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Reversed-phase chiral HPLC and LC/MS analysis with tris(chloromethylphenylcarbamate) derivatives of cellulose and amylose as chiral stationary phases

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ABSTRACT

Three polysaccharide-derived chiral stationary phases (CSP) were evaluated for the resolution of more than 200 racemic compounds of pharmaceutical interest in the reversed-phase (RP) separation mode. The population of test probes was carefully evaluated in order to insure that it covers as completely as possible all structural diversity of chiral pharmaceuticals. RP showed the highest potential for successful chiral resolution in HPLC and LC/MS analysis when compared to normal phase and polar organic separation modes. Method development consisted of optimizing mobile phase eluting strength, nature of organic modifier, nature of additive and column temperature. The newer CSPs, cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate), were compared to the commonly used cellulose tris(3,5-dimethylphenylcarbamate) in regards to their ability to provide baseline resolution. Comparable success rates were observed for these three CSPs of quite complimentary chiral recognition ability. The same method development strategy was evaluated for LC/MS analysis. Diethylamine as additive had a negative effect on analyte response with positive ion mode electrospray (ESI^+) MS(/MS) detection, even at very low concentration levels (e.g., 0.025%). Decreasing the organic modifier (acetonitrile or methanol) content in the mobile phase often improved enantioselectivity. The column temperature had only a limited effect on chiral resolution, and this effect was compound dependent. Ammonium hydrogencarbonate was the preferred buffer salt for chiral LC with ESI^+ MS detection for the successful separation and detection of most basic pharmaceutical racemic compounds. Ammonium acetate is a viable alternative to ammonium hydrogencarbonate. Aqueous formic acid with acetonitrile or methanol can be successfully used in the separation of acidic and neutral racemates. Cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) emerge as CSPs of wide applicability in either commonly used separation modes rivaling such well established CSPs as cellulose tris(3,5-dimethylphenylcarbamate). Screening protocols including these two new CSPs in the preferentially screened set of chiral columns have higher success rates in achieving baseline resolution in shorter screening time.

1. Introduction

Chiral reversed-phase (RP) HPLC and LC/MS methods of analysis are primarily developed for applications targeting (polar) compounds poorly soluble in alkanes and low molecular weight (MW) alcohols or mixtures thereof [1] and for bioanalytical applications.

Hydrogen bonding interactions are considered to be essential to chiral recognition with polysaccharide-based chiral stationary phases (CSP) [2]. Such interactions are expected to take place between analyte molecules and the CSP, mainly in the absence of strongly competing species such as water. Therefore, chiral separations on polysaccharide-based CSPs (the most widely used class of CSP) are primarily explored in the normal phase (NP) separation mode using mixtures of alkanes (e.g., hexane) and low MW alcohols as mobile phase (MP). Additional separation modes use sub- or super-critical fluids or polar organic solvents (acetonitrile or alcohols) as MP. Nevertheless, chiral recognition is possible even under conditions unfavorable for hydrogen bonding between analytes and CSP as proved with the first RP method using polysaccharide-derived CSPs reported by Ikeda et al. [3]. Ever since, analysts have explored RP chiral HPLC regarding various considerations such as sample origin, analyte solubility and/or required sensitivity, the latter necessitating the use of mass spectrometry as method of detection [4–8].

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To support drug metabolism and pharmacokinetic studies of chiral pharmaceuticals, it is necessary to combine the resolving power of HPLC with the sensitivity of mass spectrometric detection. Chiral LC/MS methods must be selective, fast (effective in high throughput laboratories), robust and sensitive to low levels of distomer in the presence of the eutomer; they must also be free of interferences from matrix components present in complex biological samples such as blood, tissue and urine. The speed of chiral LC/MS(/MS) analysis is primarily dependent on the separation efficiency of the chiral HPLC column. Advancements in column technology have made higher efficiency CSPs available for routine chiral separations [9,10]. As long as the enantiomers of interest to an assay are chromatographically resolved, further selectivity *may not* be required on part of the CSP due to the unique specificity of MS/MS detection, which allows for the simultaneous quantification of a parent drug and its metabolites in samples of biological origin. This specificity reduces the need for complete chromatographic resolution of all species of interest, leading to shorter analysis times and increased sample throughput.

Chiral RP LC applications using polysaccharide-based CSPs were reviewed by Tachibana and Ohnishi [11]. They listed close to 100 compounds reported to have been successfully resolved in both RP and some other chiral separation mode (NP or polar organic (PO)), or exclusively in RP. Hence, RP emerges as an alternative to other separation modes both when it proves to be similar or complimentary in selectivity for particular separation challenges. Most often, chaeotropic reagents are used as MP additives in RP (chiral) LC for improved resolution. Some examples are potassium hexafluorophosphate, sodium perchlorate, potassium tetrafluoroborate, sodium dihydrogenphosphate, sodium tetraborate or phosphoric acid [5]. Unfortunately, all these reagents are non-volatile, hence incompatible with atmospheric pressure ionization (API) MS. Developing fast and sensitive chiral LC separations compatible with mass spectrometric detection has remained a challenge to analysts.

