Chiral Separation of G-type Chemical Warfare Nerve Agents via Analytical Supercritical Fluid Chromatography

SHANE A. KASTEN,[1]* STEVEN ZULLI,[2] JONATHAN L. JONES,[2] THOMAS DEPHILLIPO,[3] AND DOUGLAS M. CERASOLI[1]*

[1]*Physiology and Immunology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, APG, MD*
[2]*Waters Corp., New Castle, DE*
[3]*Waters Corp., Milford, MA*

ABSTRACT   Chemical warfare nerve agents (CWNAs) are extremely toxic organophosphorus compounds that contain a chiral phosphorus center. Undirected synthesis of G-type CWNAs produces stereoisomers of tabun, sarin, soman, and cyclosarin (GA, GB, GD, and GF, respectively). Analytical-scale methods were developed using a supercritical fluid chromatography (SFC) system in tandem with a mass spectrometer for the separation, quantitation, and isolation of individual stereoisomers of GA, GB, GD, and GF. Screening various chiral stationary phases (CSPs) for the capacity to provide full baseline separation of the CWNAs revealed that a Regis WhelkO1 (SS) column was capable of separating the enantiomers of GA, GB, and GF, with elution of the P(+) enantiomer preceding elution of the corresponding P(–) enantiomer; two WhelkO1 (SS) columns had to be connected in series to achieve complete baseline resolution. The four diastereomers of GD were also resolved using two tandem WhelkO1 (SS) columns, with complete baseline separation of the two P(+) epimers. A single WhelkO1 (RR) column with inverse stereochemistry resulted in baseline separation of the GD P(–) epimers. The analytical methods described can be scaled to allow isolation of individual stereoisomers to assist in screening and development of countermeasures to organophosphorus nerve agents. *Chirality 26:817–824, 2014.* © 2014 The Authors. *Chirality* published by John Wiley Periodicals, Inc.

KEY WORDS: organophosphorus (OP); tabun (GA); sarin (GB); soman (GD); cyclosarin (GF); chiral stationary phase (CSP); Regis WhelkO1

INTRODUCTION

Organophosphorus (OP) compounds such as pesticides are typically symmetric around the phosphorus center, while chemical warfare nerve agents (CWNAs) possess a chiral center about the phosphorus. Lethal exposure to a CWNA results in irreversible inhibition of the acetylcholinesterase (AChE) enzyme, leading to cholinergic crisis in the central and peripheral nervous systems, with death often resulting from respiratory depression.[1] CWNAs are a serious threat to soldiers on the battlefield and to civilian populations from potential terrorist or other activities (e.g., Aum Shinrikyo in Tokyo, Japan 1995, or Syria 2013).[2,3] Several decades of research have been devoted to understanding the toxicology of OP nerve agents as well as to developing safe, effective, and broad-spectrum medical countermeasures, leading to the currently fielded combination of atropine, an oxime, and an anticonvulsant as a therapeutic drug regimen.

All CWNA compounds (e.g., tabun [GA], sarin [GB], soman [GD], cyclosarin [GF], and VX/VR/VS) possess a chiral phosphorus center, with undirected synthesis pathways producing racemic mixtures of enantiomers in roughly equal proportions. GD possesses a second chiral center found in its pinacolyl-R-group, giving rise to a diastereomer mix of four epimers. The toxicity of the CWNAs has been studied extensively utilizing individually isolated or stereospecifically synthesized stereoisomers of each CWNA to better understand the stereoselectivity of the AChE active site.[4] It is possible that the chirality of the CWNAs may be a major contributor to the dramatically higher toxicity of CWNAs as compared to most (structurally similar but achiral) OP pesticides. Isolation of individual stereoisomers of CWNAs from a racemic preparation has been achieved via either enzyme-mediated purification[5–9] or chiral chromatography.[4–8,10,11] Stereoisomers have been typically designated with respect to optical activity (polarimetry) such that the levorotatory (P-) enantiomers were found to be more efficient inhibitors of AChE *in vitro* and more toxic to mice *in vivo*.[3]

Chiral high-performance liquid chromatography (HPLC) is often used to separate and isolate stereoisomers from racemic mixtures. Chromatographic separation and isolation offers a process that is nondestructive, easily scalable, capable of yielding highly efficient enantioseparation, and suitable for a wide array of chemically related compounds. Our laboratory has adopted and made use of published chiral HPLC methods for both the analytical and semipreparative scale separation and/or isolation of GA and VX via a Chiral Technologies Chiralcel OD-H column.[10–12] Chiral stationary phases (CSPs) applicable for full separation of GB, GD, and GF stereoisomers by HPLC have not been well characterized.

Supercritical fluid chromatography (SFC) is a well-established chiral chromatography technology that offers several advantages over chiral HPLC.[13–15] For this study, the main advantages of SFC over HPLC include reduced reliance on organic solvents (normal phase methods), increased column efficiency, higher enantioseparation efficiency for

numerous compounds, and low solvent consumption. Individual stereoisomers can also be recovered in aqueous solvents, allowing for biological assays to be performed immediately after collection with some methods. We report here the development of chiral SFC methods for the analytical scale separation and isolation of GA, GB, GD, and GF enantiomers. All G-type CWNAs were fully resolved via a Waters (Milford, MA) SFC-MS Prep15 system using analytical Regis WhelkO1 (SS) or (RR) chiral columns in tandem with a mass spectrometer to detect and direct open-bed fraction collection of the individual stereoisomers.

