases. Using commercially relevant resolution probe compounds, comprised of non-polar to low polarity (*trans*-stilbene oxide, benzoin and tramadol), low to moderately polar (propranolol, gossypol, warfarin and alprenolol) and a highly polar (pindolol) compounds, the selectivity of the newly developed cellulose carbamate phase is compared with a traditional cellulose carbamate phase. Therefore, by design, non-polar compounds elute before the moderately polar and the moderately polar elute before the polar compounds in analytical mixtures under normal phase chromatographic conditions.

Materials and Methods

Apparatus

The LC data reported was generated using either a Waters (Milford, MA) Alliance 2695 Module equipped with a Waters 996 photodiode array detector or an Agilent (Santa Clara, CA) 1200 LC system using a 1200 Series Multiple Wavelength Detector. Data was processed using Atlas Version 8.20.2.7047, Thermo Electron Corporation (Waltham, MA). The cellulose carbamate phases used for this study were (1) 100x 4.6 mm, 5 µm Cogent EE columns from MicroSolv Technology Corporation (Eatontown, NJ) and (2) 250 x 4.6 mm, 5 µm OD columns from Chiral Technologies (West Chester, PA). LC data was collected at 254 nm using a flow rate of 1 mL/min and a column temperature of 27-30°C for this study.

Reagents

All chiral markers and mobile phase additives were used as received from Sigma (St. Louis, MO). The ethanol (denatured), isopropanol and

cyclohexane were LC grade from Sigma (St. Louis, MO) as well. The water used was USP grade from an in-house supply.

LC Method Gradients

Because of the difference in selectivity for the Cogent EE and traditional cellulose carbamate phases, they were screened using optimized gradient profiles for each column. Typically, the EE requires much less alcohol to resolve nonpolar analytes and more alcohol to elute polar analytes in order to resolve the enantiomeric pair of interest.

Representative screening gradient profiles for the EE phase are presented in Table 1. Premixed mobile phases are recommended with the EE due to the low initial alcohol content used with gradients.

Results and Discussion

EE robustness and selectivity

trans-Stilbene oxide (TSO), benzoin, propranolol, warfarin, alprenolol, and pindolol are all commonly assessed chiral targets that have been very well characterized on cellulose carbamate coated stationary phases from various manufacturers. Chiral resolution for the non-polar analyte (TSO) is obtained by eluting with a solvent system comprising of hexane (99.5%) and isopropanol (0.5%) on an EE stationary phase column. Under these conditions on a standard cellulose carbamate column, the analyte is retained and elutes only when the concentration of isopropanol is increased to 10%.

Table 2 shows solvent systems compatible to the Cogent EE phase, for the chiral separation of the compounds with well-known, traditional cellulose carbamate columns. It can be seen from these results that chiral conditions used with the traditional cellulose carbamate phase are unsuitable for the Cogent EE phase, confirming the earlier statement that non-polar compounds are eluted at much lower alcohol percentages and polar compounds at much higher alcohol percentages on the Cogent EE phase.

Why does this occur? The Cogent EE cellulose carbamate phase

Time (min)	Cogent EE	
	Mobile Phase A1 (± %modifier)	Mobile Phase B2 (± %modifier)
0	1	99
5	1	99
30	60	40
40	60	40

¹1:1 Ethanol/IPA with 0.1% trifluoroacetic acid or 0.1% isopropyl Amine or mix of both was used with this study.

²Cyclohexane with 0.1% trifluoroacetic acid or 0.1% isopropyl amine or mix of both was used with this study.

Table 1: Representative Cellulose Carbamate Gradient Profile.

Compound	Solvency	Cogent EE Cellulose Carbonate (% IPA required for elution)	Traditional Cellulose Carbonate (% IPA required for elution)
TSO	Readily dissolves in 100% hexane	0.5	10
Propranolol	Readily dissolves in 96% hexane/4% IPA	4	20
Pindolol	Insoluble in 80% hexane/20% IPA	35	20

Table 2: Relationship Between Chiral Target Solvency and LC Eluent Choice.

uses a different base silica and pore size than the pioneer cellulose carbamate phase. The Cogent EE phase also has a 500Å, instead 1000Å, pore size and a proprietary secondary amine is used to affix the cellulose carbamate to the silica rather than a primary amine used with the traditional phases. In addition, other parameters such as the coating method, coating density and the structure of the cellulose carbamate used in the stationary phase coating are all optimized to allow a close correlation of polarity and eluent polarity. These changes have altered the selectivity from the traditional stationary phase to eliminate the elution of molecules of differing polarities in a narrow solvent polarity range.

