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The use of mixed-mode ion-exchange solid phase extraction to characterize pharmaceutical drug degradation

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Abstract

Solid phase extraction (SPE) has been utilized extensively in the pharmaceutical industry for the isolation of pharmaceuticals from interfering biological matrices and the purification and concentration of impurities and degradation products present in analytical samples. The work described herein involves the novel use of mixed-mode ion-exchange solid phase extraction to characterize degradation products of several pharmaceutical drugs, thereby giving important clues to their structure and sites of reactivity. Several examples of the use of mixed-mode ion-exchange solid phase extraction to illustrate the utility of this technique are presented.

Keywords: Degradation; Mixed-mode ion-exchange solid phase extraction; Isolation; Stability; Degradation product characterization

1. Introduction

Solid phase extraction (SPE) has become a valuable tool in the pharmaceutical industry. It has been used to purify analytes of interest from interfering matrices of biological samples [1–3], to purify environmental samples [4], to purify or concentrate degradation products of interest [5–8], to purify degradation products away from interfering excipients [9] and to concentrate and isolate impurities encountered during bulk synthesis of pharmaceuticals [10].

Several types of solid phase extraction sorbent systems are known. The basic types are normal phase, reversed-phase and ion-exchange sorbent materials. Recently, solid phase sorbent technology has expanded to offer the use of mixed-mode ion-exchange media, which are combinations of reversed-phase and ion-exchange sorbents [11]. Mixed-mode sorbent systems are often more advantageous and provide better separation than standard reversed phase or ion-exchange sorbent systems alone [12–15]. Interestingly, novel sorbent systems, including "molecularly imprinted sorbents", have recently been reported [16]. Use of several different sorbent systems in tandem can provide additional separation and isolation advantages [17,18].

The basic protocol of a mixed-mode ion-exchange solid phase extraction experiment involves initial conditioning of the column, so that the sorbent bed is well solvated and can work effectively. The subsequent loading step involves the introduction of the mixture containing the analyte of interest onto the solid phase extraction column. When using ion-exchange media, a lock step is then utilized to adjust the pH of the system to ensure that ionization and ion-exchange interactions are enabled. Wash steps serve to remove unretained materials from the column and elution steps remove any retained materials. In most cases, the column is chosen so that the analyte of interest has a strong affinity for the column and the extraneous mixture components are unretained and able to be expelled from the column in the wash process. Because different steps involve different interactions of the analyte with the sorbent materials, selective elution of mixture components is possible [19] and is the basis of the work presented here.

The presented studies describe the novel use of mixed-mode ion-exchange solid phase extraction to study chemical degradation products of several common pharmaceutical compounds. A related report characterizing the acidic or basic nature of degradation products using capillary zone electrophoresis has been published, however, this characterization involved extensive manipulation of buffer electrolyte solutions and capillary polarity [8]. More recently, solid phase extraction has been incorporated into HPLC-NMR systems to make this analyti-

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cal technique even more powerful in the structural elucidation of natural products in complex mixtures [20–24]. In-line solid phase extraction columns allow for efficient concentration of the separated components such that NMR structure determination of each component of the complex mixture is greatly facilitated. While the sophisticated techniques of hyphenated HPLC-SPE-NMR analysis represents the most complete and state-of-the-art analysis available to complex mixtures, the current method presented is more ideal to gain rapid preliminary, information on degradation product mixtures for those who may not have direct, open access to HPLC-SPE-NMR systems.

As described here, ion-exchange solid phase extraction methods were creatively used to rapidly gain insight into the composition of chemical degradation product mixtures found in pharmaceutical samples. A simple, rapid (*ca.* 30 min) mixed-mode ion-exchange solid phase extraction experiment can give almost immediate information on the structural components of potential degradation products and serve to guide subsequent, more extensive analytical and isolation procedures. One of the simplest applications of this technique that can be envisioned involves hydrolysis of an amide bond, producing anionic (carboxylic acid) and cationic (amine) degradation products. Assuming that the parent compound is neutral (incapable of being ionized), application of mixed-mode ion-exchange solid phase extraction under either anionic or cationic exchange conditions will rapidly demonstrate that the products now differ in their anionic and cationic character. This process provides information on the structure and functionality of the degradation products. The data from the SPE experiment will rapidly support the researcher's initial supposition that amide bond hydrolysis may have occurred, so that other degradation pathways may be ruled out quickly. Furthermore, in its traditional role, the experiment will show that mixed-mode solid phase extraction can efficiently provide the means to rapidly separate the two degradation products from each other in one simple experiment, providing isolated samples for further elucidation by mass spectrometry, nuclear magnetic resonance spectroscopy or other analytical techniques.

In essence, mixed-mode ion-exchange solid phase extraction can be a very simple method to gain insight into the identity of any proposed degradation product in a decomposed mixture. When applied to complex degradation mixtures, such as purposeful degradation or excipient compatibility samples, this simple yet powerful technique works to deconvolute valuable structure information of all degradation products present in the mixture. This technique can determine if the degradation products have acquired new acidic or basic functionality as a result of the degradation process. This technique therefore has the ability to provide an additional level of information for every individual component of a complex degradation mixture, without the researcher being immediately belabored by tedious preparative chromatography or other separation procedures.

Several examples are presented whereby anion and cation-exchange mixed-mode solid phase extraction was used to determine the acidic or basic nature of proposed degradation products, thereby giving information on their possible structure. This process served to immediately rule out any possible product structures that were inconsistent with the extraction results. Four examples of degradation reactions involving common pharmaceutical compounds are presented. The first study details the known hydrolysis of benzocaine to 4-amino-benzoic acid in basic media to illustrate the utility of the technique using a known degradation pathway. The second study involves the hydrolysis of bezafibrate in basic media and the characterization of both the known and unknown degradation products. The third study characterizes the oxidative degradation of chlorpromazine and is used to distinguish between several possible degradation products. Finally, the fourth study illustrates the use of this technique to characterize the oxidative degradation of benzocaine with hydrogen peroxide and illustrates how the site of oxidation can be deconvoluted from the extraction results.

2. Experimental

The decomposition reactions and solid phase extraction fractions were characterized using high performance liquid chromatography (HPLC) with UV–DAD detection. Uracil was utilized in the chromatographic evaluations as an unretained marker to trace retention factors of known degradation products and components [25]. External, known standards of degradation products were utilized where available. Experimental retention factors of known standards correlated well with assigned degradation products, generally within 3–5%. Acetonitrile was utilized in place of methanol for all conditioning, wash and elution steps to avoid any reactivity with the degradation products. Quantitative recovery experiments were not considered in these qualitative assessments of degradation.

