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Mechanistic Studies of the N-formylation of Edivoxetine, a Secondary Amine-Containing Drug, in a Solid Oral Dosage Form

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ABSTRACT

Edivoxetine (LY2216684 HCl), although a chemically stable drug substance, has shown the tendency to degrade in the presence of carbohydrates that are commonly used tablet excipients, especially at high excipient:drug ratios. The major degradation product has been identified as N-formyl edivoxetine. Experimental evidence including solution and solid-state investigations, is consistent with the N-formylation degradation pathway resulting from a direct reaction of edivoxetine with (1) formic acid (generated from decomposition of microcrystalline cellulose or residual glucose) and (2) the reducing sugar ends (aldehydic carbons) of either residual glucose or the microcrystalline cellulose polymer. Results of labeling experiments indicate that the primary source of the formyl group is the C1 position from reducing sugars. Presence of water or moisture accelerates this degradation pathway. Investigations in solid and solution states support that the glucose Amadori Rearrangement Product does not appear to be a direct intermediate leading to N-formyl degradation of edivoxetine, and oxygen does not appear to play a significant role. Solution-phase studies, developed to rapidly assess propensity of amines toward Maillard reactivity and formylation, were extended to show comparative behavior with example systems. The cyclic amine systems, such as edivoxetine, showed the highest propensity toward these side reactions.

Introduction

Edivoxetine (LY2216684 HCl, Fig. 1) is a selective norepinephrine reuptake inhibitor that has been clinically evaluated for multiple central nervous system disorders. Edivoxetine is a secondary amine-containing drug that was being developed as a solid oral tablet. During formulation development studies, a new degradation product was observed that had not been previously observed during stress testing of the drug substance. Binary excipient-compatibility studies indicated that the polyvinyl alcohol–based film coating did not appear to be contributing to the degradation of the formulation and that the highest levels of this degradation product were formed with carbohydrate-containing excipients (e.g., lactose, glucose, microcrystalline cellulose [MCC], hydroxypropyl methylcellulose).

Stress testing and accelerated stability studies of the final formulated tablet, which includes MCC as a major component, showed that the degradation pathway to this new degradation product was active in the formulation, and therefore, the product was isolated and characterized (see Experimental section) as the N-formyl derivative of edivoxetine (Fig. 1). The N-formylation of primary and secondary amines is a known pathway of the Maillard reaction,[1-4] which involves the reaction of primary or secondary amines with the aldehydic ends of reducing sugars leading to a multitude of products, ultimately to a discoloration or "browning." The first step of the Maillard reaction is condensation of the amine with the aldehyde to form an N-glycosyl amine, which can have >1 form and can dehydrate to form a Schiff base (primary amine source) or an iminium ion (secondary amine source); subsequent rearrangement produces a product known as the Amadori Rearrangement Product (ARP), which exists in both cyclic and acyclic forms.[5-7] The formation of ARPs is a reversible process, as shown in Scheme 1. ARPs are typically not very stable and can degrade to multiple by-products. N-formyl products are frequently observed in Maillard processes, but the precise pathway(s) for Maillard-associated N-formylation in solid-state drug products has not yet been clearly established.

Because MCC is formally a nonreducing sugar, it was a surprise that N-formylation was an active pathway in either the drug-excipient studies or the drug product stability studies. It is worth noting that while MCC is a nonreducing sugar, MCC is known to contain low levels of free glucose, formaldehyde, and formic acid.[8]

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0022-3549/

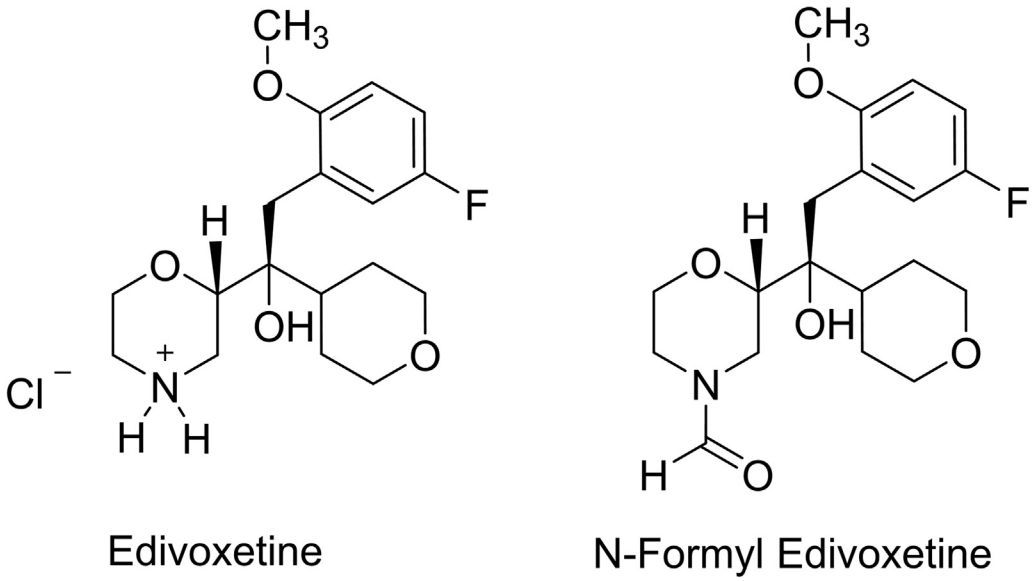
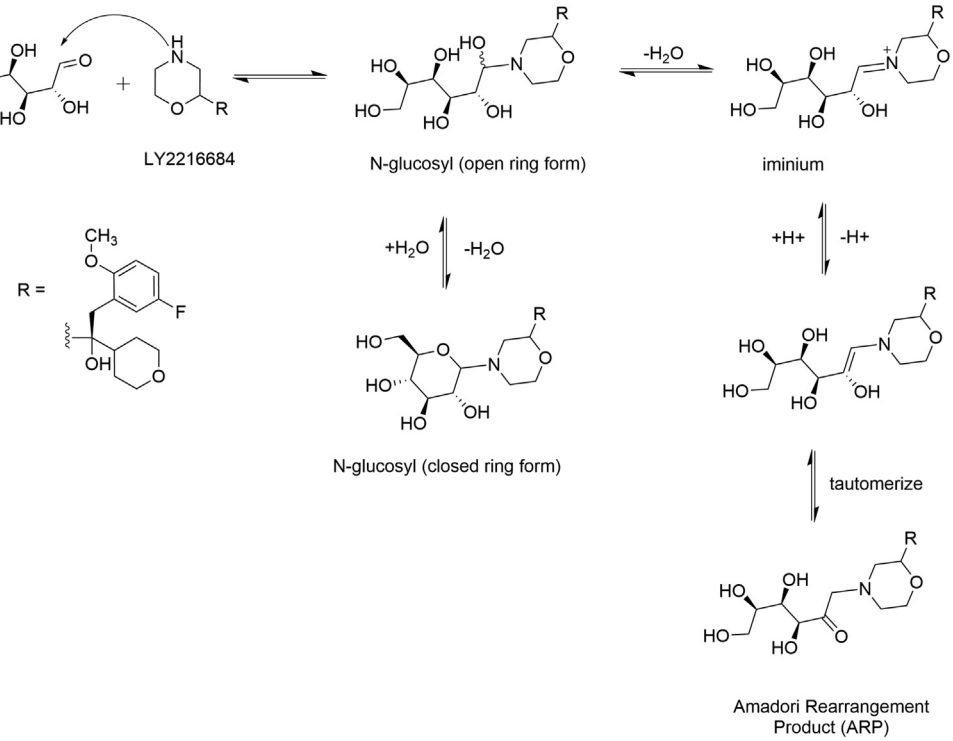


Figure 1. Chemical structures of edivoxetine, LY2216684 HCl, and the N-formyl degradation product.

Additional stability studies of edivoxetine across multiple lots/grades of MCC revealed similar N-formyl levels in all lots tested.

The degradation pathways leading to N-formylation of secondary amine-containing drugs has been documented in the literature previously, as exemplified by fluoxetine,[4] varenicline,[9] bisoprolol,[10] and duloxetine.[11] Wirth et al.[4] showed that N-formylation of fluoxetine hydrochloride could be produced through a Maillard reaction when lactose, a reducing sugar, was used in the drug product formulation. He also showed that the formation of the ARP and N-formyl derivative was base catalyzed and that both N-formyl and N-acetyl derivatives (formamides and acetamides) were formed. Citing 2 references,[12,13] Wirth et al.[4] asserted that these products were formed via 2 potential pathways: the oxidative cleavage of the glycosylamine (or the resulting ARP) or the direct reaction of the amine with a 1,2-dicarbonyl compound, glyoxal. Spiking experiments with formic acid or glyoxalic acid did not produce the N-formyl degradation product, leading to the conclusion that neither formic nor glyoxalic acids were intermediates in the pathway. Finally, Wirth et al.[4] inferred the intermediacy of the ARP in the degradation pathway to N-formyl fluoxetine by solid-state kinetic studies (heat stress of fluoxetine with lactose at 100°C, with and without magnesium stearate as a basifying agent). In the case of



Scheme 1. Reaction of reducing sugar with a secondary amine to form the ARP.

varenicline, the N-formylation was concluded to be the result of oxidative degradation of polyethylene to formaldehyde and formic acid, followed by direct reaction of varenicline with formic acid. N-methylation was also observed as a result of reaction with formaldehyde and reduction of the resulting N-hydroxymethyl derivative by formate; this N-methylation pathway is known as the Eschweiler–Clarke reaction. For bisoprolol, N-formylation was postulated to arise from oxidative degradation of the ARP resulting from reaction with reducing sugars (exemplified by glucose). In the case of duloxetine, N-formylation and N-acetylation was observed on aging in the carbohydrate-containing formulation analogous to that observed with fluoxetine; in addition, a potential complicating factor was observed during analysis of duloxetine, where an artifactual N-formylation reaction was observed during sample preparation. In this artifactual reaction, acetonitrile provided the formyl group via an oxidation reaction catalyzed by sonication and/or light in the presence of titanium dioxide. It was in this broader context that an investigation of the mechanism of N-formylation of edivoxetine in the formulated tablet was undertaken.

Experimental

Edivoxetine HCl Drug Substance and Drug Product Tablets

Drug substance and drug product were manufactured at Eli Lilly and Company. The drug substance is synthesized as a single enantiomer HCl salt, is a white to practically white solid, and is highly soluble. The synthesis of the drug substance freebase is accomplished by basification with 1 N NaOH. Drug product tablets are manufactured using a dry roller compaction process to produce varying shapes (depending on dose strength) of debossed, film-coated, immediate-release tablets. The formulation consists of edivoxetine hydrochloride drug substance, mannitol and microcrystalline cellulose fillers, croscarmellose sodium disintegrant, magnesium stearate lubricant, and varying color mixture polyvinyl alcohol–film coatings depending on the dose strength.

Table 1
UPLC/MS Method for N-Formyl Edivoxetine Degradant Characterization

Column	100 × 2.1 mm Waters BEH C_{18}, 1.7-μm particle size		
Column temperature	45°C		
Weak solvent "A"	0.1 % TFA in water		
Strong solvent "B"	Acetonitrile		
Detector	Photodiode array UV (280 nm), ES^+, ES^-		
Injection volume	2 μL		
Gradient	Time (min)	% B	Flow rate (mL/min)
	0	5	0.6
	5	80	0.6
	5.1	5	0.6
MS Parameters			
Instrument ID			ACQ-SQD L06SQD066N
Calibration file			Calibration01APR10
			ES^+
Start mass			100
End mass			1000
Data format			Centroid
Capillary			3.0 kV
Cone			25 V
Extractor			3.0 V
RF			0.1 V
Source temperature			150°C
Desolvation temperature			350°C
Desolvation gas flow			600 L/h
LM 1 resolution			22
HM 1 resolution			14.7
Ion energy 1			0.7

BEH, ethylene bridged hybrid; ES^-, electrospray$^-$; ES^+, electrospray$^+$; HM, high mass; LM, low mass; TFA, trifluoroacetic acid.

Table 2
HPLC/TOF/MS Method Used for Characterization of N-Formyl Degradant

Column	50 × 2.1 mm Agilent Extend C_{18}, 1.8-μm particle size		
Column temperature	40°C		
Weak solvent "A"	0.1% TFA in water		
Strong solvent "B"	Acetonitrile		
Detector	Photodiode array UV, ES^+		
Sample concentration	~0.3 mg/mL		
Injection volume	2.0 μL		
Gradient	Time (min)	% B	Flow rate (mL/min)
	0	5	0.6
	15	90	0.6
	15.1	5	0.6
MS Parameters			
Instrument ID			6230 Agilent TOF Tag K302913
			ES^+
Start mass			100
End mass			1700
Acquisition rate			5 Spectra/s
Data format			Centroid
Gas temperature			350°C
Drying gas			13 L/min
Nebulizer			50 psig
Sheath gas temperature			300°C
Sheath gas flow			10 L/min
VCap			4000 V
Nozzle voltage			1000 V
Fragmentor			100 V
Skimmer			65 V
OCT 1 RF Vpp			750 V
Reference masses			121.0509 *m/z*
			922.0098 *m/z*

ES^-, electrospray$^-$; ES^+, electrospray$^+$; TFA, trifluoroacetic acid; TOF, time of flight.