MATERIALS AND METHODS

CWNA was obtained from the Edgewood Chemical Biological Center (ECBC, Aberdeen Proving Ground, MD) at >98% purity per nuclear magnetic resonance (NMR) and gas chromatography mass spectrometer (GC-MS) analysis. All experiments conducted with CWNAs were performed in accordance with the strict safety standards established at the US Army Medical Research Institute of Chemical Defense for handling of these highly toxic compounds. Racemic ethyl N,N-dimethylphosphoramidocyanidate (tabun, GA) and racemic O-isopropyl methylphosphonofluoridate (sarin, GB) were diluted in ethyl acetate to 1.91 mg/mL (11.76 mM) and 1.88 mg/mL (13.42 mM), respectively, prior to use. Likewise, a diastereomer mix of pinacolyl methylphosphonofluoridate (soman, GD) and a racemic sample of cyclohexyl methylphosphonofluoridate (cyclosarin, GF) were each diluted in hexane to concentrations of 1.89 mg/mL (10.35 mM) and 1.86 mg/mL (10.35 mM), respectively, prior to use. GD stereoisomer standards were obtained from the TNO Laboratory (Rijswijk, The Netherlands) in ethyl acetate, with concentrations listed for each as C-P-, 1.54 mg/mL; C+P-, 1.69 mg/mL; C-P+, 1.72 mg/mL; C+P+, 1.6 mg/mL. Ethyl acetate (GC-grade) and hexane (GC-grade) were both purchased from Sigma-Aldrich (St. Louis, MO). Cosolvents (methanol, ethanol, 1-propanol, 1-butanol, and isopropanol) and H_2O were all purchased from Sigma-

Aldrich as Chromasolv-Plus reagents (≥99.9% pure). Bovine pancreas α-Chymotrypsin was acquired from Sigma-Aldrich. Female rabbit (New Zealand White) plasma was obtained from Bioreclamation (Westbury, NY).

Analytical scale chiral columns (4.6 mm x 250 mm, ID x L coated on 5 μm silica gel) were purchased from the following vendors: Chiral Technologies, West Chester, PA (Chiralcel OD-H and OJ-RH, Chiralpak AD-H and IB), Supelco, Bellefonte, PA (Astec CycloBond I, II, and II AC), and Regis Technologies, Morton Grove, IL (Whelk-O1 [SS]). A semipreparative Whelk-O1 (RR) column (10 mm x 250 mm, ID x L coated on 5 μm silica gel) was also purchased from Regis.

SFC/MS Conditions

CWNA and GD stereoisomers were diluted by mixing 1 part nerve agent with 3 parts hexane (GC-grade) prior to injection into an SFC system. SFC was performed on a Waters SFC-MS Prep15 system modified for the atypical very low cosolvent percentage chromatographic conditions described herein (Fig. 1). Due to the very low cosolvent percentage of the mobile phase in these separations, the sample injector was revised from "modifier stream" injection mode (standard configuration), where only the cosolvent flows through the injector, to "combined stream" mode, where the CO_2 and cosolvent premix under high pressure before flowing through the injector. Combined stream injection mode results in a faster flow rate through the injector, increasing the sweep rate of the sample from the sample loop, resulting in less band broadening as compared to the modifier stream injection technique. Novel hardware and software modifications to the sample injector were implemented to safely and accurately perform combined stream injections. Method control and data acquisition were managed via MassLynx (v. 4.1 SCN 846) software. The SFC system was equipped for analyte detection with a photodiode array (PDA) containing a flow cell modified to contain analytical lenses, increasing energy transmittance in the low UV regions of 190 nm to 220 nm to improve UV detection because of the low absorbance characteristics of the analytes, as well as a 3100 series single-quadrupole mass spectrometer (MS) fed via passive sample split. Samples were injected in combined-stream mode at 5 mL/min flow rate under 120 bar using
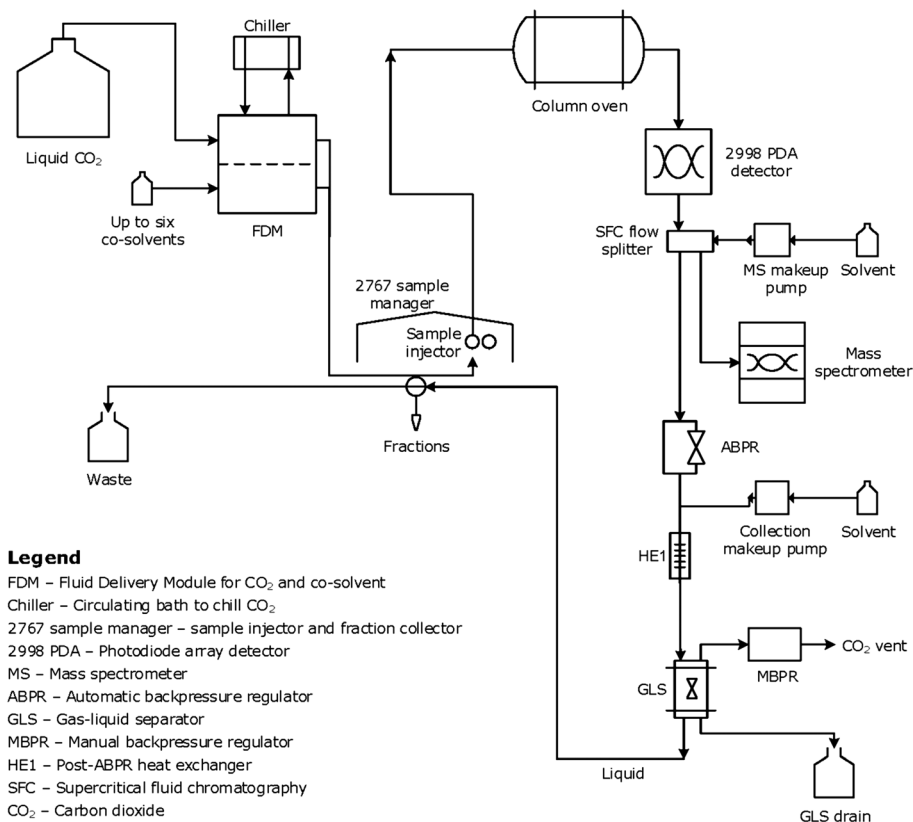