2.1. Materials

Benzocaine free base (>99+%) and uracil (99+%) were obtained from Acros, Inc. and were used without further purification. Bezafibrate, chlorpromazine hydrochloride, 4-chloro-benzoic acid, 4-amino-benzoic acid (sublimed, 99+%), sodium acetate (99+%), phosphate buffered saline buffer tablets (P4417-50TAB), 4-amino-3-hydroxy-benzoic acid, diethylamine (Reagent Plus, ≥99.5%), ethyl acetate (HPLC grade, 99.8%), acetone (Chromasolv, HPLC grade, ≥99.9%), magnesium sulfate (anhydrous, 99%) and barium hydroxide octahydrate (98+%) were obtained form Sigma–Aldrich, Inc. and were used without further purification. The solution reagent, 32% weight peracetic acid in acetic acid was obtained from Sigma–Aldrich, Inc., stored refrigerated and was used without further purification. Ethyl 4-nitrobenzoate (98%) and 27% (w/w) hydrogen peroxide aqueous solution (stored refrigerated) were obtained from Alfa Aesar, Inc. and were used without further purification. Trifluoroacetic acid, methanol (HPLC grade), acetonitrile (HPLC grade), potassium hydroxide, concentrated hydrochloric acid (36.5–38%), concentrated ammonium hydroxide (28–30%), hexanes (HPLC, 95% *n*-hexane) and concentrated sulfuric acid were Baker Analyzed® ACS Reagent grade, obtained from J. T. Baker, Inc. and

were used without further purification. Dimethyl sulfoxide-d_6 (D, 99.9%) was obtained from Cambridge Isotope Laboratories, Inc. and was used as received. Ethanol (200 proof) was obtained from Pharmaco-AAPER and was used as received. Silica gel G plates (2.5 cm × 10 cm, 250 μm thickness) containing a 254 nm fluorescent indicator (Part no. 83114) were obtained from Scientific Adsorbents, Inc. and were used as received. Silica gel 60 (0.04–0.063 μm) was obtained from EM Science and was used as received. Purified water was obtained from a U.S. Filter PureLab Plus® UV/UF system. Reaction vessels were 20 ml clear, screw cap scintillation vials obtained from VWR, Inc. and were used as received. For mixed-mode cation-exchange solid phase extraction, Waters® Oasis® MCX cartridges [6 cm^3/150 mg sorbent, 30 μm particle size, syringe barrel format, copolymer sorbent substrate, 80 Å pore size, 1 mequiv./g ion-exchange capacity (part #186000256)] were obtained from Waters, Inc. and were used as received. For mixed-mode anion-exchange solid phase extraction, Waters® Oasis® MAX cartridges [6 cm^3/500 mg sorbent, 60 μm particle size, syringe barrel format, copolymer sorbent substrate, 80 Å pore size, 0.2 mequiv./g ion-exchange capacity (part #186000865)] were obtained from Waters, Inc. and were used as received.

2.2. Instrumentation

High performance liquid chromatographic (HPLC) analysis was conducted on an Agilent® 1100 liquid chromatography system comprised of a degasser (G1322A), quaternary pump (G1311A), temperature-controlled auto-sampler (G1329A/G1330A), column compartment (G1316A) and diode array detector (G1315B). Data was processed through Agilent® Chemstation software version 10.1.

High performance liquid chromatography–mass spectrometry (HPLC–MS) analysis was conducted on the same Agilent LC system equipped with an Agilent® electrospray MS detector 1100 MSD (G1946A) operating in positive and negative ionization mode, using the chromatographic method described below. The cycle time was set to 0.95 s/cycle, the fragmentor voltage was set to 70 and a gain equal to 1.0 was used. The threshold was set at 150 with a peak width of 0.1 min and a scanning range of 100–1000 amu was utilized. A step size of 0.25 was set constant for all measurements. Data was processed through Agilent® Chemstation software version 10.1.

Solid state infrared spectroscopy was conducted on ∼5 mg sample using an IlluminatIR™ Fourier transform infrared microspectrometer manufactured by SensIR Technologies®. The system was equipped with a potassium bromide beamsplitter and a mercury–cadmium–telluride detector. A diamond attenuated total reflectance objective was used for data acquisition. Each spectrum represents 100 co-added scans using a 100 μm masking aperture, collected at a spectral resolution of 4 cm^{-1}. Sample preparation consisted of placing the sample on a standard glass microscope slide under ambient laboratory conditions. A background spectrum was acquired using the diamond attenuated total reflectance objective and subtracted from the final spectra obtained.

^1H and ^{13}C nuclear magnetic resonance (NMR) measurements were conducted on a Varian® Unity INOVA 400 MHz system powered by a Sun Microsystems® Ultra ten Creator interface.

Melting points were determined on a TA Instruments® Q-1000 differential scanning calorimeter (DSC) operating in modulated mode, with a modulation temperature amplitude of ±1 °C, a modulation of 60 s and a ramp rate of 3 °C/min.

The handheld ultraviolet lamp used for thin layer chromatography detection was the MineralLight® lamp (model UVGL-25). The rotary evaporator used for the large-scale preparative synthetic work was the Buchi® Rotavapor (model R-124), equipped with a Buchi® vacuum pump (V-500). The pH meter utilized was the mini-Lab® ISFET pH meter (model 1Q125).

2.3. General procedure for solid phase extraction using Waters® Oasis® MCX cartridges

The general procedure for conducting the cation-exchange solid phase extraction experiments was adapted from previously prescribed procedures [26]. In general, the extraction process on the mixed-mode cation-exchange resin (syringe barrel format) consisted of the following sequence of steps: condition, load, lock, wash and elute. In all cases, gravity was used to elute the solvents from the column and no vacuum system was employed. Each fraction was collected separately in a small beaker or vial. The condition step involved treatment of the column and sorbent with 5 ml of purified water: acetonitrile (1:1, v/v) mixture. Prior to loading the reaction mixture sample onto the column, the pH of the reaction mixture was first adjusted to closely match the lock solution pH (pH 7.4 in the case of chlorpromazine oxidation and pH 0.5 in the case of benzocaine oxidation) by the addition of strong (5N) and dilute (0.1N) solutions of HCl or NaOH in a drop-wise fashion. Typically, about 1 ml of the treated reaction mixture was loaded onto the conditioned column drop-wise. Based on the reported 1 mequiv./g ion-exchange capacity, the molar amount of mixture loaded onto the column was below the total ion-exchange capacity of the sorbent bed. Solid phase extraction experiments were conducted at *ca.* 10–50% of the column capacity to avoid breakthrough and obtain optimal exchange and separation. The lock step involved treating the column with either 5 ml of 0.01 M phosphate buffered saline (pH 7.4) for the chlorpromazine oxidation experiments or 5 ml of 1N HCl (pH 0.5) for benzocaine oxidation experiments. The wash step utilized 5 ml of acetonitrile to remove all unretained species from the sorbent bed. Elution was achieved by addition of 5 ml of a 0.06N barium hydroxide octahydrate:acetonitrile (1:1, v/v) mixture (for chlorpromazine oxidation study) or 5 ml of a concentrated NH_4OH (28–30%):acetonitrile (1:19, v/v) mixture (for benzocaine oxidation study). Prior to analysis, the fraction samples were acidified with a few drops of concentrated HCl, combined with the dissolving solvent [13 mM trifluoroacetic acid (pH 1.5):acetonitrile (9:1, v/v)] and a small aliquot of a 2.7 mM uracil stock solution. All samples were analyzed by reversed phase HPLC according to the method detailed below.