N-formyl Edivoxetine Degradation Product

N-formyl edivoxetine was characterized by mass spectral analysis and synthesis. A stressed edivoxetine sample containing N-formyl edivoxetine was prepared by dissolving approximately 2 mg of edivoxetine hydrochloride and 2 mg of the radical initiator 2-2′-Azobis(2,4-dimethylvaleronitrile) (VAZO52) in 4 mL of 50/50 water/acetonitrile and heating at 40°C for 16 h. Ultra performance liquid chromatography/mass spectrometry (UPLC/MS) analysis of the sample using the method given in Table 1 confirmed the presence of the proposed N-formyl edivoxetine. This sample was analyzed using a high performance liquid chromatography (HPLC) high-resolution time of flight instrument. Refer to Table 2 for the method used. The measured *m/z* value for the $[M + H]^+$ ion was 368.1867 (predicted elemental composition $C_{19}H_{27}FNO_5^+$, 0.14 ppm error) and the measured *m/z* value for the $[M + Na]^+$ ion was 390.1686 (predicted elemental composition $C_{19}H_{26}FNO_5Na^+$, 0.20 ppm error) indicating a molecular formula of $C_{19}H_{26}FNO_5$ for N-formyl edivoxetine. This formula corresponds to edivoxetine ($C_{18}H_{26}FNO_4$) plus 1 carbon atom and 1 oxygen atom.

Table 3
Preparative Liquid Chromatography Conditions to Generate the Glucose ARP

Column	Kromasil KR100, C_{18}, 50.8 mm × 25 cm, 10-μm particle size		
Weak solvent "A"	0.1% TFA in water		
Strong solvent "B"	Acetonitrile		
Detector	UV, set at 280 nm		
Injection volume	5 mL		
Gradient	Time (min)	% B	Flow rate (mL/min)
	0	5	70
	0:06	5	70
	12:00	75	70
	20:00	75	70
	20:06	5	70

TFA, trifluoroacetic acid.

Table 4
HPLC Conditions for Detection of N-Formyl Degradant

Variable	Method A			Method B		
Nominal sample concentration	0.5 mg/mL			0.5 mg/mL		
Sample diluent	50/50 0.1 N HCl/methanol			50/50 0.1 N HCl/methanol		
Column	Zorbax bonus RP, 4.6 × 75mm, 3.5-μm particle size			ACE 3 phenyl, 4.6 × 150 mm, 3-μm particle size or equivalent		
Mobile phase A	90:10 10 mM pH 5.90 ammonium acetate buffer:acetonitrile			0.1% (vol/vol) TFA in water		
Mobile phase B	5:95 10 mM pH 5.90 ammonium acetate buffer:acetonitrile			0.1% (vol/vol) TFA in methanol		
Flow rate	1.0 mL/min			1.0 mL/min		
Detection wavelength	280 nm			280 nm		
Injection volume	20 μL			40 μL (with methanol needle rinse)		
Autosampler temperature	Refrigerated			Refrigerated		
Gradient program	Time (min)	% A	% B	Time (min)	% A	% B
	0.0	100	0	0.0	60	40
	38.0	0	100	20.0	10	90
	40.0	0	100	25.0	10	90
	40.1	100	0	25.1	60	40
	48.0	100	0	30.0	60	40

TFA, trifluoroacetic acid.

The structure of N-formyl edivoxetine was confirmed by synthesis of the N-formyl derivative and comparison of retention time and mass spectrum to the proposed N-formyl edivoxetine from the stressed sample. The N-formyl edivoxetine derivative was synthesized by reacting edivoxetine free base with formyl-acetic anhydride generated *in situ* from acetic anhydride and formic acid. One milliliter of formic acid (26.5 mmol), 0.25 mL of acetic anhydride (2.7 mmol), and 3 mL of methylene chloride were combined in a vial and dried over anhydrous sodium sulfate. Approximately 203 mg of edivoxetine hydrochloride (~0.54 mmol) was dissolved in 2 mL of 50/50 water/acetonitrile, transferred to a separatory funnel along with 20 mL of pH 8 phosphate buffer, and extracted with methylene chloride. The methylene chloride extract was dried over anhydrous sodium sulfate. Over the course of ~5 min, a total of 1.1 mL of the acetic anhydride/formic acid solution was added in 0.2-mL increments. The reaction mixture was washed with 0.1 N HCl in a separatory funnel to hydrolyze any remaining anhydride and remove all acetic and formic acid. The methylene chloride layer was removed, dried over anhydrous sodium sulfate, and the solvent evaporated with a stream of nitrogen. The resulting oil was dissolved in ~2 mL of 50/50 water/acetonitrile, frozen, and lyophilized to yield a white solid. A small amount of this material was dissolved in 50/50 water/acetonitrile, analyzed using UPLC/MS, and the resulting chromatogram compared with a VAZO52 sample containing the degradant peak. Both a retention time and mass match were confirmed for the VAZO52 stressed edivoxetine sample and the synthesized N-formyl edivoxetine derivative.

Table 5
GC/MS Conditions Used for Formic Acid Analysis

Sample Diluent	1% p-toluenesulfonic acid in ethanol		
GC column	Phenomenex ZB-wax, 30 m × 0.32 mm i.d., 0.5-μm film thickness		
Carrier gas	Helium		
Carrier gas flow rate	2.5 mL/min		
Inlet temperature	170°C		
Split ratio	10:1		
Split flow	25 mL/min		
Oven temperature program	Initial	40°C	Hold for 4.2 min
	Ramp	40°C/min to 200°C	Hold for 2 min
Headspace parameters	Oven temperature	60°C	
	Equilibration time	15 min	
	Loop temperature	120°C	
	Transfer line temperature	120°C	
	Vial pressurization time	0.1 min	
	Loop fill time	0.5 min	
	Loop equilibration time	0.05 min	
	Injection volume	1 mL	
Mass selective detector parameters	Transfer line temperature	280°C	
	Source temperature	280°C	
	Quad temperature	150°C	
	Mode	Scan, 25-100 amu	

Edivoxetine—Glucose ARP

Approximately 200 mg of edivoxetine glucose adduct (ARP) was prepared by heating a mixture of edivoxetine HCl, glucose, dimethyl sulfoxide, and a small amount of pyridine. Approximately 1000 mg of edivoxetine HCl and 1000 mg of glucose were weighed into a 500-mL volumetric flask and diluted with 200 mL dimethyl sulfoxide and 4 mL of pyridine. This sample was placed in the 85°C oven for 17 h. After heating, ~10% of the ARP was formed. The ARP was isolated using preparative liquid chromatography following the method conditions listed in Table 3. The ARP was completely dried down following lyophilization. Approximately 200 mg of light brown powder

Table 6
UPLC/MS Method Conditions Used for Determination of N-Formyl Degradant

Column	100 × 2.1 mm Waters BEH C_{18}, 1.7-μm particle size		
Column temperature	45°C		
Weak solvent "A"	95/5 0.1% HOAc in water titrate to pH 5.6 with ammonium hydroxide/acetonitrile		
Strong solvent "B"	Acetonitrile		
Detector	Photodiode array UV (280 nm), ES^+		
Injection volume:	2 μL		
Gradient:	Time (min)	% B	Flow rate (mL/min)
	0	5	0.6
	5	75	0.6
	6	75	0.6
	6.1	0	0.6
MS Parameters			
Instrument ID	ACQ-SQD L06SQD066N		
Calibration file	Calibration Sep_09		
	ES^+	ES^-	
Start mass	100	100	
End mass	1000	1000	
Data format	Centroid	Centroid	
Capillary	3.0 kV	3.0 kV	
Cone	30 V	30 V	
Extractor	3.0 V	3 V	
RF	0.1 V	0.1 V	
Source temperature	150°C	150°C	
Desolvation temperature	350°C	350°C	
Desolvation gas flow	650 L/h	650 L/h	
LM 1 resolution	15	15	
HM 1 resolution	15	15	
Ion energy 1	0.5	0.5	

BEH, ethylene bridged hybrid; ES^-, electrospray$^-$; ES^+, electrospray$^+$; HM, high mass; LM, low mass.

Table 7
LC/FTMS Method Conditions Used for Determination of ^{13}C Incorporation Into N-formyl Degradant

Parameter	Setting		
HPLC parameters (Agilent 1100)			
Mobile phase A	0.1% (vol/vol) formic acid in H_2O		
Mobile phase B	0.1% (vol/vol) formic acid in acetonitrile		
Flow rate	1.0 mL/min		
Gradient	Time (min)	% A	% B
	0	90	10
	30	10	90
	30.1	90	10
	38	90	10
Injection volume	20 μL		
Column	Zorbax SB-C8, 150 × 4.6mm, 3.5 μm		
Column temperature	30°C		
PDA detection	200-400nm		
ESI FTMS conditions (Finnigan LTQ FT mass spectrometer)			
Ionization mode	Positive ion electrospray		
Scan range	80-1000 *m/z*		
Sheath gas flow (N2)	Approximately 40 (arbitrary unit)		
Source voltage	4 kV		
Capillary voltage	46 kV		
Tube lens voltage	98 kV		
Capillary temperature	250°C		
Instrument software	Xcalibur, version 2.0		

ESI, electrospray ionization; LTQ, linear trap quadrupole.

remained. The purified sample contained 98.7% ARP and 1.24% edivoxetine.

Analytical Methodology

UPLC/MS and HPLC/Time of Flight/MS Analytical Methodology for N-Formyl Edivoxetine Degradation Product Characterization

Tables 1 and 2 provide the method conditions used for characterization of the N-formyl edivoxetine degradation product.

Table 8
UPLC/MS Method Conditions Used for Determination of ARP

Column	100 × 2.1 mm Waters BEH C_{18}, 1.7-μm particle size		
Column temperature	45°C		
Weak solvent "A"	95/5 0.1% HOAc in water titrate to pH 5.6 with ammonium hydroxide/acetonitrile		
Strong solvent "B"	Acetonitrile		
Detector	Photodiode array UV (280 nm), ES^+		
Injection volume	1 μL		
Gradient	Time (min)	% B	Flow rate (mL/min)
	0	0	0.6
	5	75	0.6
	6	75	0.6
	6.1	0	0.6
MS Parameters			
Instrument ID	ACQ-SQD L06SQD066N		
Calibration file	Calibration11_08B		
	ES^+	ES^-	
Start mass	70	70	
End mass	900	900	
Data format	Centroid	Centroid	
Capillary	2.55 kV	2.77 kV	
Cone	23 V	77 V	
Extractor	1.0V	3 V	
RF	1.5 V	1.9 V	
Source temperature	150°C	150°C	
Desolvation temperature	400°C	400°C	
Desolvation gas flow	900 L/h	900 L/h	
LM 1 resolution	18	18	
HM 1 resolution	18	18	
Ion energy 1	0.54	0.64	

BEH, ethylene bridged hybrid; ES^-, electrospray$^-$; ES^+, electrospray$^+$; HM, high mass; LM, low mass.

Table 9
HPLC Method Conditions Used for Impurities Determination in Solution-Phase Experiments

Column	Zorbax SB-C8, 25 cm × 4.6 mm, × 5 mm		
Weak solvent "A"	0.1% H_3PO_4		
Strong solvent "B"	Acetonitrile		
Detector	UV, set at 250 nm		
Injection volume	10 μL		
Gradient	Time (min)	% B	Flow rate (mL/min)
	0	10	1.0
	10	90	1.0
	16	90	1.0
ARP	6.67		
Edivoxetine	6.89		
N-formyl	9.60		

Preparative Liquid Chromatography Method Conditions for Generation of Glucose ARP

Table 3 lists the summary method conditions for the preparative liquid chromatography conditions used to generate the glucose ARP.

HPLC Analytical Control Methodology for Impurities Determination

Table 4 lists the HPLC method conditions used for N-formyl degradant analysis.

Gas Chromatography/Mass Spectrometry (GC/MS) Analytical Methodology for Formic Acid Analysis

Table 5 lists the method conditions used for analysis of formic acid in various excipient and blend samples.

UPLC/MS Analytical Methodology for N-Formyl Degradant Determination

Table 6 lists the UPLC/MS method conditions used to determine N-formyl degradant levels in samples stressed with formic acid vapor.

Liquid Chromatography/Fourier Transform Mass Spectrometry (LC/FTMS) Analytical Methodology for ^{13}C Incorporation Into N-Formyl Degradant

Table 7 lists the LC/FTMS method conditions used for the determination of ^{13}C incorporation into N-formyl degradant from labeled glucose.

UPLC/MS Analytical Methodology for ARP Degradant Determination

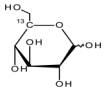
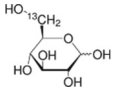
Table 8 lists the UPLC/MS method conditions used for determination of ARP in stressed samples.

Table 10
LC/MS Parameters for ^{13}C-Labeled Glucose and API Solution-State Studies

Parameter	Setting		
HPLC parameters			
Mobile phase A	0.1% (vol/vol) TFA in H_2O		
Mobile phase B	0.1% (vol/vol) TFA in ACN		
Flow rate	1.5 mL/min		
Gradient	Time (min)	% A	% B
	0	82	18
	5	82	18
	20	20	80
	20.1	82	18
	23	82	18
Injection volume	10 μL		
Column	Zorbax Bonus RP, 75 × 4.6 mm, 3.5 μm		
Column temperature	40°C		
Mass spectrometer settings			
Ionization mode	Positive ion electrospray		
Scan range	150-800 *m/z*		

TFA, trifluoroacetic acid.