As illustrated in Table 2, traditional cellulose carbamate phases elute TSO with 10% IPA and both propranolol and pindolol using 20% IPA, even though they have very different polarities and solvencies. Using the Cogent EE phase, TSO elutes and is baseline resolved at 0.5% IPA in 99.5% alkane. Propranolol elutes and is baseline resolved at 4% IPA in 96% alkane (with basic modifier). Pindolol elutes and is baseline resolved at 35% IPA in 65% alkane (with basic modifier).

The standard application of traditional cellulose carbamate phase is a 250 x 4.6 mm column packed with 5 µm particles. (3 µm particles have recently become commercially available.) The standard application of the Cogent EE cellulose carbamate stationary phase is a 100 x 4.6 mm column packed with 5 µm particles. Because of the selectivity differences, the Cogent EE column can provide comparable selectivity and rapid method performance with a third of the column length.

How does selectivity relate to solubility? From Table 2 it can be seen that TSO has high solvency in 100% alkane. Propranolol has restricted solvency in 100% alkane but high solvency in 96% alkan/4% IPA. Pindolol is a polar molecule; it is almost insoluble in 100% alkane, and very low solvency in 80% alkane/20% IPA. In this work, the alkane used was either cyclohexane, hexanes or heptane per the preference in the testing laboratories between authors. If the polarity of these targets or the solvency of these targets is considered, the results of the traditional cellulose carbamate phases show no correlation to the physical properties. Yet, Table 2 illustrates that the Cogent EE cellulose carbamate phase yields excellent correlation, to the anticipated polarity of elution for these targets. Also, the much broader range of eluent polarity should be noted. This would be of strategic use for resolving impurities or degradation products with differing polarities to the target. It is reasonable to assume that the Cogent EE would be considerably more likely to resolve these analytes from the parent target, which we discuss later, with examples.

With this wide range polarity elution, the EE should be all the more effective in the target resolution for reverse phase or normal phase non-chiral applications. This is shown in Figure 1, where the sequential elution of the enantiomer pairs for chiral compounds of great polarity difference is achieved. Typically, such a result is only achieved adding a base modifier when using the traditional cellulose carbamate phases. Also illustrated in Figure 1, is that the addition of acetonitrile as a modifier and the use of a mixture of ethanol/isopropanol, is a way to further optimize the chiral separations and avoid possible memory

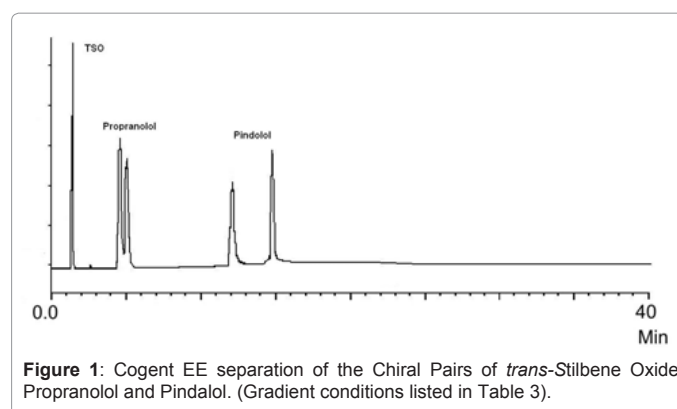


Figure 1: Cogent EE separation of the Chiral Pairs of *trans*-Stilbene Oxide, Propranolol and Pindolol. (Gradient conditions listed in Table 3).

effects from the use of acids or bases. It is possible to cover a wide polarity range, in one run, with pairs of enantiomers sequentially eluting according to their polarity increase in the normal phase mode.

The authors have found that for the EE phase, methanol should not be utilized. The stationary phase has limited stability in the presence of methanol. However, stability is achieved moving to ethanol and other higher molecular weight alcohols.

When utilizing higher molecular weight alcohols, results show that much better resolution is achieved by utilizing a mix of ethanol and isopropanol (typically 50:50), rather than using either alcohol by themselves. Using a mix of 50:50 ethanol and isopropanol has the additional advantage that this mix is fully compatible with the use of the alkanes tested over extended gradients. This mixture exhibits significantly less backpressure generation when being utilized at high alcohol percentages as compared to utilizing isopropanol as the only mobile phase alcohol.

Extensive results also show that the trace presence of acetonitrile (as in Figure 1) can, in many cases, allow no acid or base modifier to be required. This lack of modifier opens new opportunities in preparative or process applications where it is preferential to not utilize acid or base additives.