2.4. General procedure for solid phase extraction utilizing Waters® Oasis® MAX cartridges

The general procedure for conducting the anion-exchange solid phase extraction experiments was adapted from previously prescribed procedures [27]. In general, the extraction process on the mixed-mode anion-exchange resin (syringe barrel format) consisted of the following sequence of steps: condition, load, lock, wash and elute. In all cases, gravity was used to elute the solvents from the column and no vacuum system was employed. Each fraction was collected separately in a small beaker or vial. The condition step involved treatment of the column and sorbent with 5 ml of a purified water: acetonitrile (1:1, v/v) mixture. Prior to loading the reaction mixture sample onto the column, the pH of the reaction mixture was first adjusted to match the lock solvent pH (pH 6.5) by the addition of strong (5N) and dilute (0.1N) solutions of HCl in a drop-wise fashion. Typically, about 1 ml of the treated reaction mixture was loaded onto the conditioned column drop-wise. Based on the 0.2 mequiv./g ion-exchange capacity, the molar amount of mixture loaded onto the column was below the total ion-exchange capacity of the sorbent. Solid phase extraction experiments were conducted at 10–50% of the column capacity to avoid breakthrough and obtain optimal exchange and separation. The lock step involved treatment of the column with 5 ml of 50 mM sodium acetate (pH 6.5). The wash step utilized 5 ml of acetonitrile to remove all unretained species from the sorbent bed. Elution was achieved by addition of 5 ml of a 5N HCl:acetonitrile (1:1, v/v) mixture to the column. Prior to analysis, the fraction samples were combined with the dissolving solvent [13 mM trifluoroacetic acid (pH 1.5):acetonitrile (9:1, v/v)] and a small aliquot of a 2.7 mM uracil stock solution. All samples were analyzed by reversed phase HPLC according to the method detailed below.

2.5. General method for reverse phase HPLC analysis of reaction mixtures and collected SPE fractions

Reversed phase HPLC analysis was conducted on an Agilent® 1100 liquid chromatography system using diode array detection at 210 nm with a band width of 16 nm and a slit width of 4 nm. The reversed phase column utilized for all measurements was the Waters® Symmetry Shield® RP-8 (4.6 mM × 100 mM in length, 3 μm particle size (part #WAT094266)). The mobile phase utilized was 13 mM trifluoroacetic acid (pH 1.5) and acetonitrile in a gradient fashion, beginning with 13 mM trifluoroacetic acid (pH 1.5):acetonitrile (9:1, v/v) and ending with 13 mM trifluoroacetic acid (pH 1.5):acetonitrile (1:1, v/v) over a period of 34 min. Injection volume was 15 μl and the flow rate was 0.8 ml/min. Column temperature was regulated at 25 °C. Small aliquots (∼0.25 ml) of a 2.7 mM uracil standard in acetonitrile:methanol (1.5:1, v/v) was added to all HPLC samples prior to analysis as an unretained retention time marker. For reaction mixtures, HPLC sample analysis concentrations were *ca.* 0.2 mg/ml. For SPE fractions, HPLC sample concentrations were *ca.* 0.05–0.1 mg/ml.

2.6. Hydrolysis of benzocaine in 0.06N barium hydroxide

To a 20 ml clear screw cap scintillation vial containing 21 mg (0.13 mM) of benzocaine free base was added 10 ml of 0.06N barium hydroxide octahydrate and the mixture shaken slightly until all solid material had dissolved. The sealed vial was placed in a 70 °C oven for 30 min, removed from the oven and cooled. The pH of the reaction mixture was adjusted to match the lock solvent pH (pH 6.5) by the addition of strong (5N) and dilute (0.1N) solutions of HCl in a drop-wise fashion. Prior to SPE extraction, a small aliquot (∼0.5 ml) of the reaction mixture was removed, prepared and analyzed by HPLC according to the above procedure. A 1 ml aliquot was removed and utilized for the mixed-mode anion-exchange solid phase extraction experiment as described.

2.7. Hydrolysis of bezafibrate in 5N potassium hydroxide

To a 20 ml clear screw cap scintillation vial containing 20.7 mg (0.06 mM) of bezafibrate free acid was added 10 ml of 5N KOH and the mixture shaken slightly until all solid material had dissolved. The sealed vial was placed in a 70 °C oven for 3 h, removed from the oven and cooled. The pH of the reaction mixture was adjusted to match the lock solvent pH (pH 6.5) by the addition of strong (5N) and dilute (0.1N) solutions of HCl in a drop-wise fashion. Prior to SPE extraction, a small aliquot (∼0.5 ml) of the reaction mixture was removed, prepared and analyzed by HPLC and HPLC–MS according to the above procedures. A 1 ml aliquot was removed and utilized for the mixed-mode anion-exchange solid phase extraction experiment as described.

2.8. Oxidation of chlorpromazine hydrochloride with 32% peracetic acid

To a 20 ml clear screw cap scintillation vial containing 52 mg (0.15 mM) of chlorpromazine hydrochloride and a stir bar was added 5 ml of MeOH:purified water (4:1, v/v) and the reaction was chilled to 0 °C in an ice/water bath. To this mixture was added 16 ml of 32% weight peracetic acid in acetic acid. The mixture was stirred for 20 min until all solid material had dissolved and was allowed to warm slowly to room temperature. The pH of the reaction mixture was adjusted to 7.8 by the addition of 0.1N NaOH in a drop-wise fashion. A small aliquot (∼0.5 ml) of the reaction mixture was removed, prepared and analyzed by HPLC and HPLC–MS according to the above procedure. A 1 ml aliquot was removed and utilized for the mixed-mode cation-exchange solid phase extraction experiment as described.