Table 11
Summary of Materials and Associated Experiments

Description	Manufacturer	Experiment	Document Section
MCC	FMC biopolymer (Avicel® PH102)	Experimental materials used for comparison of N-formylation rates induced by MCC, lactose, and glucose excipients.	Comparison of N-Formylation Rates Induced by MCC, Lactose, and Glucose
Lactose monohydrate	Foremost Farms		
Glucose	Sigma		
LY2216684 HCl	Lilly		
Sterile water for injection	Hospira		
Syringe filters, 25 mm with 0.45-μm PVDF membrane	PALL		
Microcrystalline Cellulose Avicel® PH200	FMC Biopolymer	Experimental materials used for the assessment of formic acid in MCC, lactose, and glucose excipients.	Formation of Formic Acid in Lactose, Glucose, and MCC
Microcrystalline Cellulose Avicel® PH102	FMC Biopolymer		
D-(+)-Glucose	Sigma		
Lactose monohydrate	Foremost Farms		
LY2216684 HCl	Lilly		
LY2216684 free base	Lilly		
C1 position ^{13}C-labeled glucose 	Aldrich	Experimental materials used to assess incorporation of ^{13}C from labeled glucose into the N-formyl degradant in the solid state. Experimental materials used for the ^{13}C-labeld glucose experiments in the solution phase.	Selective ^{13}C-Labeled Glucose (C1-C6) Incorporation Into N-formyl Group ^{13}C-Labeled Glucose Experiments
C2 position ^{13}C-labeled glucose 	Isotec		
C3 position ^{13}C-labeled glucose 	Cambridge Isotope Labs		
C4 position ^{13}C-labeled glucose 	Cambridge Isotope Labs		
C5 position ^{13}C-labeled glucose 	Cambridge Isotope Labs		
C6 position ^{13}C-labeled glucose 	Isotec		
LY2216684 HCl	Lilly		
Sterile water for injection (solid state experiments only)	Hospira		
LY2216684 HCl API	Lilly	Experimental materials used for experiments to understand the selective ^{13}C incorporation into formic acid formed from ^{13}C-labeled glucose/API blends in ambient and nitrogen inerted environments.	Selective ^{13C} Incorporation Into Formic Acid Formed From ^{13}C-Labeled Glucose/API Blends in Ambient and Inert Environments
C1 position ^{13}C-labeled glucose	Aldrich, Isotec		
C2 position ^{13}C-labeled glucose	Isotec		
Sterile H_2O for injection	Hospira		
LY2216684 placebo	Lilly	Experimental materials used for investigation of the involvement of the glucose ARP in the formation of N-formyl degradant.	Role of ARP—Is It an Intermediate?
LY2216684 HCl	Lilly		
ARP	Prepared *in situ*		
LY2216684 HCl	Lilly	Experimental materials used in the solution phase investigation of reducing sugars and edivoxetine drug substance.	Reducing Sugar and Edivoxetine Drug Substance Evaluations
Fluoxetine.HCl	Siegfried		
Vortioxetine.HBr	ARK Pharm, Inc.		
4-Phenyl piperazine	Alfa Aesar		
Glucose	Aldrich		
Arabinose	Aldrich		
Fructose	Aldrich		
Maltose	Aldrich		
Lactose	Aldrich		

(*continued on next page*)

Table 11 (*continued*)

Description	Manufacturer	Experiment	Document Section
LY2216684 HCl Magnesium stearate KOH NaCMC (70K and700K) MCC Glucose	Lilly Aldrich Mallincrodt Aldrich Aldrich Aldrich	Experimental materials used for the investigation of excipient effects in the solution phase.	Effects of Excipients
LY2216684 free base Glyoxal Methyl glyoxal Formic acid Citric acid Glucose	Lilly Aldrich Aldrich Aldrich Aldrich Aldrich	Experimental materials used for the spiking study reaction investigations with formic acid, methyl glyoxal, and glyoxal in the solution phase.	Spiking Reactions With Formic Acid, Methyl Glyoxal, and Glyoxal
LY2216684 HCl Lysine Arginine Arginine.HCl Glucose	Lilly Aldrich Aldrich Aldrich Aldrich	Experimental materials used for the investigation of Maillard and acid scavengers in the solution phase.	Maillard and Acid Scavengers

PVDF, polyvinylidene difluoride.

HPLC Analytical Methodology for Solution-Phase Experiments
Impurity Determination

Table 9 lists the UPLC/MS method conditions used for determination of ARP in stressed samples.

LC/MS Analytical Methodology for Solution-Phase Experiments Investigating ^{13}C Incorporation Into N-Formyl Degradant

Table 10 lists the LC/MS method conditions used to determine the ^{13}C incorporation into N-formyl degradant for solution-state studies.

Materials

Table 11 lists a summary of the experimental materials used for the experiments discussed earlier.

Results and Discussion

As mentioned in the Introduction, stress testing and accelerated stability studies of edivoxetine tablets showed evidence of an active degradation pathway to 2 major degradation products, originally observed during excipient compatibility studies (see Fig. 2 for representative HPLC chromatogram of a stressed sample). The 2 degradation products were isolated and characterized (see Experimental) as N-formyl edivoxetine and glucose Amadori Rearrangement Product (glucose ARP), consistent with an active Maillard degradation pathway. As part of the analytical control strategy development, an investigation was undertaken to help understand the mechanism of formation and potential variables to control the degradation pathway.

Solid-State Studies

Comparison of N-formylation Rates Induced by MCC, Lactose, and Glucose

Experiments were conducted using binary blends of the active pharmaceutical ingredient (API) and MCC, lactose or glucose at a 1:50 ratio (wt/wt) of API:excipient, similar to the ratios that would be expected in the unit dose formula. Samples were prepared by mixing the API and excipient with a mortar and pestle and storing

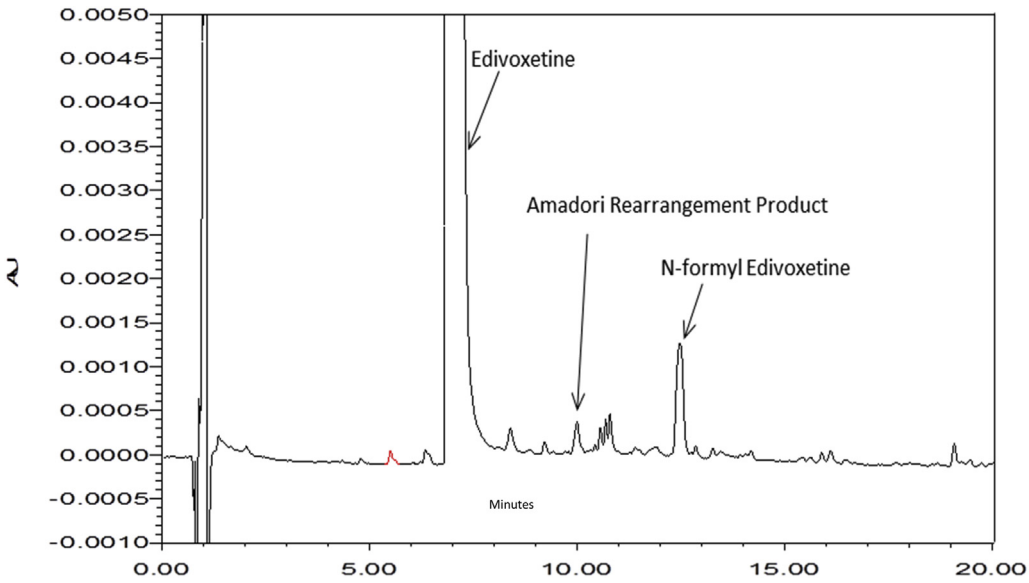


Figure 2. Representative HPLC-UV chromatogram for the analysis of an edivoxetine tablet after exposure to 70°C/75% RH for 36 h (method A, Experimental).

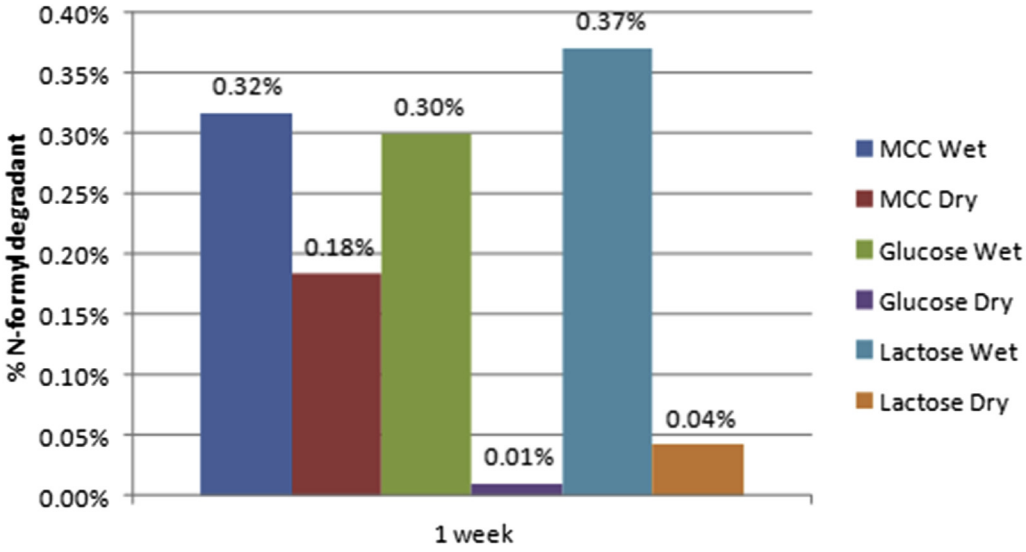


Figure 3. Amount of N-formyl (%) found in API:excipient (MCC, glucose, lactose) blends after 1 week at 70°C (closed container). Legend top to bottom equals bars left to right.

at 70°C/ambient relative humidity (RH) in vials with a tightly closed lid, with (referred to as "wet" hereafter) or without (referred to as "dry" hereafter) addition of water (40% wt/wt). After 1 week of storage, samples were analyzed by HPLC (refer to HPLC method B in Experimental section), and the results are shown in Figure 3.

The presence of the high level of moisture spiked onto the samples resulted in much higher levels of N-formylation. The surprisingly high levels of N-formylation in the presence of the MCC (which is a nonreducing sugar) suggested that known MCC impurities, such as free glucose, formic acid, or formaldehyde, might be involved in the reaction.[8,14] Experiments were, therefore, conducted to evaluate the potential role of formic acid and/or formaldehyde in the N-formylation pathway.

Spiking Studies With Formic Acid and Formaldehyde

Studies were performed to evaluate the potential of formic acid, a known breakdown product of glucose and other reducing sugars and a known contaminant of many carbohydrate-based excipients, to be the N-formylating reagent. Because of the potential for suppressing the reaction by dramatically lowering the pH from direct addition of formic acid to the formulation (protonating the secondary amine and thus dramatically reducing nucleophilicity), exposure of edivoxetine hydrochloride to formic acid vapors was considered.

Approximately 100 mg of the placebo formulation was mixed with ~5 mg of edivoxetine API, ground into a fine powder with a mortar and pestle, and placed into a 20-mL scintillation vial. In addition, a 4-mL uncapped scintillation vial containing formic acid was placed in the same 20-mL scintillation vial. The 20-mL scintillation vial was then capped and stored at 40°C for 12 days. After 12 days, the sample was analyzed on the UPLC/MS system following the method conditions found in Table 6. The level of N-formyl was found to be >4% after 12 days, indicating that formic acid (present either as a formulation impurity or generated upon aging) can react

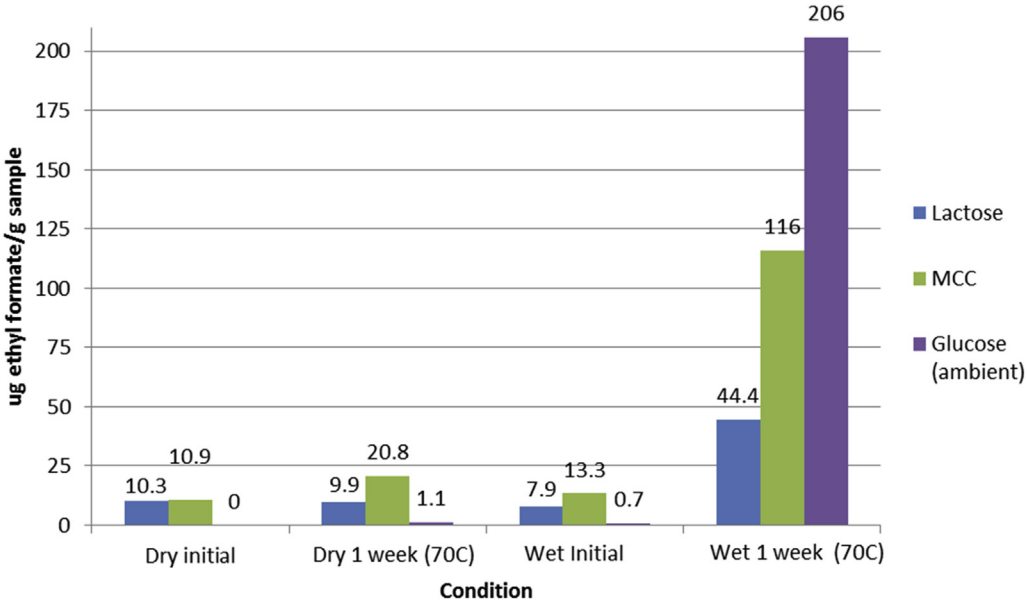


Figure 4. Amount of formic acid (detected as the ethyl formate derivative) found in lactose, MCC, and glucose before and after 1 week at 70°C for both dry and wet samples.