Fig. 1. Block diagram of the modified Waters Prep15 SFC configuration. The Prep15 instrument was modified to run in combined-stream mode in which the cosolvent and CO_2 are mixed under high pressure prior to injection of sample.

either 0% or 1% cosolvent during isocratic method development. Sample injection used sample sandwiching in which 80 µl hexane (presolvent) was added to the injection syringe followed by 95 µl of sample and then 20 µl hexane (postsolvent) immediately prior to injection onto a 100 µl wide bore loop (0.04″ i.d.). An in-line mobile phase mixer was installed inside the column oven upstream of the column in the flow path. The additional homogenization of the mobile phase resulted in lower baseline noise in the chromatograms, increasing the signal-to-noise (S/N) of the peaks. The column oven was held at a constant temperature of 40 °C. When tandem columns were used, a second column was connected to the first column using 5 cm of 0.02" (i.d.) stainless steel tubing. A sample fraction was split off the main line and infused with MS makeup solvent (90/10/0.1; MeOH/H_2O/Formic Acid, v:v) at a flow rate of 0.4 mL/min prior to entering the electrospray ionization (ESI) source. Main line flow was further supplemented with makeup solvent, which was used to carry the sample into a passive heat exchanger before entering the gas-liquid separator (GLS) following CO_2 evaporation. Make-up solvent was either filtered H_2O (18 ohm, 0.2 µm filter) at a flow rate of 1 mL/min for method development or Chromasolv-Plus H_2O at a flow rate of 4 mL/min when individual stereoisomers were isolated. Test injections were made and used to simulate fraction collection via the FractionLynx simulator prior to individual stereoisomer collection. Collection of fractions was triggered by *m/z* intensity thresholds with open-bed fraction collection of separated stereoisomers (collection parameters as per Waters recommendation). Optimized parameters for ESI-MS were as follows: Positive ion mode; capillary, 5.0 kV; Cone, 25 V; Extractor, 1 V; RF lens, 0.1 V; Source Temperature, 150 °C; Desolvation Temperature, 350 °C; N_2 gas flow, Desolvation (400 L/hr) and Cone (60 L/hr). Chromatographic analyses were performed using either original extracted ion chromatograms or versions that were smoothed via a Savtzky Golay algorithm (window size, 1.0; smoothing iterations, 40) with background subtracted (polynomial order, 1; below curve %, 0.1; tolerance, 0.01); no significant differences were observed in the parameters derived from original and smoothed chromatograms. For presentation purposes only smoothed chromatograms are shown.

Chromatographic Characterization

Chromatography parameters were determined by drawing lines tangent to the leading and trailing edge of each peak to approximate the four-sigma peak width to estimate the theoretical plates (N), retention factor (*k*), separation factor (α), and resolution (R_S).[16] Parameters were used to verify both the enantioselectivity and the separation efficiency of various CSPs for G-type CWNAs. Theoretical plates were assessed using the following formula:

$$N = 16 \left(\frac{t_R}{W_t} \right)^2$$

in which the peak width (W_t) was taken as the difference in time between the tangent line intersections with the baseline for each peak. The average retention time, t_R, was determined by averaging the time to peak maxima from subsequent injections. Calculation of *k* was carried out using the following formula:

$$k = \frac{t_R - t_0}{t_0}$$

where t_0 is the column void time (set equal to 1 min for all chromatograms). The α was calculated as:

$$\alpha = \frac{k_2}{k_1}$$

with k_1 and k_2 as the respective retention factors for two adjacent peaks. Resolution (R_S) between adjacent peaks was determined as:

$$R_s = \left(\frac{\sqrt{N}}{4} \right) \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k}{k+1} \right)$$

in which the N for the second peak was used for the equation. Methods that give full baseline resolution between peaks ($R_S \geq 1.6$) allow for the

most accurate quantitation of stereoisomers and provide the best yield and enantiopurity when isolation is required.

Stereoisomer Elution Order

The elution order of stereoisomers was determined using various published methods and designated with respect to optical activity by analogy.

The elution order of GA enantiomers was determined by incubating α-chymotrypsin with racemic GA, which has previously been established to preferentially bind the P(+) isomer of GA.[6] Chymotrypsin was resuspended in 1 mM HCl/2 mM $CaCl_2$ at a concentration of 100 mg/mL. Racemic GA was diluted 1/10 in 50 mM 4-morpholinepropanesulfonic acid (MOPS) buffer (pH 7.4) with reaction initiated by mixing 1200 µl diluted GA with 250 µl of chymotrypsin suspension and conducted by incubation at room temperature. Several 200-µl aliquots were collected at various times (0, 0.5, 1, 2, 5 min) and mixed with 1000 µl hexane to partition the unreacted GA into the organic phase following vortexing for 30 s and 1 min of centrifugation. Approximately 900 µl of organic phase was removed and placed in a 4-mL SFC vial prior to analysis.