2.9. Oxidation of benzocaine free base with 27% hydrogen peroxide

To a 20 ml clear, screw cap scintillation vial containing 10.1 mg (0.06 mM) of benzocaine free base was added 5 ml of 27% H_2O_2 and the mixture shaken slightly until all solid material had dissolved. The sealed vial was placed in an oven at 50 °C for

19 h. After 19 h, the vial containing the slightly yellow reaction mixture and precipitate was removed from the oven and cooled. Prior to solid phase extraction, a 1 ml aliquot of the reaction mixture was combined with 1 ml of acetonitrile to completely dissolve the precipitate. The pH of this sample was adjusted to match the lock solvent pH (0.5) by the addition of a 5N HCl solution in a drop-wise fashion. Prior to SPE analysis, a small aliquot (∼0.5 ml) of the reaction mixture was removed, prepared and analyzed by reversed phase HPLC and HPLC–MS according to the above procedure. The remaining amount of this mixture (∼1 ml) was utilized for the mixed-mode cation-exchange solid phase extraction experiment as described.

2.10. Large-scale preparation and characterization of chlorpromazine oxidative degradation product

To a 500 ml round bottom flask containing a magnetic stir bar at 0 °C was added 2 g (5.6 mM) of chlorpromazine hydrochloride in 250 ml of MeOH:water (4:1, v/v) and 1.2 ml of 32% weight peracetic acid in acetic acid. The ice/water bath was removed and the mixture was stirred for 1 h and allowed to warm to room temperature. After stirring, the reaction mixture was basified slowly with the additional of 50 ml of 5N KOH and extracted three times with ethyl acetate (3× 200 ml) using a 500 ml separatory funnel. The combined ethyl acetate fractions (∼600 ml) were dried over magnesium sulfate, concentrated on a rotary evaporator and dried under high vacuum to yield 1.3 g (70%) of a sticky film. The film was analyzed by HPLC, HPLC-mass spectrometry, solid state IR analysis, thin layer chromatography, ^{1}H and ^{13}C NMR analysis.

Isolated film ^{1}H NMR (400 MHz, DMSO-d_6) *d* 1.7–1.89 (m, 2H), 2.11 (s, 6H), 2.22–2.38 (m, 2H), 4.31 (t, J = 7.06 Hz, 2H) and 6.94–7.24 (m, 7H); ^{13}C NMR (400 MHz, DMSO-d_6) d 139.72, 138.30, 133.85, 133.67, 131.91, 124.81, 123.42, 122.89, 122.18, 117.27, 116.74, 56.35, 40.17, 36.96 and 24.59; Solid state IR (neat, cm^{-1}): 2901.45, 2830.31, 2725.1, 1580.07, 1570.24, 1459.54, 1448.26, 1416.95, 1246.82, 1041.50, 1022.47, 920.06, 841.85, 795.21 and 754.61 cm^{-1}; HPLC/MS (positive ionization) *m/z* (relative intensity): 338.9 (2), 337.9 (7), 336.9 (37), 335.9 (19), 334.9 (M^+, 100).

Predicted ^{1}H NMR spectra were generated using ACD Labs software [28] for the proposed oxidative degradation products, chlorpromazine phenothiazine-N-oxide and chlorpromazine sulfoxide proposed degradation products and were used in the determination of the structure of the isolated material. Chlorpromazine phenothiazine-N-oxide (^{1}H NMR, *predicted*): *d* 1.98–2.05 (m, 2H), 2.24 (t, J = 1.5 Hz, 6H), 2.38–2.45 (m, 2H), 3.93–4.20 (m, 2H) and 7.1–8.25 (m, 7H). Chlorpromazine sulfoxide (^{1}H NMR, *predicted*): d 1.70–1.77 (m, 2H), 2.13–2.18 (m, 2H), 2.23 (t, J = 1.49 Hz, 6H), 3.40 (t, J = 7.07 Hz, 2H) and 7.15–7.90 (m, 7H).

Thin layer chromatography experiments were conducted using silica gel G glass plates in two solvent systems, acetone:diethylamine (9:1, v/v) and ethyl acetate:methanol:diethylamine (7:2:1.5, v/v/v). The plates were spotted several times with a micropipette from a 6 mg/ml stock solution of the isolated material in methanol and dried with a stream of nitrogen. Plates were introduced into a glass chamber containing the solvent system and a large piece of filter paper (for atmospheric saturation). The plates were visualized using a hand-held UV light operating at 254 nm and retention factors were determined.

2.11. Large-scale preparation and characterization of benzocaine oxidation product, 4-amino-3-hydroxy-ethyl benzoate

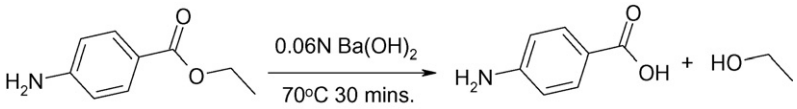
In an esterification procedure based on the process of Anzalone and Hirsch [29], 0.5 g of 4-amino-3-hydroxy-benzoic acid (3.3 mM) was weighed into a 50 ml round bottom flask containing a stir bar. To this solid, 40 ml of ethanol (200 proof) and 3 ml of concentrated sulfuric acid were added. The mixture was refluxed for 24 h and the course of the reaction was followed by HPLC using the same chromatographic method described previously. When complete, the reaction was carefully poured into 100 ml of 0.05 M sodium hydroxide and the entire mixture was added to a separatory funnel. The aqueous portion was extracted with 4× 100 ml of ethyl acetate and the combined ethyl acetate fractions were dried over anhydrous magnesium sulfate. The combined ethyl acetate fractions (∼400 ml) were dried over magnesium sulfate, concentrated on a rotary evaporator and dried under high vacuum to yield 0.5 g of a dark red solid. The solid was purified on silica gel using a hexanes:ethyl acetate (2.3:1, v/v) elution mixture, where the product had a retention factor (R_f) of 0.35 ± 0.01. A total of 0.2 g of a reddish solid was isolated to give an overall yield of 33%. The melting point range was determined to be 94.1–97.9 °C. ^{1}H NMR (400 MHz, DMSO-d_6) d 1.21 (t, J = 7.06 Hz, 3H), 4.15 (q, J = 7.06 Hz, 3H), 5.35 (broad s, 2H), 6.56 (d, 1H), 7.23 (m, 2H) and 9.4 (broad s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6) d 166.66, 143.46, 142.97, 123.16, 117.18, 115.1, 113.11, 60.19 and 15.01; LC/MS (positive ionization) *m/z* (relative intensity): 223 (acetonitrile adduct, 23), 183 (7), 182 (M^+, 100), 160 (12).