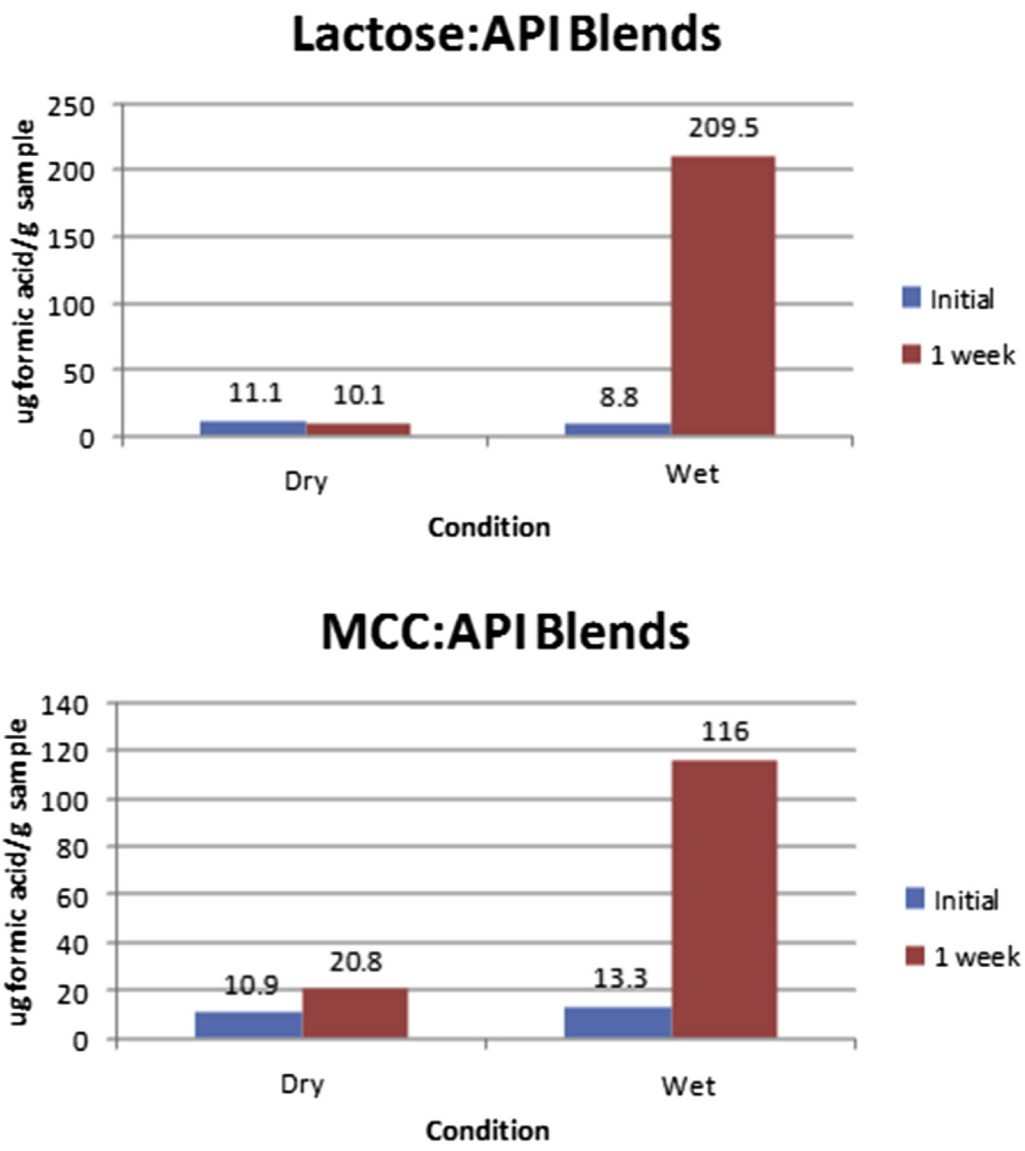


Figure 5. Amount of formic acid (detected as the ethyl formate derivative) formed in blends of lactose and edivoxetine and MCC and edivoxetine before and after 1 week at 70°C.

with edivoxetine to form the N-formyl product. Similar spiking studies with formaldehyde did not result in formation of N-formyl edivoxetine, suggesting that formaldehyde is not participating in the N-formylation.

Formation of Formic Acid in Lactose, Glucose, and MCC

Because spiking studies demonstrated that formic acid can react with edivoxetine to form the N-formyl product and because formic acid is a known degradation product of reducing sugars, the potential for formation of formic acid in lactose, glucose, and MCC from degradation was investigated. The investigation involved stressing of these common excipients at 70°C at low humidity and with added water (20% wt/wt), both alone and in the presence of edivoxetine. The amount of formic acid was assessed by GC/MS analysis (see Table 5 for method conditions) using a derivatization method, detecting formic acid as the ethyl formate derivative, as previously described.[8]

As shown in Figure 4, after 1 week at 70°C, there was a low level of residual formic acid (ca. 10 μg/g, detected as the ethyl formate derivative) in unstressed lactose that increased to over 40 μg/g for lactose that has water spiked into the sample (wet), whereas the levels for the sample without spiked water (dry) actually decreased modestly. In the case of MCC, the measured levels of formic acid more than doubled after 1 week of stress (from an initial level of ca. 11 to roughly 25 μg/g) for the dry sample and increased substantially for the wet sample. Glucose shows a much more dramatic increase in formic acid levels, increasing from <1 to >200 μg/g after 1 week at 70°C under wet conditions; very little increase of formic acid was observed under dry conditions.

When blended with edivoxetine, however, the levels of formic acid increased much more dramatically on 1 week of stress for all 3 excipients in the presence of added moisture (wet samples), suggesting a significant role of edivoxetine in the reaction to form formic acid (see Figs. 5 and 6). It is worth noting here that the effect of oxygen was studied in the case of glucose (both alone and with blends of edivoxetine) by conducting the study either in the presence of air (21% oxygen) or under a nitrogen-inerted atmosphere (samples were prepared in a glove box in a nitrogen atmosphere, tightly sealed in a glass vial). As seen in Figure 6, nitrogen inerting modestly decreases the yield of formic acid (approximately 36% for glucose alone, 17% for dry glucose:edivoxetine blend, and 31% for wet glucose:edivoxetine blend after 1 week). These results indicate

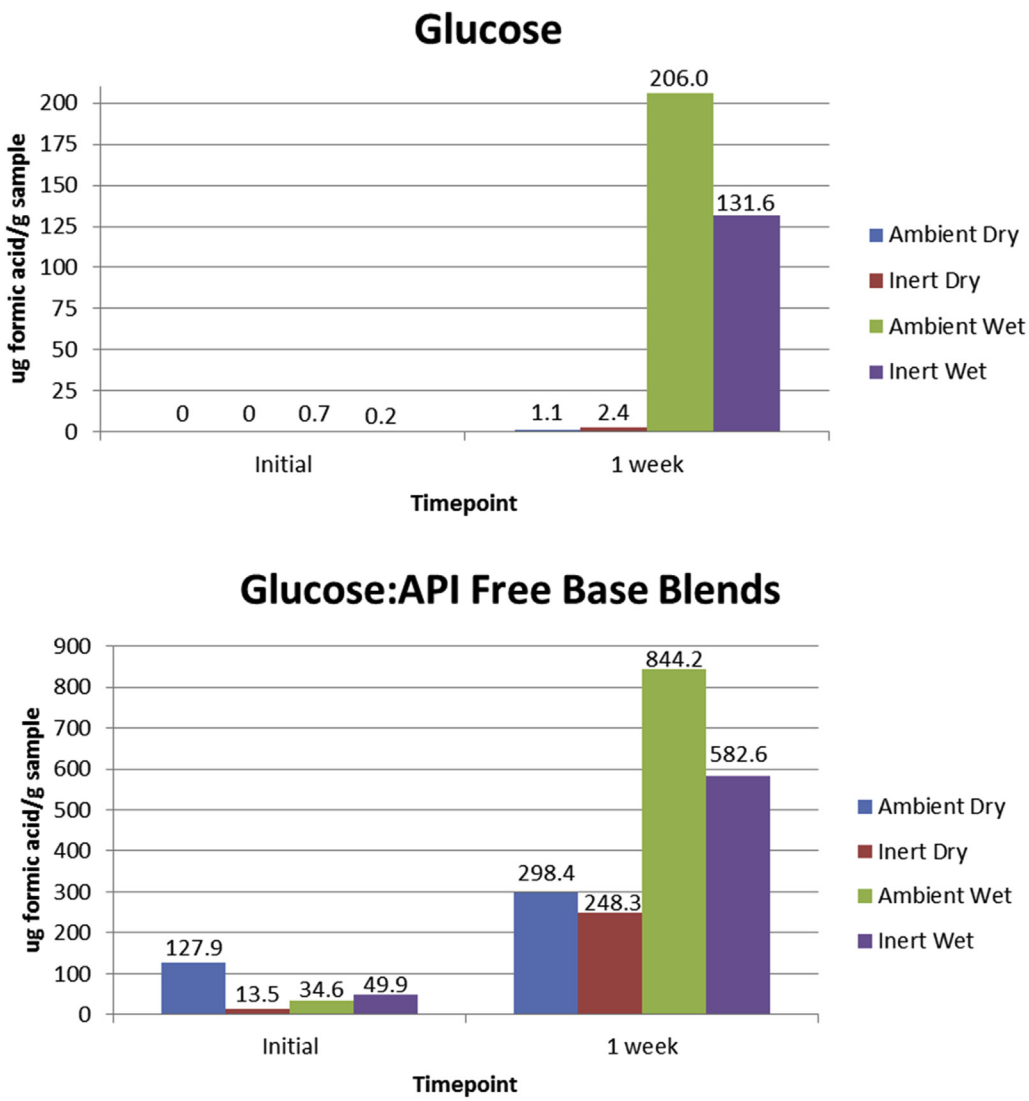


Figure 6. Amount of formic acid (detected as ethyl formate derivative) found in glucose and blends of glucose and edivoxetine free base prepared in air (ambient) and nitrogen (inert) conditions before and after 1 week stored at 70°C.

that oxygen moderately increases the rate of formation of formic acid from glucose, implying that formic acid can be formed from both oxidative and nonoxidative pathways. These results are consistent with chemistry associated with the Maillard reaction that has been described in the literature previously.[15-17]

Selective ^{13}C-Labeled Glucose (C1-C6) Incorporation Into N-formyl Group

Experiments were conducted with ^{13}C-labeled glucose to delineate the source of the carbon incorporated into the N-formyl degradant. Binary blends (1:1, wt/wt) of ^{13}C-labeled glucose isotopomers (single ^{13}C labels at positions 1 through 6; C1−C6 positions) and edivoxetine API (5 mg of API:5 mg glucose:100 μL water) were prepared and stressed at 70°C for 1 week in a closed container. See Table 11 (Materials section) for materials used. The resulting formation of N-formyl degradant was measured using HPLC method B (see Table 4). The amount of ^{13}C incorporation from each carbon position in glucose was measured using the LC/FTMS method (see Table 7). All 6 ^{13}C-labeled glucose:API wet blends showed formation of significant and comparable levels of N-formyl (0.2-0.45%). Results from this study indicated a preferential incorporation of C1 transfer from glucose into the N-formyl carbon.

Table 12
Isotopic Abundance Ratios of N-Formyl Degradant Produced by Stressing Edivoxetine API With ^{13}C-Labeled Glucose

Position of ^{13}C Label in Glucose	*m/z* 390 Intensity	*m/z* 391 Intensity	m/z 391/390 Intensity Ratio	Solid-State % Enrichment[a]
C1	667620	752726	112.75	48
C2	1309442	300593	22.96	2.4
C3	1429573	328194	22.96	2.4
C4	1245994	267019	21.43	0.9
C5	670340	140381	20.94	0.4
C6	966217	259708	26.88	6.0

[a] Calculation: [391 counts − (390 counts × 0.205)]/[391 counts − (390 counts × 0.205) + 390 counts)] × 100.

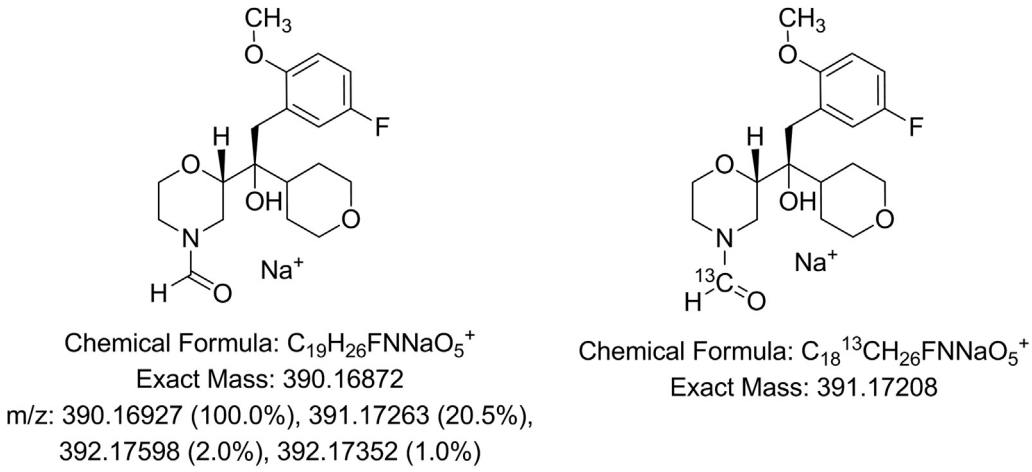


Figure 7. Structures of sodiated N-formyl degradation product with natural isotopic abundance listed (left) and ^{13}C N-formyl degradation product (right).

Table 12 lists the isotopic abundance analysis by LC/FTMS (Table 7) for wet glucose:API blend samples. The structures and isotopic abundance information of sodiated N-formyl degradant and ^{13}C N-formyl degradant (measured ions) are shown in Figure 7. The relative ratio of the *m/z* 391 peak to the m/z 390 peak was measured for the N-formyl peak in each sample. In non–^{13}C-enriched N-formyl degradant peaks, the intensity of the *m/z* 391 peak should be 20.5% of the *m/z* 390 peak (Fig. 7). If the carbon in the N-formyl product is coming from one or more of the carbons in glucose, the intensity of the *m/z* 391 peak should be >20.5% of the m/z 390 peak. As seen in Table 12, the data indicate that ^{13}C was incorporated into N-formyl primarily from C1 of glucose, and to a much lesser extent, C6.