Racemic GB was incubated with rabbit plasma to determine the elution order of the enantiomers, as it has been shown that rabbit plasma preferentially hydrolyzes the P(+) isomer of GB.[4] A reaction was initiated by mixing 50 µl of rabbit plasma with 1000 µl of racemic GB (diluted 1/10 in saline) and was then incubated at room temperature for 20 min. Four 200-µl aliquots were removed at various times (0, 2, 10, and 20 min), and unreacted GB was extracted with 1000 µl of hexane using the same procedure described above prior to injection onto the tandem WhelkO1 (SS) columns.

An analytical chiral GC-MS method for the baseline separation of enantiomers of GF was duplicated in our laboratory and used to determine the elution order and optical activity of the enantiomers separated on the WhelkO1 (SS) column via the SFC.[17] SFC-isolated enantiomers were each injected into an Agilent 6890/5973 GC-MS fitted with a GammaDEX™ 225 (Supleco, Bellefonte, PA) column operated in EI mode using the referenced method. Individual enantiomers of GF were isolated via the SFC using a minimum intensity threshold to trigger collection of peak fractions based on detection of the *m/z* 181 ion. Enantiomers were isolated in Chromasolv-Plus H_2O and extracted with ethyl acetate at a volume ratio of 1:0.5 (enantiomer:ethyl acetate). Individually extracted enantiomers at a concentration of roughly 100 µM were injected (1 µl) into the GC-MS to determine relative retention times. The elution order of the enantiomers from the SFC was correlated to the elution order from the GC and by analogy used to assign optical activity to the enantiomers based on the referenced method.[17]

Stereoisomer standards of GD described above were provided with defined optical activities. The individual isomers were diluted (1 part isomer with 3 parts hexane, v:v), and 95 µl was injected onto the WhelkO1 (SS) tandem columns to determine elution order and respective retention times for each. The stereoisomer standards had slight contamination with the antipode epimers, with the C + P + isomer having the most contamination.

RESULTS
G-Agent Electrospray Ionization - Mass Spectrometry

Detection of G-agent stereoisomers required a mass spectrometer since no spectral signature could be identified for the G-agents even when using the PDA module modified to afford enhanced sensitivity. ESI-MS parameters were established for detection of GF ions using the Intellistart module (Masslynx) via direct infusion. Both the protonated molecular ion and the fragment ion(s) abundances were further optimized by manual adjustment of ion source parameters. Optimized parameters determined for GF also provided suitable ionization and detection of GA, GB, and GD ions. Ions detected in positive mode included the protonated molecular ion $[M+H]^+$, characteristic fragment ions, and proposed methanol adducts of molecular fragments (Fig. 2). Fragment ions were typically the most abundant ions
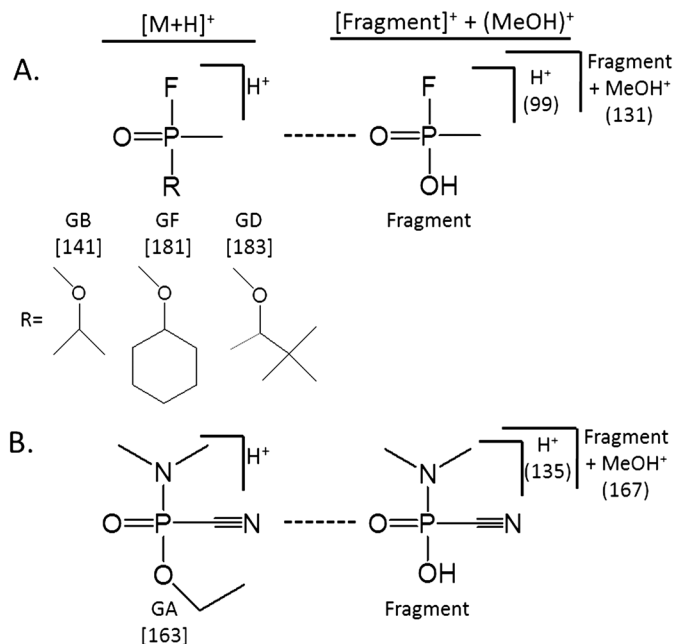
A.

$[M+H]^+$

$[Fragment]^+ + (MeOH)^+$

GB [141]   GF [181]   GD [183]

Fragment

Fragment + MeOH$^+$ (131)

H$^+$ (99)

R=

B.

H$^+$

GA [163]

Fragment

Fragment + MeOH$^+$ (167)

H$^+$ (135)

Fig. 2. Ions corresponding to the protonated molecular ion ($[M+H]^+$), fragment, and fragment-methanol adduct were detected for GA, GB, GD, and GF in positive ion mode. Fragmentation and adduction patterns were similar with m/z ratios specified in parentheses for each ion detected.

detected for each agent. Ion scans of GF ranging from m/z 50 to 200 included detection of the protonated molecular ion (m/z 181), a fragment ion (m/z 99), and a proposed methanol-fragment adduct ion (m/z 131). Adduction of fragment ions with a solvent molecule is rare under ESI conditions, but such adducts have been reported for acetonitrile adducted to G-agent fragments.[18] An ionization profile similar to that obtained for GF was also detected for GA, GB, and GD. Detected ions included the $[M+H]^+$ ion and fragment ion(s) for GA (m/z 135), GB (m/z 99), and GD (m/z 99; also m/z 85 resulting from fragmentation of the alkoxy R-group but not depicted in Fig. 2) along with proposed methanol-fragment adducts of m/z 167, 131, and 131 for GA, GB, and GD, respectively. Chiral separation of the G-agent stereoisomers was monitored under positive mode using a scan range from m/z 50 to 200 with chiral chromatography parameters determined using extracted ion chromatograms (EIC) for fragment ions only. Fraction collection of individual stereoisomers was directed by mass detection of either the protonated molecular ion or characteristic product ions.