3. Results and discussion

3.1. Study 1: hydrolysis of benzocaine in 0.06N barium hydroxide

The ester hydrolysis of benzocaine in basic media is well described in the pharmaceutical literature [30,31]. The authors detail the formation of 4-amino benzoic acid and ethanol as the main degradation products of the reaction (Scheme 1).

When the reaction was analyzed using reversed phase high performance liquid chromatography after heating at 70 °C for 30 min, the benzocaine parent (RT 10.8) and a component with RT 3.1 min are observed (Fig. 1), with approximately 30% degradation of the parent. The ethanol by-product of the reaction was not observed under these analytical conditions and was not considered further in these investigations. The identity of the RT 3.1 min component was confirmed to be 4-amino-benzoic acid by correlation of retention time, UV absorption spectra, HPLC–MS trace and retention factor to an external standard of the material.



Scheme 1. Ester hydrolysis of benzocaine in basic media.

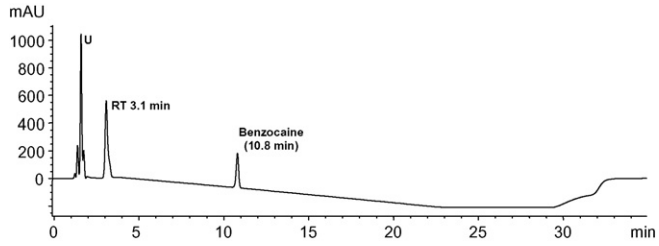


Fig. 1. HPLC chromatogram of the benzocaine hydrolysis reaction mixture. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

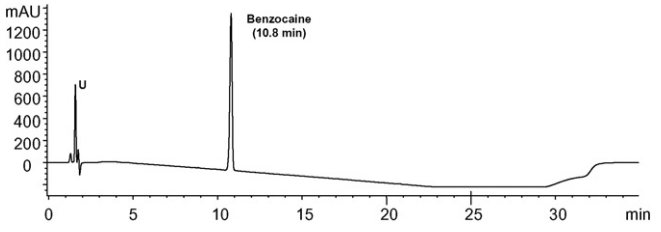


Fig. 2. HPLC chromatogram of the anion-exchange solid phase extraction wash fraction of the benzocaine hydrolysis reaction. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

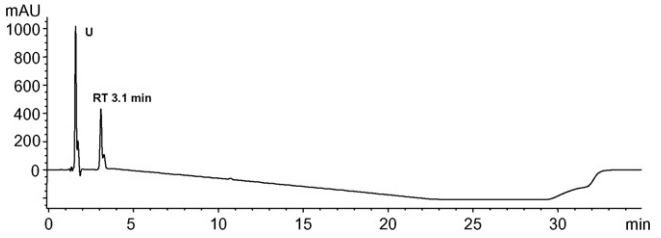


Fig. 3. HPLC chromatogram of the anion-exchange solid phase extraction elute fraction of the benzocaine hydrolysis reaction. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

Prior to the application of SPE to study this reaction, the pK_as of the reactant and products were estimated using software provided by Advanced Chemistry Development, Inc. [32]. Benzocaine was calculated to have a pK_a value of 2.51 ± 0.10 (protonated amine). The degradation product, 4-amino benzoic acid, was calculated to have pK_a values of 4.86 ± 0.10 (carboxylic acid) and 2.51 ± 0.10 (protonated amine).

A mixed-mode anion-exchange SPE experiment was chosen to separate 4-amino-benzoic acid from the unreacted parent. Based on the above estimation of pK_a values for the reactant and products, a lock buffer of pH 6.5 was utilized to render the analyte of interest, 4-amino-benzoic acid, negatively charged and capable of being retained on the anion-exchange column. Under these conditions, the benzocaine parent remained unionized.

The anion-exchange SPE experiment was completed and all collected fractions of the solid phase extraction process (condition, load, lock, wash and elute fractions) were captured and analyzed using reversed phase high performance liquid chromatography (HPLC). Examination of the wash fraction revealed the presence of the benzocaine parent (Fig. 2).

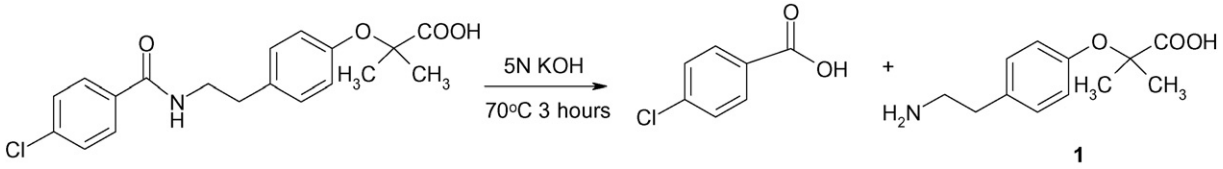
Examination of the elute fraction revealed the presence of 4-amino-benzoic acid (Fig. 3). Other collected fractions did not contain evidence of either the benzocaine parent or 4-amino-benzoic acid.

As expected from its known structure and pK_a value, the unionized benzocaine parent was present in the acetonitrile wash of the extraction process and was unretained on the anion-exchange sorbent. As predicted, the degradation product with RT 3.1 min, which was assigned to 4-amino-benzoic acid, was retained on the anion-exchange sorbent under pH 6.5 conditions and released under the acidic conditions of the elution process. In this case, SPE was used to further confirm the degradation product's identity as 4-amino-benzoic acid, as its behavior in the anion-exchange extraction experiment was entirely consistent with its known structure and predicted pK_a values.

Using this known degradation reaction, where the main degradation product and parent differed in their acidic and basic nature, the utility of this SPE technique to study chemical degradation was demonstrated. Additionally, the 4-amino-benzoic acid degradation product was able to be readily and completely separated from the unreacted parent compound under the conditions of the rapid, simple solid phase extraction experiment.

3.2. Study 2: hydrolysis of bezafibrate in 5N potassium hydroxide

The decomposition of bezafibrate in basic media was not documented in the pharmaceutical literature but was proposed to involve simple amide hydrolysis as detailed in Scheme 2.



Scheme 2. Proposed amide hydrolysis of bezafibrate in basic media.