The most widely used polysaccharide-based CSPs are cellulose tris(3,5-dimethylphenylcarbamate), amylose tris(3,5-dimethylphenylcarbamate) and cellulose tris(4-methylbenzoate) [12–15]. These CSPs demonstrate wide chiral recognition ability, good chemical stability and high loadability in all common separation modes: NP, PO, supercritical fluid (SFC) and RP chiral LC [15]. These CSPs differ in their utility in separating chiral compounds at large, with amylose and cellulose tris(3,5-dimethylphenylcarbamate)s being the most successful ones [12–14,16]. Current screening protocols targeted at identifying combinations of CSP/MP conditions capable of providing adequate resolution can be dramatically improved by including chloromethylphenylcarbamates of cellulose and amylose in the preferentially screened set of chiral columns [9,17–19].

The tris(halomethylphenylcarbamate) derivatives of cellulose and amylose were first proposed and evaluated as chiral selectors in HPLC by Chankvetadze et al. [20–23]. Such CSPs were first made commercially available under the brand name Sepapak (Sepaserve, Muenster, Germany) and more recently as Lux chiral HPLC columns (Phenomenex, Torrance, CA, USA). Sepapak and Lux columns have been the focus of several investigations conducted in NP, PO or RP separation modes [9,17,24,25]. Lux Cellulose-2 (cellulose tris(3-chloro-4-methylphenylcarbamate)) and Lux Amylose-2 (amylose tris(2-chloro-5-methylphenylcarbamate)) demonstrate wide chiral recognition ability, similar to the aforementioned dimethylphenylcarbamate derivatives, as well as significant complementarity in all commonly used separation modes. Similar conclusions were reached in a recent study comparing the selectivity of cellulose and amylose tris(chloromethylphenylcarbamate) derivatives to other polysaccharide-based CSPs in NP and PO [19].

To our knowledge, no comprehensive evaluation of the performance of cellulose and amylose tris(chloromethylphenylcarbamate) derivatives in reversed-phase LC has been published to date. Zhou et al. recently published a study on various CSPs, including cellulose tris(3-chloro-4-methylphenylcarbamate), using a small number of *neutral* racemates as test probes in NP and RP [25]. Also, these CSPs have not been compared to the commonly used cellulose and amylose tris(dimethylphenylcarbamate)s in regards to their success in resolving racemic mixtures at large.

In this work we explore the performance of cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) CSPs and compare them to cellulose tris(3,5-dimethylphenylcarbamate) in regards to ability to resolve racemic compounds in reversed-phase mode using MPs compatible with MS and/or UV detection. These CSPs were screened in a number of RP MPs for the separation of over 200 racemates (mostly generic APIs). Performance was evaluated based on success rates in *baseline* resolving members of this diverse group of chemical compounds. The intense screening effort serving as basis for this report resulted in clear patterns that lend themselves to devising expeditive screening protocols and method optimization with chloromethylpehnylcarbamates of cellulose and amylose as CSPs.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile, methanol and water were purchased from Burdick & Jackson (Morristown, NJ, USA). ACS-grade formic acid, acetic acid, ammonium acetate, ammonium hydrogencarbonate and diethylamine (DEA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All screened racemates were obtained from Sigma–Aldrich, except for benzodiazepines and ketamine and its derivatives, which were purchased as 1.0 mg/ml stock solutions from Cerilliant.

Stock solutions of racemic compounds were prepared in methanol at a concentration of 1–5 mg/ml (depending on detector response) and diluted to 100–500 μg/ml for UV detection and 500 ng/ml for MS detection. The injection volume was 1–5 μl depending on detector response.

2.2. Instrumentation

An Agilent HP1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) was used for chromatographic separations. A Synergi column selector (Phenomenex, Torrance, CA, USA) accommodating 6 HPLC columns was used with good success for expediting chiral screening.

Chiral chromatographic separations followed by UV detection were performed using Lux Cellulose-1, Lux Cellulose-2 and Lux Amylose-2 HPLC columns with the dimensions 250 mm × 4.6 mm i.d. packed with 5 μm particles (Phenomenex, Torrance, CA, USA). Typically faster LC/MS analyses were performed on columns with the dimensions 150 mm × 2.1 mm i.d. packed with 3 μm particles (same source). The acidic MP consisted of 0.1% acetic, formic, or trifluoroacetic acid in water (solvent A) and acetonitrile or methanol (solvent B). It was used for resolving acidic or neutral racemates. Ammonium hydrogencarbonate (5–20 mM) or ammonium acetate (5–20 mM), with or without the addition of 0.1% DEA (solvent A) in mixture with acetonitrile or methanol (solvent B), was used for separating basic or neutral racemates. All chiral separations were attempted in isocratic elution mode at room temperature at flow rates of 1.0 or 0.2 ml/min with UV or MS detection, respectively, and in MP of various eluting strength. The UV detector was set at 220 nm.