Chiral Column Screening for the Separation of GF Enantiomers by SFC

Several analytical SFC columns were screened based on published results and recommendations from column manufacturers (for safety reasons, CWNA could not be sent out for vendor screening services). Parameters that influence chiral separation include flow rate, cosolvent composition and percentage, cosolvent modifier (basic/acidic), column temperature, column length, system back pressure, and CSP type. Preliminary screening of various CSPs was initiated using only 1% isopropanol (IPA) cosolvent until a column capable of at least partial resolution (R_S ~1.0) of the enantiomers of GF was discovered. As mentioned above, a single Chiralcel OD-H was found to be capable of resolving the enantiomers of GA with 0.3% cosolvent via HPLC, although GA enantiomers

could not be resolved with 0% or 1% cosolvent on the SFC with a single OD-H column inline, nor could the enantiomers of GB, GD, or GF be resolved under these conditions (data not shown). Various other polysaccharide-type chiral columns (Chiralpak AD-H, Chiralcel OJ-RH, Chiralpak IB) were tested along with the OD-H, but none afforded detectable separation of GF enantiomers. Slight separation (R_S ~0.6) was detected for two different unmodified cyclodextrin columns, CycloBond I (β-cyclodextrin) and CycloBond II (γ-cyclodextrin). A diacylated γ-cyclodextrin HPLC column, CycloBond II AC, was also screened primarily based on similarity to the GammaDex 225 GC capillary column (stationary phase also diacylated γ-cyclodextrin) capable of resolving the enantiomers of GF by gas chromatography.[17] No separation was detected with the CycloBond II AC column inline in the SFC. The only CSP found to be able to provide considerable baseline resolution ($R_S = 1.0$) of the GF enantiomers was the Regis pirkle-type WhelkO1 (SS) column. Further chiral method development centered on establishing full baseline separation ($R_S \geq 1.6$) of the stereoisomers of GF followed by separation methods for the stereoisomers of GA, GB, and GD using the WhelkO1 (SS) column. Raising the cosolvent percentage to >1% decreased the retention time of the GF enantiomers on the WhelkO1 (SS) column but also drastically reduced the separation efficiency, such that method development with isocratic mobile phases was restricted to abnormally low cosolvent percentages of either 0% or 1%. It should be noted that the current version of Masslynx software only supports programming whole-number cosolvent percentages; as such, fractions of a percent were not assessed during isocratic method development.

The separation efficiency of racemic GF was evaluated on the WhelkO1 (SS) column with either 0% or 1% of various cosolvent alcohols (Table 1) to determine the best separation method for the isolation of individual stereoisomers. Cosolvents commonly used in chiral SFC method development include methanol, ethanol, and isopropanol because of polarity differences that can affect separation efficiency and analyte retention for a given CSP. Two other cosolvents, *n*-propanol and *n*-butanol, were also included with polarity ranging from high to low: methanol > ethanol > n-propanol n-butanol > isopropanol for the whole group. Full baseline

TABLE 1. Separation efficiency of GF using different cosolvents

Cosolvent[a]	WhelkO1 (SS)[b]	k_1	k_2	α	R_S
None	x1	15.3	17.3	1.13	1.7
	x2	39.0	43.7	1.12	2.2
Methanol	x1	6.2	6.8	1.10	0.7
	x2	13.1	14.2	1.08	1.0
Ethanol	x1	7.5	8.4	1.12	0.9
	x2	15.2	16.8	1.11	1.2
n-Propanol	x1	8.5	9.5	1.12	1.1
	x2	22.6	24.6	1.09	1.2
n-Butanol	x1	7.8	8.6	1.11	0.8
	x2	18.0	19.7	1.09	1.3
Isopropanol	x1	8.7	9.9	1.13	1.0
	x2	21.9	24.6	1.12	1.7

[a]Methanol has the highest cosolvent polarity, with isopropanol having the least (Methanol > Ethanol > *n*-Propanol > *n*-Butanol > Isopropanol).
[b]1x indicates a single column inline or 2x for analysis utilizing a second column connected in series.

resolution ($R_S = 1.7$) was achieved with 0% cosolvent when a single WhelkO1 (SS) column was inline. The addition of 1% cosolvent reduced the retention factor ~2–fold, allowing for shorter retention times. However, GF enantiomers were no longer baseline resolved with any of the cosolvents screened.