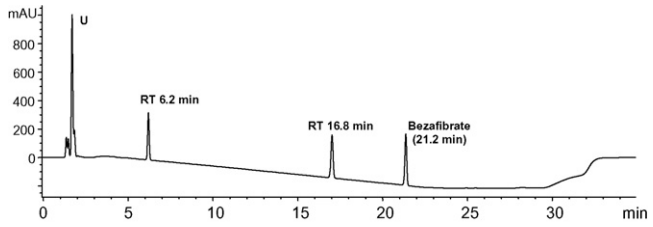


Fig. 4. HPLC chromatogram of the bezafibrate reaction mixture following decomposition in basic media. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

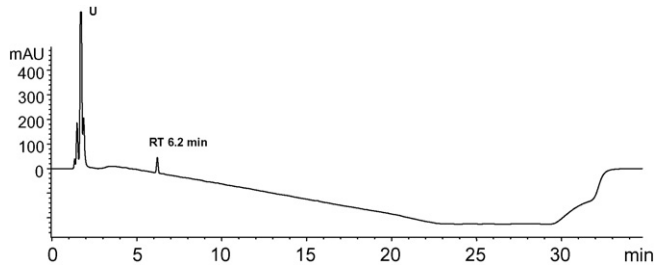


Fig. 5. HPLC chromatogram of the anion-exchange solid phase extraction load fraction of the bezafibrate reaction mixture. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

Degradation of bezafibrate in 5N potassium hydroxide at 70 °C for 3 h afforded nearly 50% decomposition of the bezafibrate parent and produced two degradation products as evidenced by HPLC analysis (Fig. 4). Both degradation products appeared more polar than the parent, with retention times (RTs) of 6.2 and 16.8 min.

The identity of the component with RT 16.8 min as 4-chloro-benzoic acid was confirmed by comparison of retention time, UV absorption spectra, HPLC–MS trace and retention factor to an external standard. The product with RT 6.2 min was proposed to be the corresponding compound **1** in Scheme 2.

The calculated pK_a values of bezafibrate (3.29 ± 0.10) and 4-chloro-benzoic acid (3.97 ± 0.10) were determined using ACD Lab software. The proposed degradation product (**1**) was also examined and the compound was predicted to be zwitterionic in character, with pK_a values of 3.23 ± 0.10 (carboxylic acid) and 10.05 ± 0.10 (protonated amine).

Based on these pK_a values, a mixed-mode anion-exchange SPE experiment was chosen to separate 4-chloro-benzoic acid and the unreacted bezafibrate parent from the analyte of interest, the proposed degradation product **1**. A lock buffer of pH 6.5 was utilized to render the bezafibrate and 4-chloro-benzoic acid negatively charged and capable of being retained on an anion-exchange column. At this pH, the carboxylic acid and amine moieties of product **1** were both ionized, such that the product should behave as a neutral entity, having no net charge. In this case, the product should not be retained on the SPE column and should be capable of being washed and collected from the column.

The anion-exchange SPE experiment was completed and all collected fractions of the solid phase extraction process (condition, load, lock, wash and elute fractions) were captured and analyzed using reversed phase high performance liquid chromatography (HPLC). Examination of the lock fraction revealed only the presence of the RT 6.2 min product, indicating that the component was not retained on the column (Fig. 5).

Examination of the elute fraction revealed the presence of 4-chloro-benzoic acid and the unreacted bezafibrate parent (Fig. 6), which was consistent with their calculated pK_a properties and acidic functionality. No evidence of bezafibrate, 4-chloro-benzoic acid or the proposed product **1** was found in other collected SPE fractions.

While the solid phase extraction results proved consistent with the proposed structure of degradation product **1**, the rapid extraction and isolation of the product allowed for further characterization of the analyte by HPLC–MS analysis. Analysis of the isolated lock fraction using positive ionization electrospray HPLC-mass spectrometry indicated the isolated product had a MH^+ ion of m/z 224.1 amu, which proved consistent with its tentative structural assignment.

In this case, the SPE extraction experiment was able to confirm structural information for the proposed degradation product and demonstrated that the degradation product contained functional groups that rendered it effectively neutral at pH 6.5, as originally predicted. The results provided rapid support of the proposed degradation pathway of amide hydrolysis, which was beneficial because amide hydrolysis is a very common degradation pathway in many pharmaceutical systems [33–35]. Additionally, application of the SPE technique provided the benefit of rapid and efficient isolation of the unknown for further analytical characterization.

3.3. Study 3: oxidation of chlorpromazine with peracetic acid

Oxidative degradation is often the most difficult of degradation patterns to determine and the site of oxidative reactivity can be difficult to predict on many complex pharmaceutical molecules [36]. The ion-exchange solid phase extraction procedure can be readily utilized in the determination of sites of oxidative reactivity, as illustrated for the oxidation of chlorpromazine hydrochloride (Fig. 7).

When chlorpromazine hydrochloride was subjected to oxidative stress with 32% peracetic acid at 0 °C, significant degradation (~33%) of the parent was observed. Under this oxidative stress, chlorpromazine decomposed to a single, more

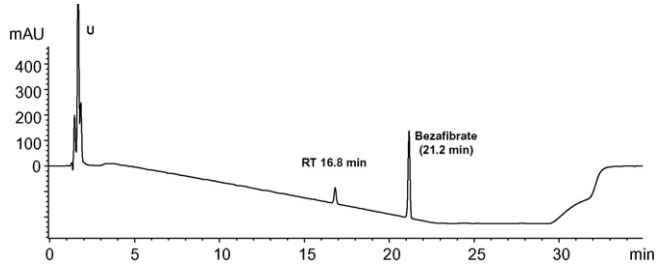


Fig. 6. HPLC chromatogram of the anion-exchange solid phase extraction elute fraction of the bezafibrate reaction mixture. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

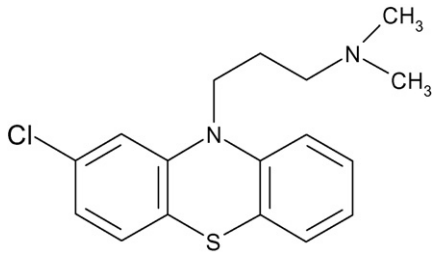


Fig. 7. Structure of chlorpromazine.

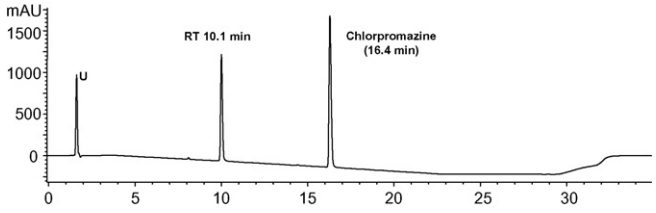


Fig. 8. HPLC chromatogram of the chlorpromazine reaction mixture following decomposition in 32% peracetic acid. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

polar degradation product (RT 10.1 min) as observed by HPLC analysis (Fig. 8).