Ability to reproducibly change pH modifiers from acid to base and back: A study in robustness

Figure 2 (included as supplementary data) shows replicate injections of basic, acidic and neutral chiral probes on the same EE column using an extended 1% to 60% polar gradient (Table 1). This procedure was then repeated for multiple injections with same targets, then switched to and repeated for all targets shown, including many additional targets not shown. The Cogent EE phase rapidly re-optimizes from acid to basic modifiers. This can be very advantageous, as can be seen in Figure 2 (included as supplementary data), where modifier addition can radically affect the retention of readily ionizable basic or acidic compounds (2A to 2D), while having little effect on less ionizable compounds, such as benzoin (shown in Figure 2E (included as supplementary data)) and TSO (examined but not shown). In searching for a generic additive, TFA and DEA/TEA may not be utilized, as they are not suitable for evaporation when conducting preparative or process chiral work. The results suggest that utilizing a mix of 50:50 ethanol:isopropanol, with a mix of TFA:IPAmine may have potential as a true generic mix for the EE phase, where acid and base addition can be tolerated. This could reduce the number of optimization steps when utilizing the Cogent EE column as opposed to traditional cellulose carbamate columns.

Time	Flow (mL/min)	%A	%B	%C	%D
0.0	1	99.5	0.5	0	0
2	2	90	5	0.5	4.5
5	2	60	19	2	19

(A) Hexane, (B) Ethanol, (C) Acetonitrile and (D) Isopropanol

Table 3: Gradient conditions for Figure 1.

Gossypol analysis

Gossypol (gossypol [1,1',6,6',7,7'-hexahydroxy-3,3'-dimethyl-5,5'-bis(1-methylethyl)-2,2'-binaphthalene-8,8'-dicarboxaldehyde]) is a natural product exhibiting profoundly enantiospecific antitumor and male antifertility action for the different optical isomers [17,21]. For this reason, there has been significant interest in the resolution of the gossypol optical isomers. Although methods are available to resolve gossypol by derivatization methods, direct resolution without derivatization is always desirable. The EE phase illustrates column orthogonality in that the phase can operate selectively in both reversed phase and normal phase modes, while we were not able to resolve gossypol using the traditional cellulose carbamate phase in normal phase. Figure 3 illustrates both the reversed phase chiral separation of gossypol and the analysis of a partially degraded / impure gossypol sample. The high-pressure capability (2000 psi) for EE cellulose carbamate phase can be utilized to increase flow and minimize analysis time. This high-pressure capability, unique selectivity and general modifier robustness, all enhance the preparative and process application potential for chiral and non-chiral applications. While many commercial cellulose carbamate stationary phases have difficulty with chiral resolution of gossypol, with the EE phase even anhydrogossypol isomers can be resolved in the same run utilizing an extended gradient in the normal phase.

Commercial Application of the EE phase for tramadol analysis and preparation

The improved selectivity of the EE cellulose carbamate coated stationary phases is demonstrated with the chiral analysis of tramadol. Like TSO, tramadol is a molecule that is soluble in alkane diluents with little alcohol necessary to dissolve the drug. Tramadol is an analgesic drug where the individual enantiomers exhibit differing pharmacological properties. The (-)-tramadol enantiomer preferentially inhibits noradrenaline [22] uptake while the (+)-tramadol enantiomer inhibits serotonin uptake and binds to opioid receptors [23]. The selectivity of the EE phase, as illustrated in Figure 4, readily resolves the enantiomers of tramadol rapidly with minimal polar modifier and no need for acid or base additives. This not only is advantageous in terms of laboratory throughput, but would aid in yielding efficient preparative scale isolation if needed.

Commercial application of the EE phase to separate diastereomers

Our laboratory was asked to investigate the purity of a proprietary diastereomer. Using a 98% n-hexane/2% (IPA/EtOH, 50/50 mix) isocratic mobile phase, the Cogent EE yielded the profile shown in Figure 5. Repeating the analysis using a 1 to 60% (IPA/EtOH, 50/50) gradient,

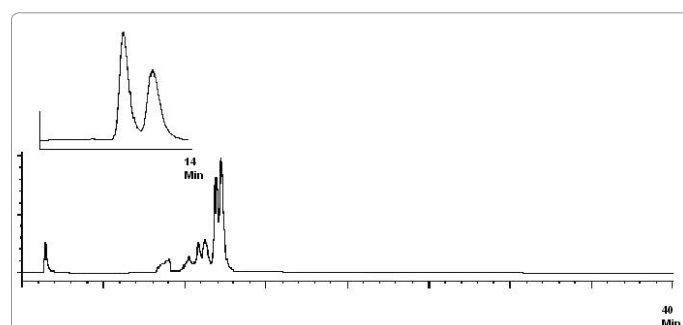


Figure 3: Routine (Reversed Phase) Separation of Gossypol and Extended (Normal Phase) Separation of Gossypol and Degradation Products using the EE Phase.