Since inclusion of cosolvent decreased resolution, a second WhelkO1 (SS) column was connected to the first column to determine how doubling the column bed length might enhance the separation of the enantiomers. The resolution of the GF enantiomers increased to an R_S value of 2.2 with 0% cosolvent utilizing two WhelkO1 (SS) columns in tandem (Fig. 3). Only the isopropanol cosolvent allowed full baseline separation ($R_S = 1.7$) on tandem columns, whereas the structural isomer, *n*-propanol, did not allow full resolution. Resolution increased as cosolvent polarity decreased when two columns were connected in series. The R_S of 2.2 with 0% cosolvent is more than adequate to allow for the isolation of pure enantiomers of GF and is also very amenable to a semipreparative scaling of the process. Despite full baseline separation of the enantiomers with 1% isopropanol, recovering isolated stereoisomers devoid of alcohol cosolvents was determined to better allow easy sample manipulation postisolation and also to provide for a more biologically relevant background for immediate isomer-specific enzyme-based assays.

Separation Efficiency of GA, GB, and GD Stereoisomers on WhelkO1 (SS)

Based on the results obtained with GF, chiral separation methods were evaluated and developed for GA, GB, and GD using single and tandem WhelkO1 (SS) columns. Chiral separation parameters for both single and tandem columns are listed in Table 2, with representative fragment EICs shown in Fig. 4 (A, GA; B, GB; C, GD) for tandem column separation only. Enantiomers of GA, GB, and GD could not be fully resolved ($R_S \leq 1.4$) on a single WhelkO1 (SS) column. Cosolvents screened with GF were also screened with GA, GB, and GD (data not shown); as was the case with GF, the best resolution was achieved with 0% cosolvent. Enantiomers of GA ($R_S = 1.9$) and GB ($R_S = 2.0$) were completely baseline resolved on tandem columns. The first two stereoisomers of GD to elute were baseline resolved, while the remaining two stereoisomers to elute were only partially separated (Fig. 4C).

Elution Order of Stereoisomers on SFC WhelkO1 (SS)

Optical activity has often been used to distinguish stereoisomers from one another based on rotation of plane polarized light. Stereoisomers that rotate the light counterclockwise are called levorotatory or (–), while those that
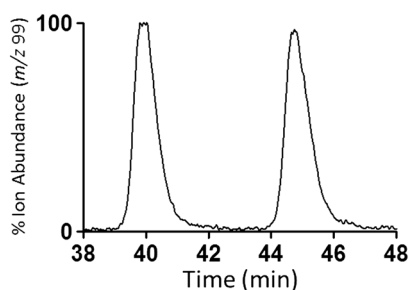
TABLE 2. Separation parameters of G-agents

WhelkO1(SS)	1x[a]			2x[a]		
G-Agent	GA	GB	GD	GA	GB	GD
k_1	11.4	7.8	7.6	25.7	17.7	16.5
k_2	12.9	9.6	9.1	28.5	21.6	19.6
k_3			11.1			23.2
k_4			11.1			24.3
α_1	1.13	1.23	1.2	1.11	1.22	1.18
α_2			1.22			1.2
α_3			1.0			1.05
R_{S12}	0.8	1.1	1.4	1.9	2.0	2.2
R_{S23}			1.2			2.7
R_{S34}			0			0.7

[a]1x indicates a single column inline and 2x for analysis utilizing a second column connected in series.
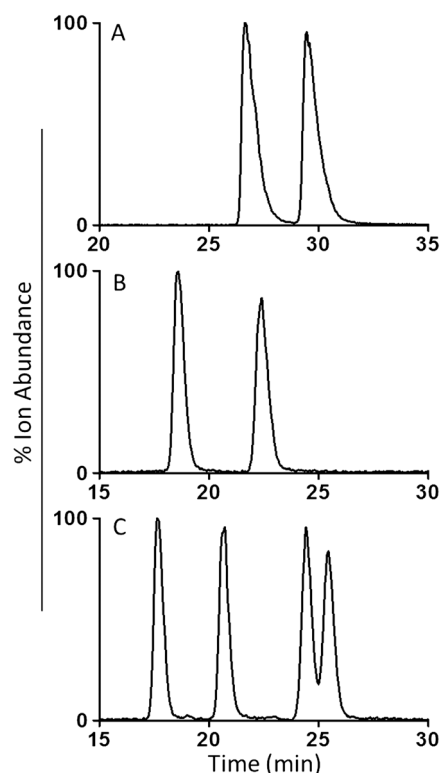

Fig. 4. Separation efficiency profiles for GA (**A**), GB (**B**), and GD (**C**). Stereoisomers were separated using 2x WhelkO1 (SS) columns with 0% cosolvent.

Fig. 3. EIC showing the separation profile of GF enantiomers on 2x WhelkO1 (SS) columns with 0% cosolvent.

rotate the light clockwise are called dextrorotatory or (+). While the individual stereoisomers were not assessed by polarimetry directly in this study, several previously developed methods were used here to determine the elution order of the stereoisomers on the WhelkO1 (SS) column, with optical activity assigned by analogy.

Bovine pancreas chymotrypsin has been shown to be enantioselective for the hydrolysis of the P(+) enantiomer of GA, allowing for the isolation of the P(–) enantiomer at up to 99% optical purity.[7] Racemic GA was incubated with α-chymotrypsin to determine the rate of hydrolysis of each enantiomer. By analogy, the enantiomer with the higher rate of hydrolysis can be assigned as the P(+) enantiomer of GA. Several aliquots were removed from the reaction mixture

over the incubation period, and unreacted GA was extracted into hexane prior to injection onto the SFC with two WhelkO1 (SS) columns in tandem. Extracted ion chromatograms for each aliquot were overlaid to demonstrate enantioselective degradation of the P(+) enantiomer. Based on the hydrolysis profile seen in Fig. 5, the enantiomer with the shorter retention time on the WhelKO1 (SS) column was exclusively hydrolyzed over the observed time course. By analogy the enantiomer that was first to elute from the WhelkO1 (SS) column was the P(+) enantiomer (light gray fill), and the second eluting enantiomer was the P(−) enantiomer (dark gray fill) of GA.