Selective ^{13}C Incorporation Into Formic Acid Formed From ^{13}C-Labeled Glucose/API Blends in Ambient and Inert Environments

Experiments were conducted with ^{13}C-labeled glucose to investigate the mechanism of formation of formic acid from glucose degradation. The previous study for incorporation of ^{13}C into N-formyl showed preferential incorporation from the C1 position. In this study, ^{13}C at the C1 and C2 positions of glucose were investigated for their contribution to formic acid formation. Binary blends (1:1, wt/wt) of ^{13}C-labeled glucose isotopomers (selectively labeled on either C1 or C2 positions) and API were prepared and stressed at 70°C for 1 week in a closed container. These samples were also used to examine the role that oxygen may play in the degradation mechanism (prepared either in a glove box under a nitrogen blanket [inert] or under normal atmospheric conditions [in air]). For glucose alone, approximately 100 mg of material was weighed into a 10-mL headspace vial and capped tightly. Binary blends made up of approximately 1 g ^{13}C-labeled glucose (selectively labeled on either C1 or C2) and 1 g API were prepared under both inert (under nitrogen) and ambient conditions using a mortar and pestle. Approximately 200 mg of each blend was weighed into separate 10-mL headspace vials and capped. Water (20 μL) was added to the samples (to increase reaction rate) before storing in an

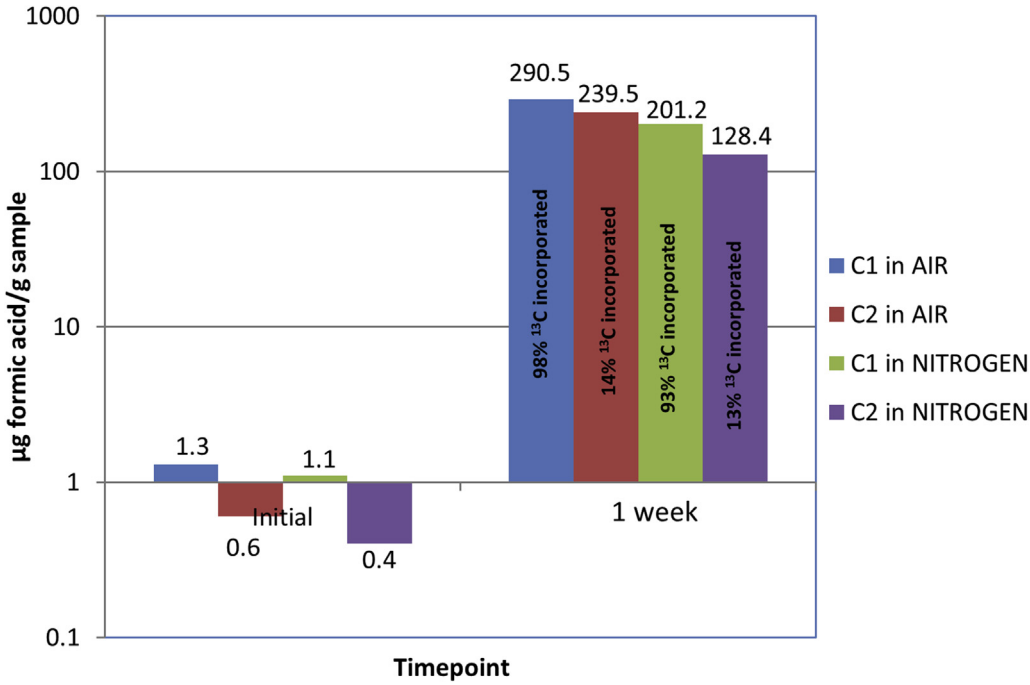


Figure 8. Amount of formic acid (detected as the ethyl formate derivative) in glucose before and after 1 week at 70°C under air and nitrogen (logarithmic scale).

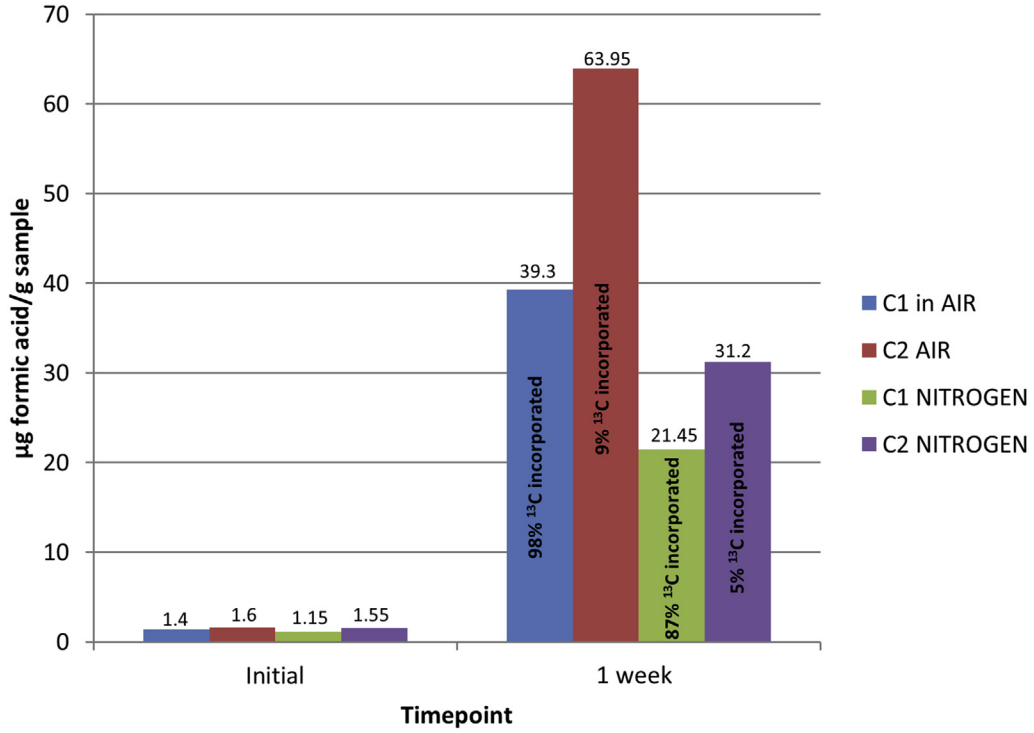


Figure 9. Amount of formic acid (detected as the ethyl formate derivative) in glucose:edivoxetine blends after 1 week at 70°C in air and nitrogen illustrating the percent of ^{13}C incorporation from C1- and C2-labeled glucose.

oven at 70°C for 1 week. The amount of formic acid was determined by GC/MS analysis (see Table 5).

Figures 8 and 9 show the microgram formic acid/g glucose or glucose:edivoxetine blend, respectively, on a logarithmic scale with the amount of ^{13}C incorporation labeled on each bar. In both cases (either glucose stressed alone or glucose stressed in the presence of edivoxetine API), the samples stressed in air had slightly higher amounts of formic acid than those stressed under a nitrogen environment. The formic acid produced in the C1-labeled samples stored in air incorporated ^{13}C almost quantitatively (98% incorporation), clearly implicating the C1 position of glucose in formic acid formation. C1-labeled samples stored under nitrogen also showed

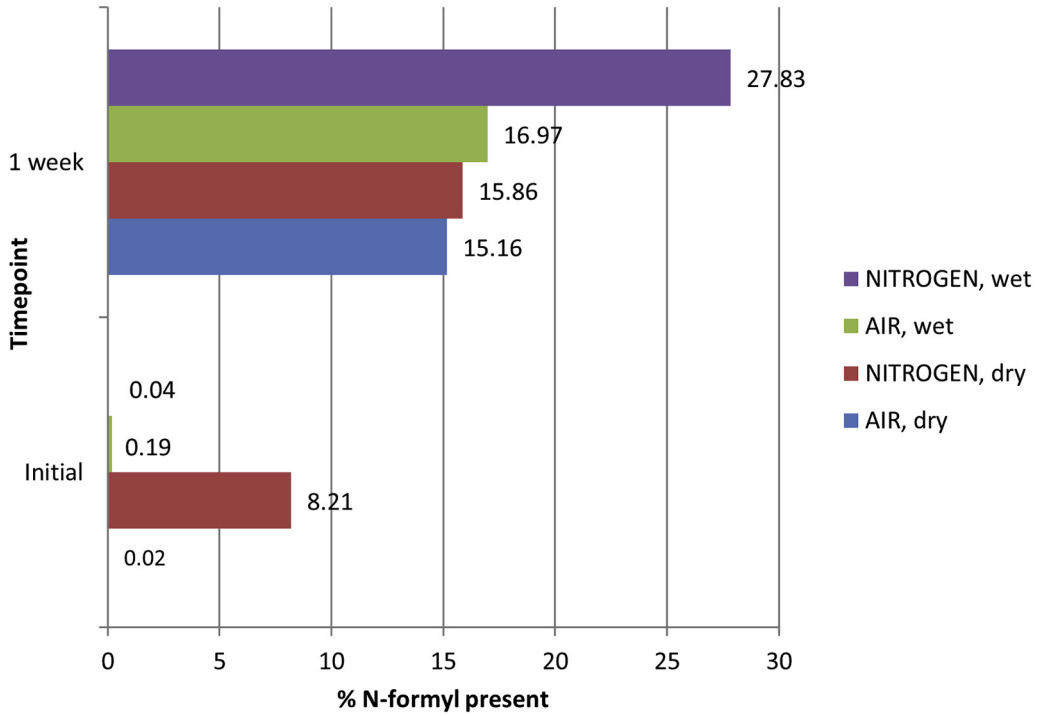


Figure 10. Amount of N-formyl degradant formed in wet and dry edivoxetine free base:glucose blends prepared under air and nitrogen and stored at 70°C for 1 week.

Table 13
DOE Study of the Solution-Phase Reaction of Edivoxetine With Glucose

Entry	KOH (Equivalent)	pH	Glucose (Equivalent)	Time (h)	ARP (Area %)	N-Formyl Degradant (%)
1	0	4.00	2	96	0	0.0
2	0.25	6.75	2	96	3.18	0.89
3	0.50	7.50	2	24	4.72	0.54
4	0.75	8.02	2	24	4.43	0.98
5	1.06	NR	2	24	3.22	2.25
6	1.25	9.07	1	20	1.36	5.05
7	1.50	NR	2	24	1.17	10.11
8	1.50	9.28	0.5	20	5.58	4.77
9	1.5	13	0.0	96	0	0.0
10	0	9.3	2.0	23	3.99	2.12

NR, not recorded.

high levels of ^{13}C (87% and 93% incorporation). The formic acid produced in the C2-labeled samples stored in air incorporated only low levels of ^{13}C (ranging from 5% to 14% incorporation), indicating that the C2 position of glucose is a minor contributor to formic acid formation.

The Role of Oxygen in the N-Formylation Reaction

Having established that oxygen moderately increases the formation of formic acid from glucose on aging, the role of oxygen in the N-formylation reaction was investigated. The free base of edivoxetine was chosen to allow higher levels of N-formyl formation in a shorter period of time versus the more stable edivoxetine HCl salt. Thus, 1:1 (wt/wt) blends of glucose and edivoxetine-free base were prepared either under air (normal laboratory conditions) or under nitrogen, and samples were stored for 1 week at 70°C. A small amount of water (20%, wt/wt) was added to half of the samples to increase reaction rates. These are referred to as wet samples and the others referred to as dry samples.

HPLC method B (see Table 4) was used to determine the level of N-formylation. Figure 10 shows that all samples had significant levels of the N-formyl degradant formed after 1 week (ranging from 15% to 28%); surprisingly, the levels formed under nitrogen

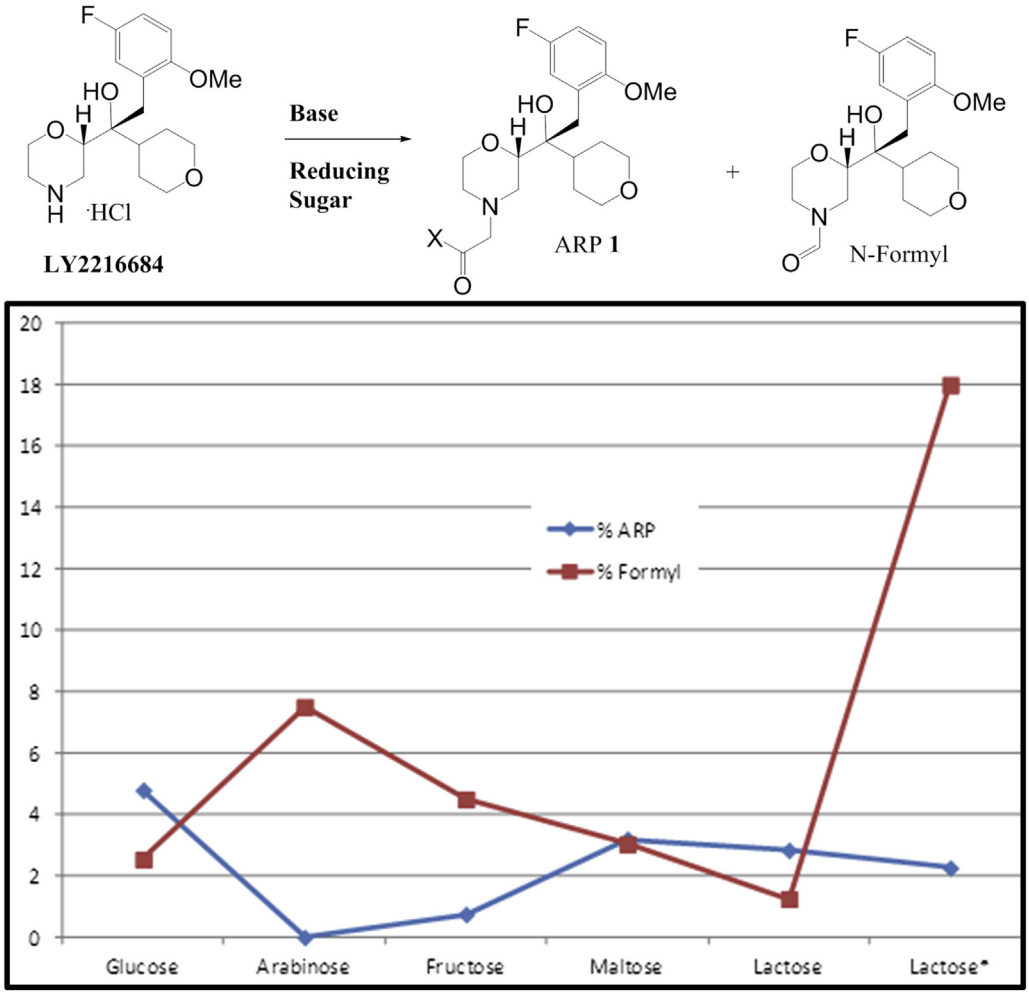


Figure 11. Reducing sugar Maillard reactivity: 2 equivalent reducing sugar with edivoxetine HCl, 1.0 equivalent base, and 24 h reaction time at 60°C. "*", 1.25 equivalent base added.