Prior to application of SPE to study the decomposition, the pK_a values for chlorpromazine and the potential degradation products (Fig. 9) were examined. For the unreacted chlorpromazine parent (Fig. 7), ACD Labs software [32] predicted a value of 9.41 ± 0.28 for the protonated propyl chain amine and a pK_a value of 1.28 ± 0.20 for the protonated phenothiazine amine. If the site of oxidation occurred at the most basic, propyl chain nitrogen (**2**, Fig. 9), ACD Labs software predicted a pK_a value of 4.69 ± 0.40 for the N-oxide and -1.67 ± 0.20 for the protonated amine of the phenothiazine nitrogen center. If the site of oxidation occurred at the less basic, phenothiazine nitrogen center (**3**, Fig. 9), pK_a values of 2.01 ± 0.20 for the N-oxide and 8.89 ± 0.20 for the protonated amine of the propyl chain nitrogen were predicted. If the oxidative stress conditions formed the sulfoxide degradation product (**4**, Fig. 9), pK_a values of 9.37 ± 0.28 for the protonated propyl chain amine and -3.36 ± 0.20 for the protonated phenothiazine amine were expected. If the oxidation conditions formed the sulfone product (**5**, Fig. 9), pK_a values of 9.37 ± 0.28 for the protonated propyl chain amine and -5.20 ± 0.20 for the protonated phenothiazine amine were expected.

Cation-exchange solid phase extraction was specifically employed to determine the site of oxidative reactivity in the molecule. Examination of the retention characteristics of the single degradation product of the reaction following cation-exchange solid phase extraction at a pH of 7.4 was employed to determine if the most basic nitrogen was involved in the oxidative degradation process. Investigation of potential N-oxide formation was based on previous reports of the reaction of amines with peracids to form N-oxide degradation products [37,38]. If oxidation had occurred at the most basic propyl chain nitrogen center, the degradation product (**2**) would have a significantly lower pK_a value and would not be retained on the column under the pH conditions chosen for the cation-exchange process. Alternatively, oxidative reactivity at the less basic, phenothiazine nitrogen or the sulfur atom would enable the product to retain strong basicity and would therefore be retained under the chosen SPE cation-exchange conditions.

Completion of the cation-exchange solid phase extraction procedure revealed that the single oxidation product was present in the elute fraction (Fig. 10), along with unreacted chlorpromazine parent. This result confirmed that the degradation

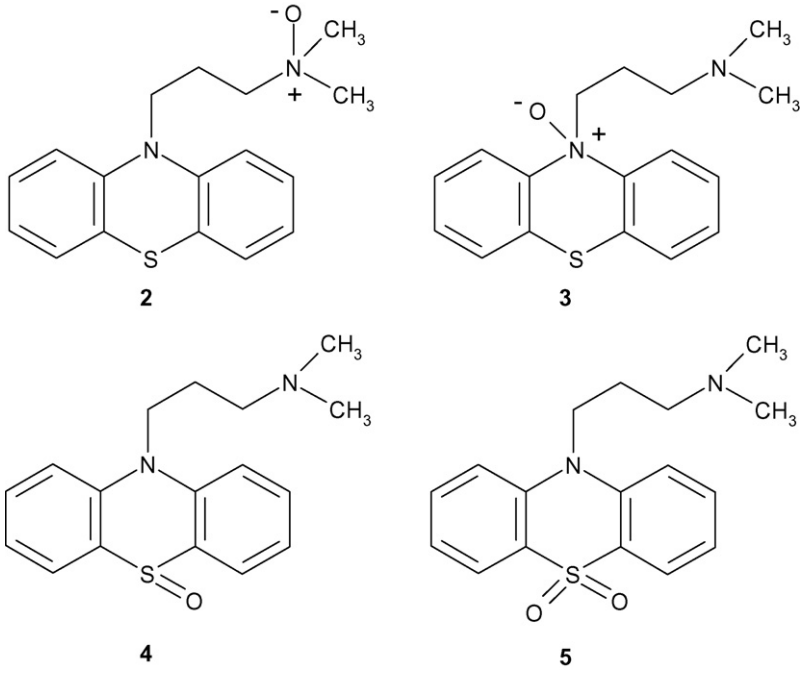


Fig. 9. Structures of possible chlorpromazine oxidation products.

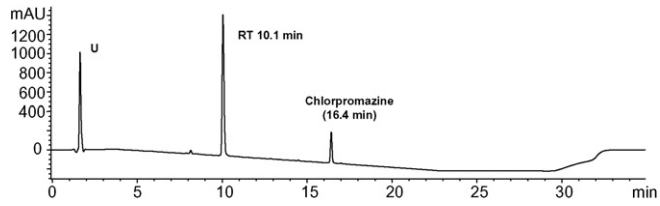


Fig. 10. HPLC chromatogram of the cation-exchange solid phase extraction elute fraction of the chlorpromazine oxidation reaction mixture. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

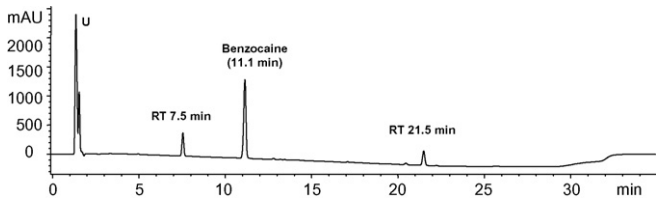


Fig. 11. HPLC chromatogram of the benzocaine reaction mixture following decomposition in 27% hydrogen peroxide Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

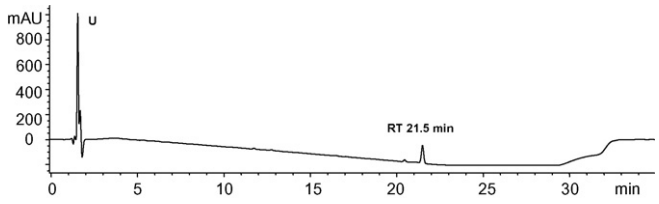


Fig. 12. HPLC chromatogram of the cation-exchange solid phase extraction wash fraction of the benzocaine oxidation reaction mixture. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.

product (RT 10.1 min) was positively charged at the extraction pH (pH 7.4, strongly suggesting the site of reactivity was not the strongly basic nitrogen atom. Based on this evidence, the site of oxidative reactivity was concluded to be remote from this nitrogen center.

Following the initial application of SPE to rule out one of the potential degradation products, a larger scale preparation and isolation of the unknown oxidative degradation product was conducted to further characterize its structure. Positive ionization electrospray HPLC-mass spectrometry of the isolated degradation product revealed a mass to charge (m/z) ratio of 335 amu for the molecular ion ($M\mathrm{H}^+$), an increase in mass of 16 amu over the chlorpromazine parent, effectively ruling out the sulfone degradation product (**5**, Fig. 9). ^{1}H NMR data was consistent with the assignment of the degradation product as chlorpromazine sulfoxide (**4**, Fig. 9) and was found to be inconsistent with predicted ^{1}H NMR spectra [28] expected from oxidation on the less basic, phenothiazine nitrogen atom (**3**, Fig. 9).