The elution order of GB enantiomers from the WhelkO1 (SS) SFC column along with optical activity assignment was determined in a manner similar to that described above for GA. It has been demonstrated that rabbit plasma is highly enantioselective, with a marked preference for degrading the P(+) enantiomer of GB.[7] To determine the enantiospecific elution order of GB enantiomers, racemic GB was incubated with rabbit plasma and various reaction endpoints were collected. Extracted samples were injected onto the WhelkO1 (SS) column, and the results indicated enantioselective hydrolysis (Fig. 6). The enantiomer eluting first (light gray fill) was hydrolyzed preferentially, such that by analogy this enantiomer is the P(+) GB enantiomer followed by elution of the P(−) enantiomer (dark gray fill).

Chiral separation of GF enantiomers has been published where a Supleco GammaDex 225 GC capillary column was used and optical activity was correlated with isomer elution order.[17] In this study, the P(−) enantiomers of GF were found to elute prior to the P(+) enantiomers on the GammaDex 225 column. Individual enantiomers of GF were separated on the SFC using the WhelkO1 (SS) column, with each enantiomer isolated by open-bed fraction collection. The isolated enantiomers were then extracted into ethyl acetate and injected onto a GammaDex 225 GC column (see Fig. 7) using a method virtually identical to that in the work referenced above. Based on the published elution order, the isolated enantiomer found to elute first from the WhelkO1 (SS) SFC column was the P(+) enantiomer of GF, as it was the second enantiomer to elute from the GammaDex 225 GC column (light gray fill). As with GA and GB, the P(+) enantiomers of GF have shorter retention on the WhelkO1 (SS) column, while the P(−) enantiomers are more strongly retained.

The elution order of the four GD diastereomers was also determined for the WhelkO1 (SS) column using individual
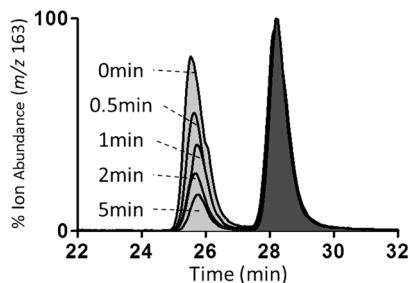

Fig. 5. Racemic GA was incubated with bovine pancreas chymotrypsin, and aliquots were removed over time and injected onto 2x WhelkO1 (SS) columns. The first peak (light gray shading) to elute from the SFC was preferentially degraded by chymotrypsin, while the second peak (dark gray shading) remained intact over the 5-min incubation period. Based on chymotrypsin enantioselectivity the elution order of GA enantiomers from the WhelkO1 (SS) column was P(+) followed by the P(−) enantiomer.
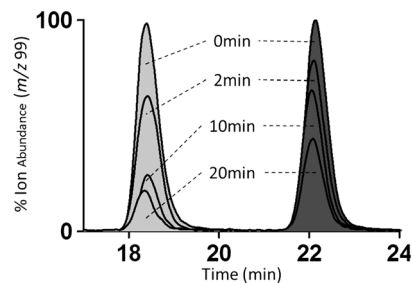
Fig. 6. GB enantiomer elution order was determined by incubating racemic GB with rabbit plasma. Several aliquots were removed and injected onto 2x WhelkO1 (SS) columns. The first peak to elute was determined to be the P(+) enantiomer (light gray shading) followed by elution of the P(−) enantiomer (dark gray shading) of GB based on the enantiopreference of rabbit plasma.
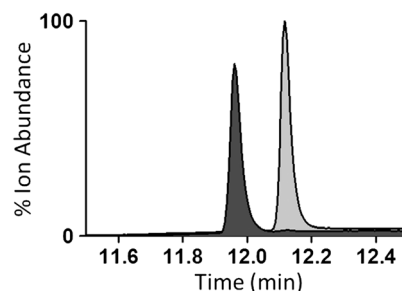

Fig. 7. GF enantiomers were separated on 2x WhelkO1 (SS) columns, collected, and individually injected onto a GammaDex 225 GC capillary column to determine the relative elution order of the isolated enantiomers. An EIC of the elution of each enantiomer on the GammaDex 225 GC column is shown. By analogy, the first enantiomer of GF to elute from 2x WhelkO1 (SS) columns was the P(+) enantiomer followed by the P(−).