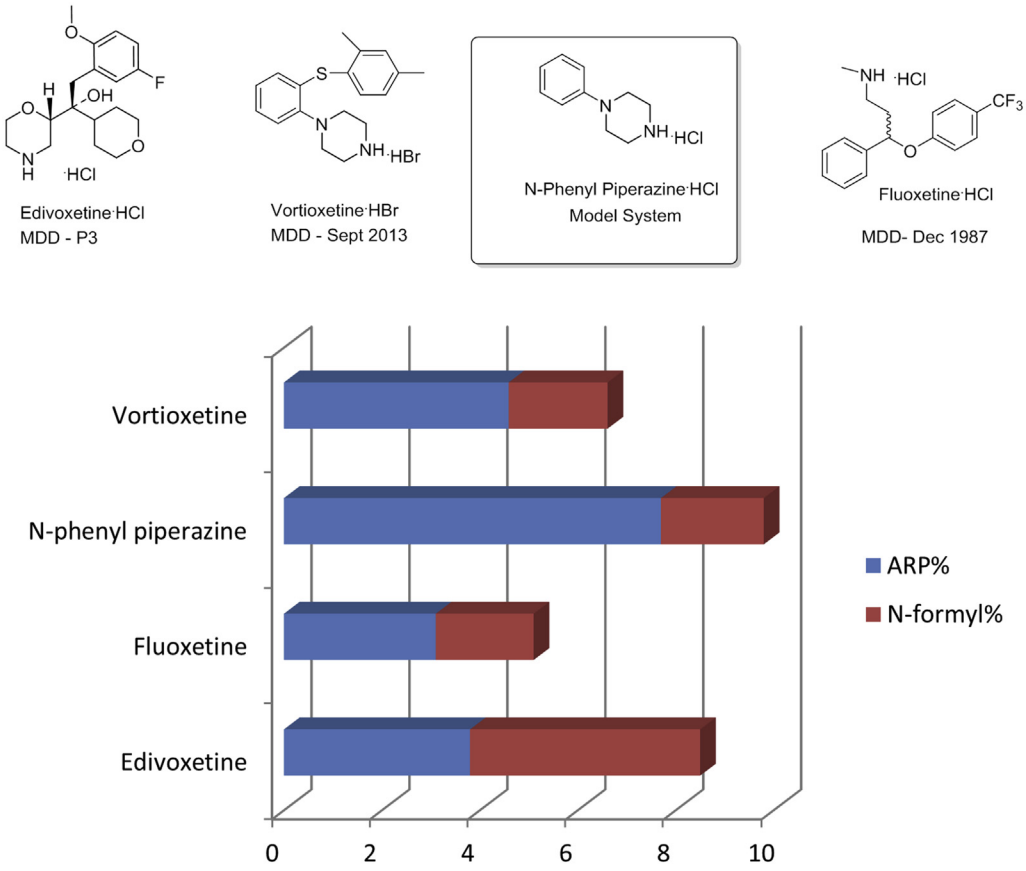


Figure 12. Maillard reaction substrate screen: 2 equivalent glucose, 1 equivalent substrate, 5 vol water, and 24 h reaction time at 60°C.

were higher than those formed in air, especially for the wet samples. This is the opposite effect of what was observed for the formation of formic acid discussed earlier, where the presence of oxygen increased the yield. This suggests that the formation of formic acid does not require an oxidative process and may not be the only pathway to the N-formylation of edivoxetine because the mechanisms (i.e., of N-formylation and formic acid formation) do not perfectly overlap with regard to the effect of oxygen. This result is consistent with the results of the solution-based investigation (see sections Effects of Excipients and Spiking Reactions With Formic Acid, Methyl Glyoxal, and Glyoxal), where evidence suggested that oxygen is not needed for the N-formylation reaction.

Role of ARP—Is It an Intermediate?

The glucose ARP was prepared and purified (see Experimental) for the purpose of investigating whether it could be a direct intermediate leading to the N-formyl degradant. The purified ARP was exposed to various stress conditions in the presence of the placebo formulation of edivoxetine drug product while monitoring the formation of N-formyl. In addition, as a control, edivoxetine was exposed to the same stressed conditions. Approximately 550 mg of placebo formulation and 23 mg of ARP were placed into a pestle and ground up with a mortar to homogenize the sample. Additionally, ~560 mg of placebo formulation and 24 mg of edivoxetine were placed into a pestle and ground up with a mortar to homogenize the sample. The ARP and edivoxetine samples were stressed for a total of 6 days at 70°C/20% RH and 70°C/75% RH. The UPLC/MS method found in Table 8 was used for the analysis.

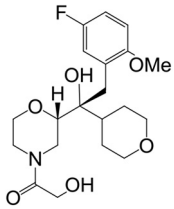
After stressing for 6 days, the ARP converts back to edivoxetine to a level of >60%. Only a very small amount of N-formyl was formed over the same 6 days of stressing (0.4%). If the ARP were an intermediate enroute to the N-formyl product, much higher levels of N-formyl formation would be expected; these results indicate

Table 14
Solution Phase Edivoxetine Hydrochloride Reaction With Glucose in the Presence of Drug Product Excipients

Entry	Base (Amount)	pH	Sugar (equiv)	Time (h)	ARP (%)	N-Formyl Degradant (%)
1	Magnesium stearate (1.2 equiv)	6.5	Glucose (2.0)	72	20.1	0.56
2	Magnesium stearate (0.25 equiv)	6.0	Glucose (2.0)	18	10.0	0.35
3	Magnesium stearate (0.10 equiv)	6.0	Glucose (2.0)	21	8.0	0.50
4	KOH (1.38 equiv)	9.3	MCC (1.0 kg/kg)[a]	23	0.0	0.41
5	NaCMC (1.0 kg/kg LR) 70 K	7.0	Glucose (2.0)	23	1.38	0.0
6	NaCMC (1.0 kg/kg LR) 700K	7.0	Glucose (2.0)	27	0.0	0.0
7	FB (1.0 equiv)	9.3	Glucose (2.0)	23[a]	3.99	2.12

Equiv., equivalent; FB, free base.
[a] Experiment run at 60°C; all other experiments run at 70°C.

Table 15
ARP Conversion to N-formyl Edivoxetine



Compound 2 "Glycolate amide"

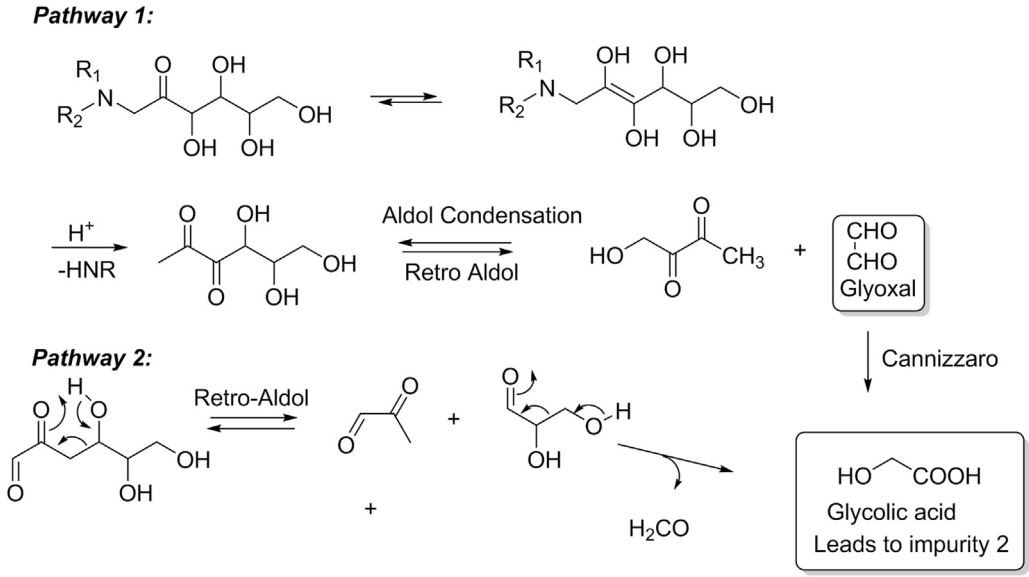
Entry	Condition	ARP (%)	Edivoxetine	N-formyl Degradant (%)	Compound 2 (%)
1	T = 0 at 20°C	92.0	5.60	0.90	0.40
2	0.5 h at 20°C	92.2	5.85	0.97	0.29
3	18 h at 20°C	91.8	6.01	0.92	0.32
4	2h at 60°C	89.9	7.36	1.00	0.71
5	22 h at 70°C	13.9	75.9	1.87	4.36
2N KOH added					
6	2.25 h at 60°C	88.8	8.4	1.36	0.78
7	2.5 h at 60°C	75.1	16.9	3.82	0.73
8	3.5 h at 60°C	39.0	45.0	8.97	4.24
9	6 h at 60°C	7.8	62.6	12.0	7.39

that the ARP does not appear to be a direct intermediate leading to the N-formyl under these conditions, and the results are similar to what is seen in solution-phase experiments (see sections Effects of Excipients and Spiking Reactions With Formic Acid, Methyl Glyoxal, and Glyoxal).

Solution-Based Maillard Studies

Because of the lengthy times required for studies of the N-formylation reaction in the solid state, a rapid solution phase test was desired which would assess a substrates propensity toward Maillard reactivity. To assess the feasibility of the solution phase approach, glucose was evaluated as a model reducing sugar (Table 13). A design of experiments was conducted, primarily evaluating basicity and glucose concentration. The standard conditions used were dissolution of edivoxetine in 10 volumes of 80% water/20% ethanol followed by addition of glucose and adjustment of the pH with 2N KOH at 60°C. Two control experiments were performed, one without added base (entry 1, Table 13) and the other with edivoxetine in the free base form (entry 10). The amount of ARP and N-formyl were measured by HPLC analysis (see Table 9). All experiments were run in an Argonaut 2050 parallel reactor under inert conditions, unless otherwise specified.

All experiments where base and glucose were added produced measurable quantities of both ARP and N-formyl impurity within 24 h. The primary variable affecting N-formylation levels was pH, where at pH ≥9 significant increases in N-formylation were observed. Even with as low as 0.5 equivalents of glucose, ~5% N-formyl could be produced over a ~24-h period (Table 13, entry 8, pH 9.28). At pH ≤9, the ARP is observed at significantly higher levels (3-10×) than the N-formyl degradant (Table 13, entries 1–4).



Scheme 2. Proposed reaction pathways for formation of compound 2.

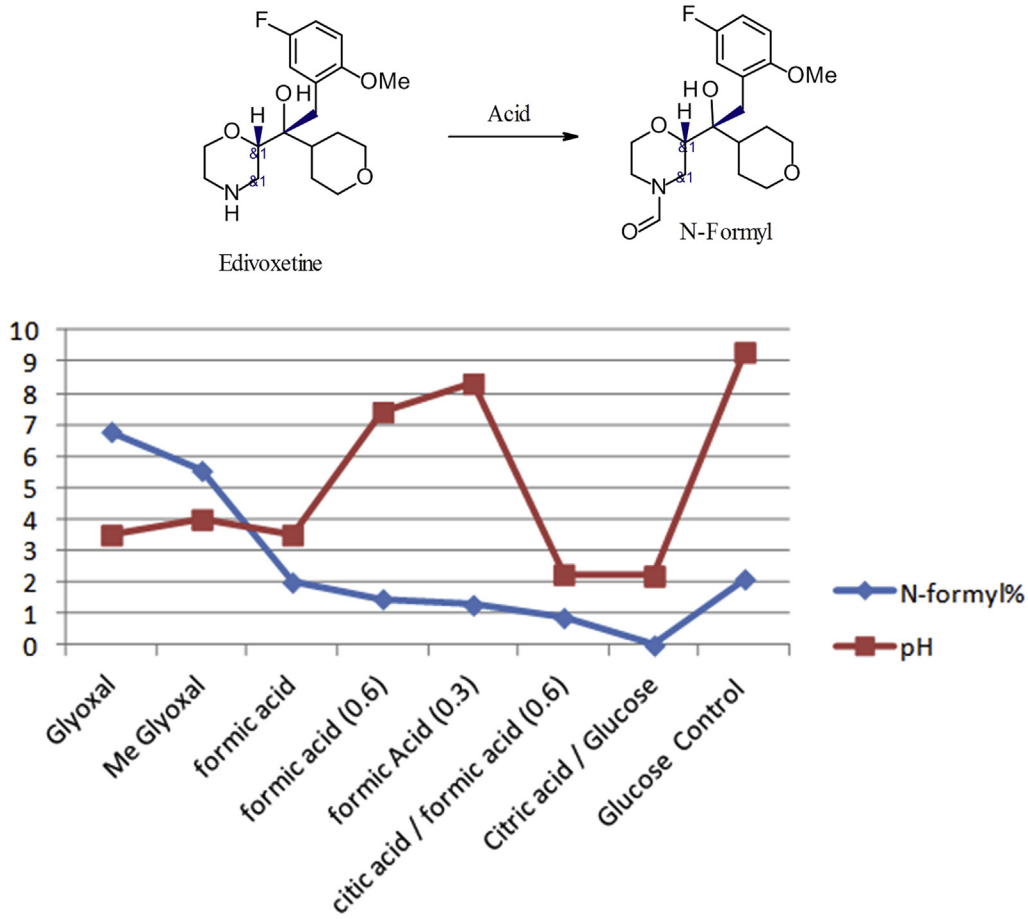


Figure 13. Formylation control experiments with glyoxal, methyl glyoxal formic acid, formic and citric acid, citric acid and glucose, and glucose.