Several additional characterization studies were conducted to further confirm the assignment of the degradation product as chlorpromazine sulfoxide. Solid state IR analysis of the recovered material revealed a strong absorption band at 1022.5 cm^{-1}, indicative of the presence of a sulfoxide group [39]. The product was consistent with the known, thin layer chromatography retention factors on silica gel G of chlorpromazine sulfoxide [40]. Retention factors (R_f) were found to be 0.67 ± 0.01 in a acetone:diethylamine (9:1) solvent system and 0.65 ± 0.01 in an ethyl acetate:methanol:diethylamine (7:2:1.5) solvent system, compared with reported values of 0.68 and 0.65, respectively.

In this case, application of cation-exchange SPE was utilized to rapidly evaluate the initial reaction mixture and to gain insight into the nature of the chemical degradation. The process proved useful in guiding further analytical work to confirm the site of oxidative reactivity in the chlorpromazine molecule.

3.4. Study 4: oxidation of benzocaine with hydrogen peroxide

Benzocaine is simple pharmaceutical drug containing a single aniline group, with a predicted pK_a value of 2.51 ± 0.10 [32]. Oxidation of benzocaine with 27% hydrogen peroxide at 50 °C over 19 h produced a mixture of unreacted parent and two main degradation products (Fig. 11). The two major degradation products had retention times of 7.5 and 21.5 min. The parent benzocaine molecule was observed at 11.1 min.

Having no upfront information about the potential degradation products of this reaction, the solid phase extraction evaluation was designed based on the known, acidic character of the parent molecule. Utilizing this approach, the degradation products could be classified as having similar ionization character as the parent or it could be determined that the ionization character of the degradation product had changed in the course of the degradation process, thereby giving information as to the possible sites of reaction.

Application of cation-exchange solid phase extraction at pH 0.5 (~2 pK_a units below the pK_a of the parent) to the reaction mixture gave immediate information on each reaction product. The product with RT 21.5 min was present in the wash fraction (Fig. 12) and suggested that this degradation product no longer contained the basic aniline functionality. In this case, it was proposed that the oxidative decomposition had occurred on or near the basic aniline nitrogen, to render this degradation product non-cationic at the acidic SPE extraction conditions.

Examination of the elute fraction (Fig. 13) of the cation-exchange SPE experiment revealed the presence of unreacted benzocaine parent and the degradation product with retention time 7.5 min. The presence of the degradation product in the elute fraction indicated that the component had retained its basic functionality during the oxidative degradation process. Based on the extraction information, it was concluded that decomposition

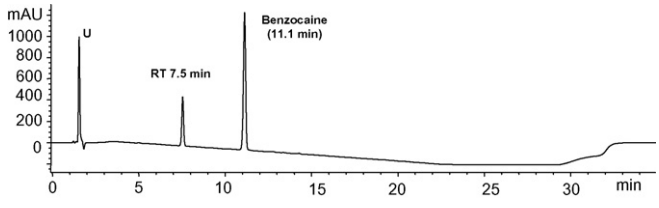
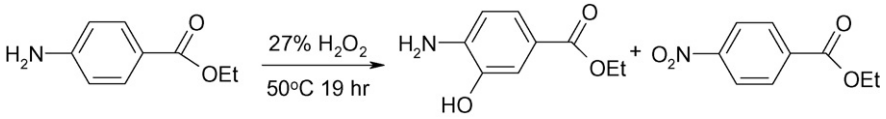


Fig. 13. HPLC chromatogram of the cation-exchange solid phase extraction elute fraction of the benzocaine oxidation reaction mixture. Retention times (RT) are listed for each component. Uracil (U) is the unretained standard.



Scheme 3. Degradation of benzocaine in hydrogen peroxide solution.

did not occur at the basic aniline nitrogen of this product and that oxidation occurred elsewhere on the molecular scaffold.

Further structural elucidation of the products from the degradation experiment was conducted to confirm and validate the SPE results. While no HPLC–MS information was readily available for the RT 21.5 min due to poor ionization, the SPE data served to confine the potential structures of this component to ones in which the aniline nitrogen was oxidized and no longer basic in nature. Thus, it was readily determined that the RT 21.5 min degradation product was 4-nitro-ethyl benzoate by correlation of retention time and UV absorbance spectrum to an authentic, external standard.

Similarly, the SPE data acquired on the RT 7.5 min product revealed that this product was still basic in nature, suggesting oxidation had occurred in such a way as to retain the basic properties of the molecule. Positive ionization electrospray HPLC–MS analysis revealed a mass to charge (m/z) ratio of 182.1 amu for the molecular ion ($M\mathrm{H}^+$), an increase in mass of 16 amu over the benzocaine parent, which suggested the addition of one oxygen atom to the molecule. Utilization of both the SPE and HPLC–MS data led to the proposal that oxidation may have occurred on the aromatic ring, which provided possible structures that were consistent with both the extraction and analytical results. Final identification of the degradation product as 4-amino-3-hydroxy-ethyl benzoate, by correlation to an authentic standard, was thereby greatly facilitated by the information provided from the solid phase extraction experiment and HPLC–MS analysis. The overall degradation of benzocaine in 27% hydrogen peroxide was therefore determined and is illustrated in Scheme 3.

4. Conclusions

The examples cited herein display the utility of the ion-exchange SPE technique to study drug degradation processes. The protocols revealed can serve to provide rapid, ancillary structure support information to proposed pharmaceutical degradation products and in its traditional role, provide an efficient, simple isolation method for degradation products. This technique may prove most valuable in the early stages of degradation investigation, where it may rapidly validate certain proposed degradation pathways and rapidly rule out others. This tool may be utilized for degradation product characterization during forced degradation studies of drug substances and drug products, examination of degradation during excipient compatibility screening, characterization of impurities during the manufacture of bulk drug substances and even to aid in the characterization of metabolite structures. Use of this technique with SPE columns in tandem may serve to fully characterize a reaction mixture rapidly and provide isolated, individual samples for further characterization. The availability of many configurations of high throughput SPE formats also makes this technique extremely amenable to rapid automated characterization of degradation.

While this technique is not purported to replace standard analytical characterization (MS, NMR, etc.), it is intended to be used as a collaborative tool in the study of degradation. Overall this report serves to introduce mixed-mode ion-exchange solid phase extraction as not only an extraction and isolation tool, but also as a structure elucidation aid.

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