isomer standards (nearly enantiopure), as previously used in our laboratory to determine the GD isomer elution order on a TA GC capillary column.[19] As described in the preceding section, two analytical-scale WhelkO1 (SS) columns were joined together, giving ample separation of the GD diastereomers for individual identification. The retention times of the isolated standard epimers were correlated with the retention times of the individual stereoisomers separated from a diastereomer mix of GD, as shown in Fig. 8. The P(+) isolated standards were retained the least on the WhelkO1 (SS) columns, with the C(−)P(+) isomer (solid line, light gray fill) eluting first followed by the C(+)P(+) isomer (dashed line, light gray fill) as seen in the Fig. 8B overlay (both P(+) isomers had modest contamination with the antipode P(−) isomers). Both P(+) isomers were completely baseline resolved from one another and from the two P(−) isomers (Table 2). The P(−) enantiomers were more tightly retained on the WhelkO1 (SS) column, with the C(+)P(−) isomer (solid line, dark gray fill) eluting immediately prior to the C(−)P(−) isomer (dashed line, dark gray fill), resulting in incomplete resolution even when tandem columns were used. The elution order of the four GD isomers was C(−)P(+) followed by C(+)P(+), then C(+)P(−) followed shortly thereafter by C(−)P(−), based on the retention times of the isolated isomer standards (Fig. 8A). Three semipreparative WhelkO1 (SS) columns were connected in series, but the P(−) epimers of GD still could not be fully resolved (data not shown). As an alternative, a single semipreparative WhelkO1 column was purchased from Regis with the reverse stereochemistry
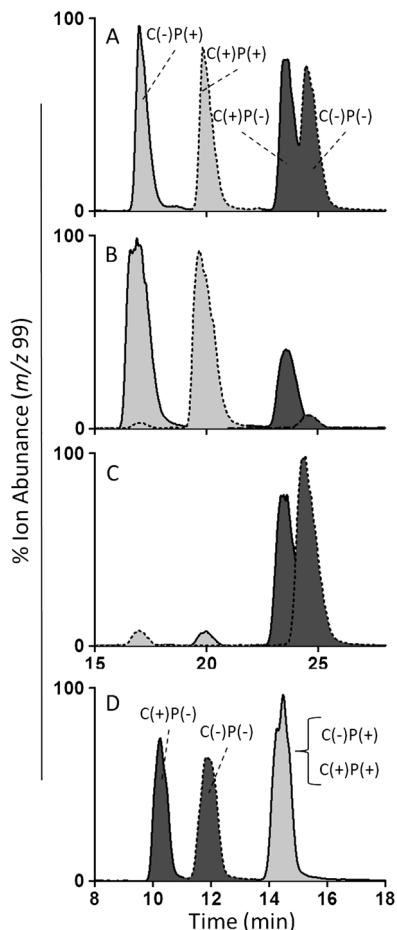
Fig. 8. (**A**) Separation profile of diastereomic GD on 2x WhelkO1 (SS) columns. Sets of enantiomers, (**B**) P(+) and (**C**) P(–), of GD were injected onto 2x WhelkO1 (SS) columns to determine the relative elution order of the four GD stereoisomers. The elution order based on retention time was determined for each stereoisomer and represented in panel **A**. (**D**) Elution order of GD stereoisomers from a single WhelkO1 (RR) column showing the reverse enantioselectivity of the antipode stationary phase resulting in elution of the P(–) enantiomers prior to the P(+) enantiomers.

(WhelkO1 (RR)) and was tested to determine how reversing the stereochemistry of the functional group would influence the elution order of the four GD diastereomers. Chiral separation on a single (RR) column resulted in a reversed order of elution but almost identical elution profile for the GD epimers (Fig. 8D). As with the single (SS) column, the first two stereoisomers eluted with near baseline resolution, but on the (RR) column these two stereoisomers were the C(+)P(–) followed by the C(–)P(–) isomer. Addition of a second (RR) column in tandem is anticipated to result in the full resolution of the two P(–) stereoisomers of GD. Based on these results, the four diastereomers of GD can be isolated to enantiopurity using tandem WhelkO1 (SS) columns for isolation of the P(+) isomers and tandem WhelkO1(RR) columns to isolate the P(–) isomers.

CONCLUSION

The methods presented in this study demonstrate the analytical chiral separation of GA, GB, GD, and GF via an SFC-MS using the Regis WhelkO1 type column. The Regis WhelkO1 (SS) column was capable of full baseline separation of the enantiomers of GA, GB, GF, and the P(+) enantiomers of GD, while the WhelkO1 (RR) column separates the P(–) enantiomers of GD. Separation of the enantiomers of VX was also possible using the WhelkO1 (SS) column (data not shown). Chiral SFC methods are currently being developed for VX and other dialkylphosphonothiolate compounds and will be described in a subsequent article. Changes in column temperature and system back pressure were two parameters that were not tested during method development but are currently being evaluated as ways to further optimize chiral separation.

Preliminary scale-up and method development on the semipreparative scale has already proven successful, potentially allowing for isolation of pure enantiomers on the milligram scale. We are currently developing semipreparative methods for such isolation. We are also attempting to confirm the assignment of individual stereoisomers by using vibrational circular dichroism to determine absolute configuration (R/S assignment) of each stereoisomer in solution. These efforts will allow assessment of racemization rates of CWNAs in different buffer conditions, as well as determination of spontaneous and oxime-mediated reactivation rates of AChE inhibited by defined individual CWNA stereoisomers.

Understanding the stereochemistry of CWNAs is critical for understanding the biology and biochemistry of these compounds *in vivo*. Countermeasures such as stoichiometric or catalytic bioscavengers, or protein-based drugs designed to intercept and destroy nerve agents in the circulatory system, require activity against the more toxic stereoisomers of CWNAs to be effective. The capacity to easily produce enantiomerically pure isomers of CWNAs will aid in the development of medical countermeasures against these highly toxic compounds by allowing high-throughput screening of candidate enzymes for activity against the more toxic CWNA isomer(s). These reagents will also allow rapid determination of the absolute kinetic parameters of candidate bioscavenger enzymes for isolated stereoisomers, accelerating the down-selection process of these enzymes prior to *in vivo* protective efficacy studies.

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