It is noteworthy that without added base (Table 13, entry 1) the observed pH was 4, and under these conditions no N-formyl or ARP products were observed in the presence of glucose. When edivoxetine free base was used rather than the hydrochloride salt (with no pH adjustment), the observed pH was 9.3 (Table 13, entry 10) and significant levels of ARP and N-formyl product were formed. Overall, the solution-phase approach revealed a response for Maillard and formylation reactivity within 24 h, indicating that solution studies could be a useful predictive model.

Reducing Sugar and Edivoxetine Drug Substance Evaluations

With basic pH established as the important variable governing production of N-formyl and ARP from edivoxetine in the solution-phase system, the effects of different reducing sugars (glucose, fructose, arabinose, and lactose) on the production of N-formyl and ARP formation were investigated (Fig. 11). Fructose and arabinose had 2× and 3× higher N-formylation reaction rates than glucose, whereas lactose had a 0.5× rate of N-formylation. However, even for the lactose system, the rate of formylation could be dramatically accelerated by increasing the basicity of the system (pH >9) by the addition of secondary base, which resulted in >10× rate increase of N-formyl production. We next studied the propensity of other secondary amine drug substances, such as antidepressants fluoxetine.HCl and vortioxetine.HBr, toward the Maillard reaction (Fig. 12). The total amount of ARP and N-formyl by-product for edivoxetine was 2× the levels observed for fluoxetine. Interestingly, ARP was slightly enriched for vortioxetine, but the formylation level was about half of what was observed in edivoxetine. The model system 4-phenyl piperazine was evaluated and produced the highest overall amount of combined ARP and N-formyl by-product, indicating that this could potentially be a useful model substrate for excipient Maillard propensity.

Effects of Excipients

The effects of the presence of the excipients used in the edivoxetine drug product (magnesium stearate, MCC, and

Table 16
Solution-Phase ^{13}C Isotopic Label Study

Carbon Label (^{13}C)	*m/z* 390 Intensity (%)	*m/z* 391 Intensity (%)	m/z 391/390 Intensity Ratio	Solution Phase % Enrichment[a]
C1	51.1	48.9	95.6	42.9
C2	69.4	30.6	44.1	19.1
C3	65.7	34.3	52.2	24.1
C4	64.7	35.3	54.5	25.3
C5	67.7	32.3	47.7	21.4
C6	59.0	41.0	69.4	32.9

[a] Calculation: [391 counts − (390 counts × 0.205)]/[391 counts − (390 counts × 0.205) + 390 counts)] × 100.

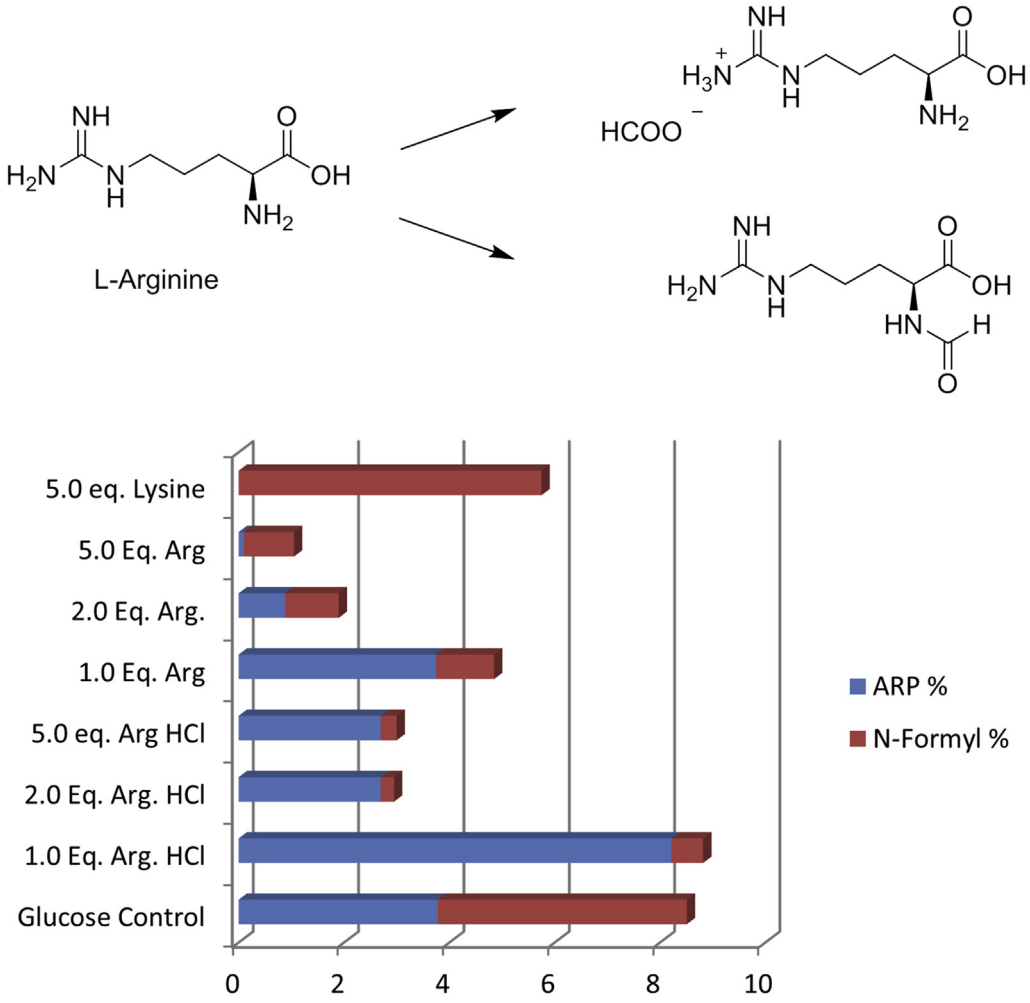


Figure 14. Maillard reaction/formic acid scavengers: 2 equivalent glucose, free base API, 60°C.

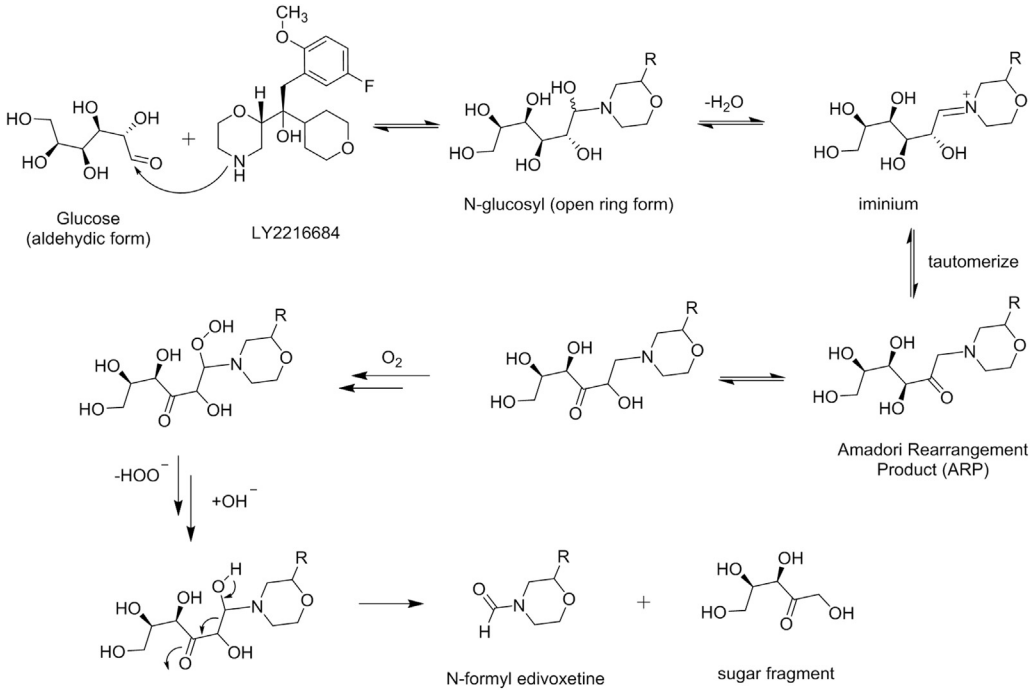
sodium croscarmellose) in the solution-phase system (see Table 14) were also investigated. The main finding was that the lubricant magnesium stearate produced a high level of ARP (10-20%) and more modest level of N-formyl (0.35-0.56%) in the presence of 2.0 equivalents of glucose. A key observation was that magnesium stearate increased the pH in the solution-phase system from 4 to 6.5, so it supports the hypothesis that deprotonation of edivoxetine is a key step in the Maillard mechanism. For example, the ARP/N-formyl product distribution is comparable when 0.25 equivalents of magnesium stearate (Table 14, entry 2) or KOH are used (Table 13, entry 2). Further confirmation of the deprotonation hypothesis is observed when edivoxetine is stirred with magnesium stearate for 24 h; edivoxetine free base was isolated with a yield of 15% after filtration and extraction.

The effects of incorporation of various excipients into the solution system were also performed in the presence of glucose. Sodium croscarmellose 700K polymer in the presence of glucose did not produce any ARP or N-formyl product and sodium croscarmellose 70K monomer in the presence of glucose produced only a minor amount of ARP over 23 h (1.3%). The lack of reactivity of edivoxetine in the croscarmellose systems may be because of the low solubility of sodium croscarmellose in this media. From the previous studies (Table 13), soluble base was the dominant parameter governing the formation of ARP and N-formyl. In addition, although the solubility of sodium croscarmellose was not formally measured, thick suspensions were noted. MCC in the absence of added glucose produced 0.41% of N-formyl degradant after 23 h without detectable ARP (Table 14, entry 4). Together, these results are consistent with the conclusions of solid-state testing that the ARP is likely not a direct intermediate in the N-formyl pathway.

To more directly test the potential intermediacy of the ARP in the pathway to N-formyl, the ARP was isolated by chromatography in ~95 % purity. ARP, 20 mg, was dissolved into a 1.0-mL solution of 40% aqueous ethanol. The solution was heated and then analyzed by HPLC at different time intervals.

The principle reaction observed was reversion to edivoxetine, which was >50% complete after 4 h at 70°C. Interestingly, N-formyl was ~1% at the initial time point and only grew to ~2% after 22 h. However, after 22 h, a new impurity was observed, referred to as compound 2, which was characterized as the glycolate amide (Table 15). A mechanism is proposed for compound 2 (Scheme 2) which is derived from glycolic acid which could be derived from a Cannizzaro reaction with glyoxal.

The experiment was repeated, this time monitoring degradation of ARP at 20°C before heating to 60°C (Table 15). N-formyl was present at 1.0% at the initial time point and did not increase after 18 h at 20°C followed by 2 additional hours at 60°C. The glycolate amide impurity was measured at ~0.4% at $t = 0$ min and increased

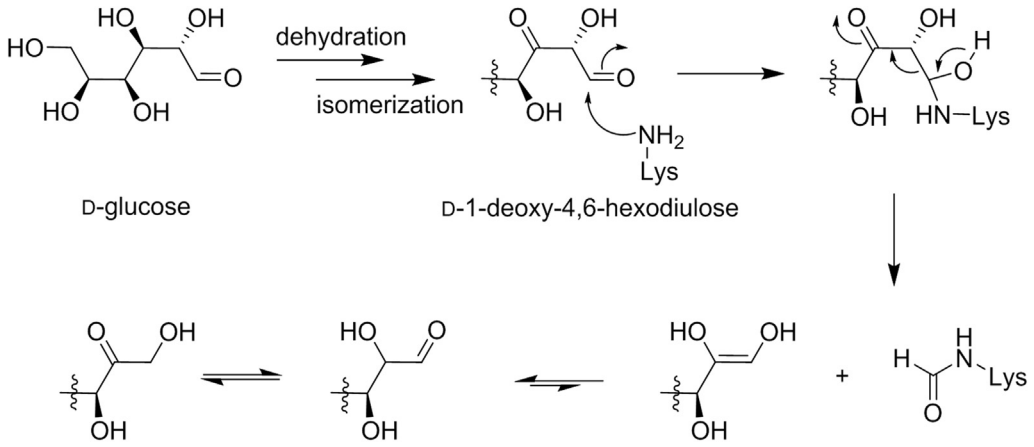


Scheme 3. Possible oxidative pathway to N-formyl edivoxetine. This pathway was ruled out based on experiments conducted under inert (nitrogen) atmosphere.