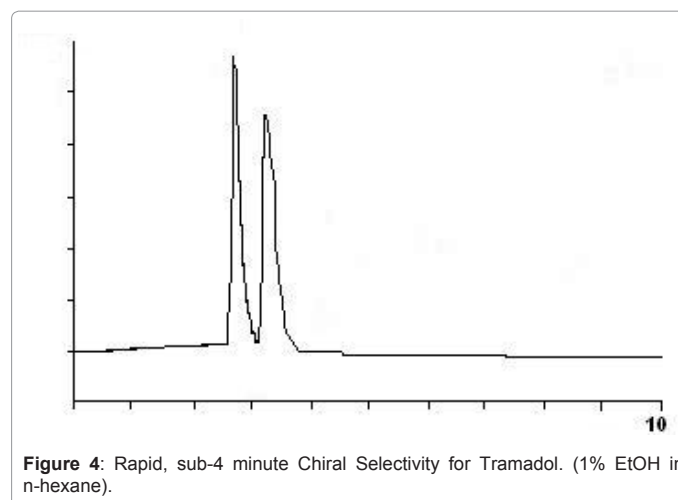


Figure 4: Rapid, sub-4 minute Chiral Selectivity for Tramadol. (1% EtOH in n-hexane).

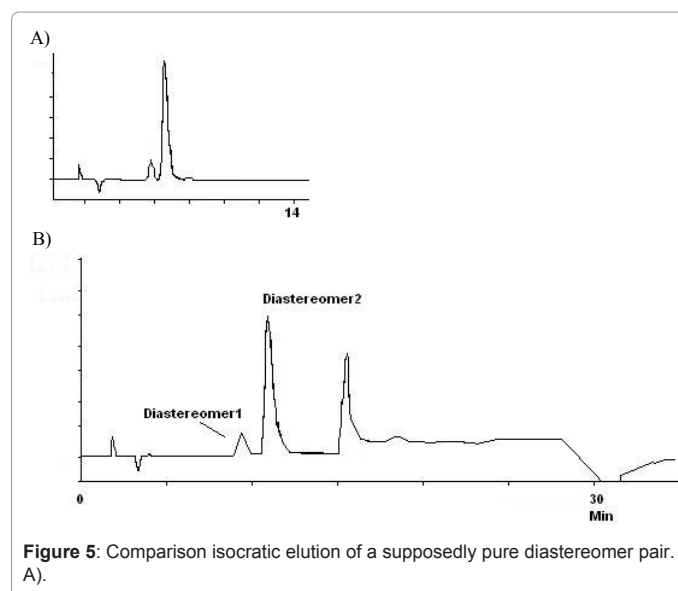


Figure 5: Comparison isocratic elution of a supposedly pure diastereomer pair. A).

the Cogent EE revealed an unknown polar achiral contaminant. The ability to have sufficient selectivity to recognize the presence of such totally unexpected, but real, contaminations has considerable significance in any bioactivity related study. This contaminant was not seen when utilizing a traditional cellulose carbamate column.

Conclusion

The EE coated cellulose carbamate stationary phase presented here has been shown to give a unique selectivity as compared to the traditional cellulose carbamate stationary phase tested. Traditional cellulose carbamate phases often exhibit narrow polarity band selectivity performance using coated and bonded cellulose chiral columns. The Cogent EE stationary phase separates the target compounds over a much broader range of alcohol additive over the traditional cellulose carbamate stationary phase, which gives more sensitivity in the separations. [Range of Cogent EE: 0.5 to 35% alcohol vs Range of OD column: 10-20] When it comes to predicting elution order, the Cogent EE phase has shown a novel ability to match eluent choice to target solvency and polarity. This should be a significant asset in laboratory or process preparative chromatography applications. The

expanded range of polarity response for the Cogent EE phase opens up previously unexplored possibilities of utilizing coated cellulose phase such as the determination of impurity profiles and degradation pathways for structural isomers, achiral and chiral compounds alike. The ability to utilize trace amounts of acetonitrile instead of using any acid or base modifier could have significance along with its unique selectivity for laboratory and process preparative applications of chiral, diastereomer and achiral applications.

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