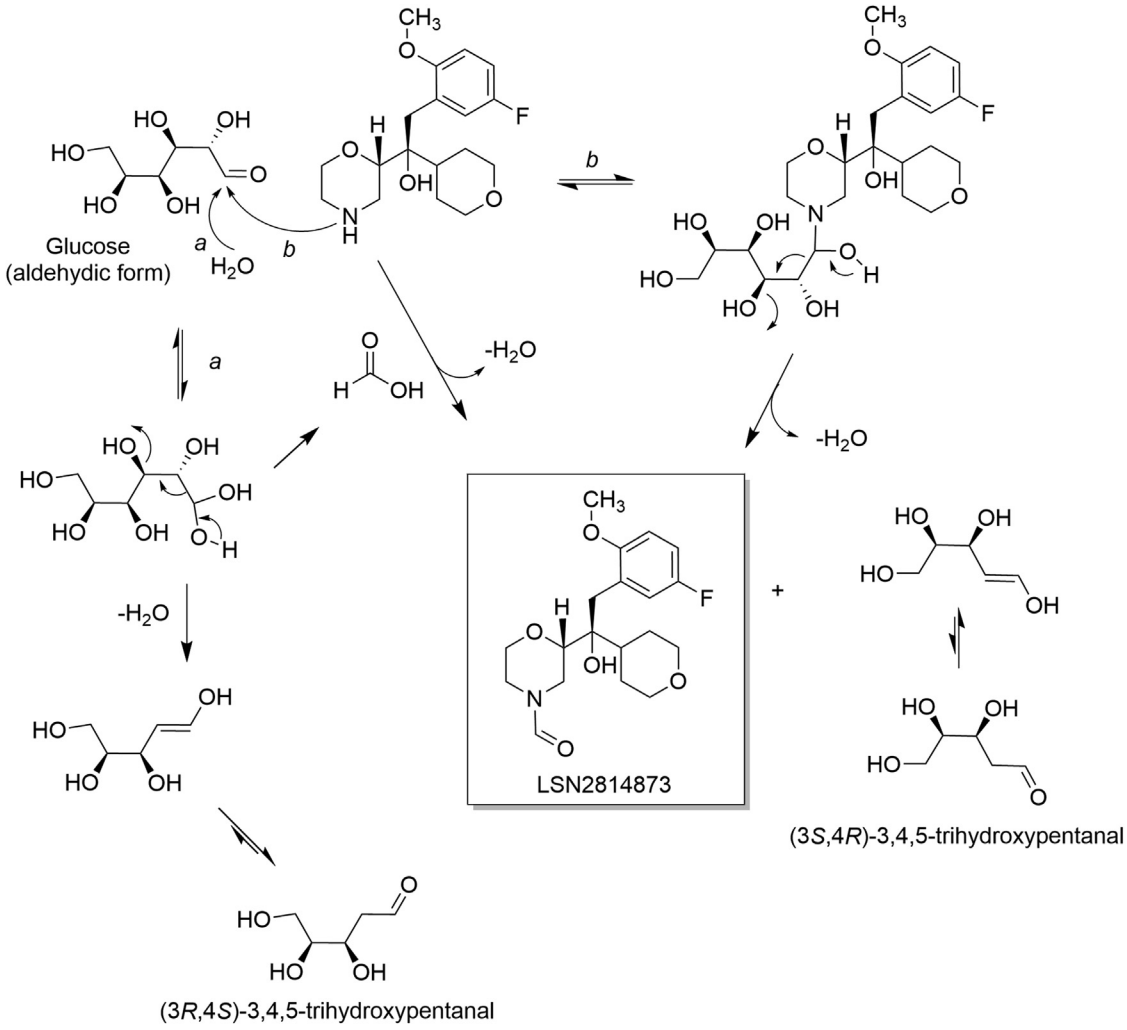
slightly to ~0.70% after 2 h at 60°C (Table 15). After the ARP was nearly fully consumed, the mixture was then basified with 2N KOH, which resulted in high levels of N-formyl (12.0%) and compound 2 (7.4%). The increase in compound 2 is likely due to a Cannizzaro side reaction that is accelerated at high pH, which appears to be a new mechanistic pathway promoted by high base levels (Scheme 2). Summarizing, at pH values <8, ARP converts mainly back to edivoxetine; at pH values >8, both edivoxetine and N-formyl are produced. Because compound 2 was not observed in any of the solid-state studies, we do not believe that the pathways shown in Scheme 2 are active in the drug product. No significant differences were observed when the reaction was run under nitrogen inerting or in the presence of oxygen (ambient air). Overall, the solution system experimental results at pH ≤8 are consistent with the solid-state experimental results, supporting the hypothesis that the ARP is not a direct intermediate leading to N-formylation under conditions relevant to the edivoxetine drug product.

Spiking Reactions With Formic Acid, Methyl Glyoxal, and Glyoxal

The solution system was also used to investigate the ability of formic acid to react directly with edivoxetine to form the N-formyl product. Control experiments were performed in solution with edivoxetine-free base with formic acid, formic and citric acid, glucose and citric acid, or glucose, and the results showed production of N-formyl product at rates similar to the glucose system (Fig. 13). Additional spiking studies were done using glyoxal and methyl glyoxal which are also simple acids that can lead to N-formylation and other by-products.[4,18] All 3 spiking experiments resulted in formation of N-formyl edivoxetine, demonstrating that multiple pathways can produce N-formyl. Additional experiments were run with or without the presence of oxygen (atmospheric air vs nitrogen-inerted system), and the results did not show a significant difference in product distribution, consistent with the hypothesis that N-formylation of edivoxetine does not involve an oxidative step (Fig. 13).



Scheme 4. Maillard pathway for N-formylation of lysine described in the literature.[15] This pathway is disfavored for edivoxetine in the solid-state tablet formulation due to the requirements for glucose to dehydrate and isomerize to the 1,3-beta-dicarbonyl.



Scheme 5. Proposed pathways for the N-formylation of edivoxetine in the presence of glucose (or any reducing sugar). These pathways are supported by the studies documented in this article, although no attempts were made to detect the proposed sugar byproducts shown.

^{13}C-Labeled Glucose Experiments

Experiments were conducted with selectively labeled ^{13}C-labeled (C1-C6) glucose to delineate the source of the carbon incorporated into the N-formyl degradant (analogous to the studies performed in the solid state, vide supra). All 6 ^{13}C-isotopically labeled glucose isomers were purchased for solution-phase experiments and the following procedure used. Samples of 1 g edivoxetine HCl and 2.0 equivalents of ^{13}C1 D-glucose were dissolved in a solution of 5 mL water and 3 mL ethanol. A 20% aqueous KOH (0.81 g, 1.07 equivalents) solution was added and then heated to 60°C. After 24 h, the solution was cooled to room temperature then analyzed by HPLC (see method in Table 9). The same procedure was used for the other 5 ^{13}C glucose labels. HPLC analysis of the ^{13}C-glucose experiments showed formation of N-formyl at a level of ca. 1.8%. Isotopic abundance information obtained by LC/MS analysis (see method conditions in Table 10) revealed incorporation for the 6 glucose ^{13}C experiments (Table 16). As was observed in the solid-state ^{13}C experiments (refer to Table 12), the data obtained from the solution system revealed that the highest ^{13}C incorporation was from C1; however, levels of label incorporation C2-C6 in the solution system were significant, indicating that N-formylation in the solution system is more complex than in the solid state and must involve multiple pathways. This complexity is likely a result of the solution-mediated dehydration, fragmentation, and tautomerization of glucose, which has been described elsewhere.[19,20]

Maillard and Acid Scavengers

With formic acid identified as a significant intermediate in the N-formylation process, alternate approaches to reducing this by-product were evaluated using acid scavengers (Fig. 14). It was found that arginine HCl was effective at reducing N-formylation by ~75%, although some ARP still formed under these conditions. Arginine and lysine (nonsalted forms) were sufficiently basic to promote formylation. Overall, the results suggest that arginine HCl could be a useful excipient to suppress Maillard/formylation reactivity.

Mechanistic Discussion

The overall pathway for the N-formylation of edivoxetine in the drug product can be discussed in the context of previous research (literature precedence) and the results of the investigations described in this article. More than 1 pathway is possible, and

possible pathways along with the overall proposal based on the results of all the experiments are discussed subsequently.

As discussed in the Introduction section, there is literature precedence for N-formylation of primary and secondary amines as a result of the Maillard reaction, which encompass a complex cascade of multiple products, ultimately leading to discoloration and browning.[5-7,9] Both oxidative[16] (involving oxidation of glucose to glucosone, a 1,2-dicarbonyl) and nonoxidative[15] pathways have been proposed to account for N-formylation of amines under various conditions; the proposals involve direct reaction of amines with the reducing sugar ends of carbohydrates. An alternate oxidative pathway has also been proposed via oxidation of the ARP,[10] although no mechanism was proposed. Because the carbon alpha to the nitrogen in ARP is activated for oxidation via radical-mediated oxidation, the oxidative pathway in Scheme 3 can be proposed. Oxidative pathways were, however, ruled out for the N-formylation of edivoxetine based on multiple experiments under inert atmosphere that showed no dependence on the presence of oxygen; furthermore, multiple experiments were conducted with ARP that were consistent with the conclusion that ARP is not an intermediate in the edivoxetine tablet N-formylation reaction (vide supra).

A few nonoxidative N-formylation pathways can be envisioned (Schemes 4 and 5). The most obvious pathway is a condensation reaction between the amine and formic acid. Wirth et al.[4] had previously discounted a direct reaction with formic acid based on the results of spiking studies. Waterman et al.[9] attributed the N-formylation of varenicline to formic acid produced by oxidative degradation of polyethylene, but the actual formylation reaction mechanism was not studied. A Maillard reaction pathway (represented in Scheme 4) has been suggested by Smuda et al.[15] for N-formylation of lysine by direct reaction with an isomer of glucose (1-deoxyhexo-2,3-diulose, a 1,3-beta-dicarbonyl), which can arise from glucose via dehydration and tautomerization. Smuda et al.[15] also described the competing formation of formic acid (among other carboxylic acids) from analogous attack of water on the 1,3-beta-dicarbonyl; based on the comparatively high yield of the N-formylation reaction, it was postulated that nucleophilic attack of the amine of lysine on the aldehyde leading to N-formylation competes favorably with the hydrolysis reaction that leads to formic acid.

The results of our investigations of N-formylation of edivoxetine are compatible with the proposed pathway by Smuda et al. (Scheme 4); however, although we cannot rule out the Scheme 4 pathway, we propose the pathways shown in Scheme 5 because the 1,3-beta-dicarbonyl pathway (Scheme 4) requires glucose to undergo dehydration and isomerization before reaction with the amine and is, therefore, more complex, especially when considering the solid state where molecular mobility is limited. We are proposing pathways shown in Scheme 5 as plausible alternatives; the direct pathway (pathway b) involves nucleophilic attack of the amine nitrogen on the aldehydic carbon (ring-opened anomeric carbon of glucose or other reducing sugars), leading to fragmentation of the sugar backbone and N-formylation. Alternatively, the nucleophile can be water (pathway a), leading to the analogous fragmentation and formation of formic acid, which was experimentally demonstrated to be a formylating reagent in this report.

In summary, we propose that there are 2 major degradation pathways for the N-formylation of edivoxetine in the solid-state formulation: (1) hydrolysis of glucose (via attack of water on the aldehydic carbon) to formic acid, which reacts with edivoxetine by nucleophilic attack of residual unprotonated secondary amine, and (2) nucleophilic attack of the unprotonated secondary amine on the aldehydic carbon of glucose followed by C1-C2 fragmentation. The ratio of these 2 pathways leading to N-formylation is not known. The glucose fragments resulting from these pathways, which could provide additional mechanistic insight, have not yet been explored.

Conclusions

The N-formylation of amines in solid oral dosage forms is an important degradation pathway involving Maillard chemistry that has been broadly observed in the pharmaceutical industry, yet surprisingly, little is known about the mechanism of this pathway. The investigation has used the observed N-formylation in a solid oral dosage form of edivoxetine to study the mechanism in both the solid state and in a newly developed solution-based system. The results of the experiments conducted are consistent with the N-formylation degradation pathway resulting from a direct reaction of edivoxetine with (1) formic acid (generated from decomposition of MCC or residual glucose) and/or (2) the reducing sugar ends (aldehydic carbons) of either residual glucose or the MCC polymer. The results of labeling experiments using isotopically ^{13}C-labeled glucose indicate that the primary source of the formyl group is the C1 position from reducing sugars. The presence of water or moisture accelerates this degradation pathway. Surprisingly, investigations in the solid and solution states support that the edivoxetine–glucose Amadori Rearrangement Product (glucose ARP) does not appear to be a direct intermediate leading to N-formyl degradation of edivoxetine. Additionally, oxygen does not appear to play a significant role in the N-formylation of edivoxetine based on multiple experiments under inert atmosphere that showed no dependence on the presence of oxygen.

These results have implications for the development of stable pharmaceutical formulations of primary or secondary amines. The N-formylation of edivoxetine is clearly dependent on the presence of reducing sugars in the formulation, although even nonreducing sugars, such as MCC, lead to low but significant levels of the N-formylation. It is noteworthy that MCC typically contains low levels of free glucose and reducing sugar (aldehydic) carbons on the end of every MCC chain. There appears to be several factors to enable control/minimization of this pathway: (1) keeping the microenvironmental pH of the formulation slightly acidic, for example, by adding an acidic excipient such as citric acid or removal of a basic excipient, such as sodium croscarmellose; (2) use of nonreducing sugars in the formulation; (3) use of excipients that have low formic acid content; (4) addition of “reducing sugar”/formic acid scavengers, such as arginine HCl; (5) control of moisture content and use of a desiccant to keep the formulation dry; and (6) use of activated carbon to adsorb volatile formic acid. It is noteworthy that the use of activated carbon in the container of capsules has shown modest promise in lowering the N-formylation reaction for several pharmaceutical products prone to N-formylation.[21] It is hoped that continuing research on this important degradation pathway, including use of the solution-based system to rapidly investigate the reaction and potential control strategies, will lead to effective control strategies to ensure the stability and purity of amine-containing drug products.

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