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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 359–367
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Minimization of ion suppression in LC–MS/MS analysis through the application of strong cation exchange solid-phase extraction (SCX-SPE)

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Received 28 July 2004; received in revised form 6 October 2004; accepted 28 October 2004
Available online 25 December 2004

Abstract

Ion suppression of drug response is a major source of imprecision for bioanalytical analysis using LC–MS/MS. Endogenous phospholipids cause ion suppression in both positive ESI and negative ESI modes and must be removed or resolved chromatographically. Three types of ion-exchange solid-phase extraction mediums were evaluated to determine their abilities to remove phospholipids. It was determined that although mixed mode phases fulfills the requirements of retaining both analytes and diverse metabolites, reverse phase retention mechanisms are detrimental in eliminating ion suppression caused by late eluting phospholipids. If an analyte and its metabolites can be retained using an ion-exchange mechanism alone, mixed mode extraction phases should be avoided.

Keywords: LC–MS/MS; Ion suppression; SPE; SCX-SPE; Mixed mode SPE; Phosphatidylcholine; LysoPC

1. Introduction

Today, liquid chromatography and tandem mass spectrometry (LC–MS/MS) is the method of choice for the quantitation of analytes in biological matrices. The combination of LC and MS/MS offers unparalleled sensitivity and specificity few other techniques can match [1,2]. It comes as no surprise that for a period of time following the introduction of LC–MS/MS instrumentation, sample cleanup and high-resolution chromatography were considered superfluous [3,8]. With their importance deemphasized, sample cleanup typically involved acetonitrile precipitation of plasma proteins and chromatographic separation was achieved by the use of short analytical columns (typically 3 cm and less) with steep gradients or short isocratic runs, often less than 2 min. However, it became apparent that inadequate sample cleanup and chromatography often led to ion suppression that caused irreproducible results for some bioanalytical methods [4,5]. The effect of ion suppression is observed primarily in electrospray ionization (ESI) whereby analyte signal is attenuated by competition from the ionization of bulk ions inside the solution droplets [15,16]. Although these disturbances are not visible in the analyte MRM channels of interest, the precision and accuracy of bioanalytical methods can be compromised by sample-to-sample differences in matrix ion concentrations and components competing with the ionization of analyte. A number of techniques to visualize ion suppression have been proposed in the literature. The most common method involves a post-column infusion of the compound of interest while the MRM transition is recorded during an injection of blank matrix sample [7], producing chromatograms similar in appearance to IR spectra, ion suppression is revealed as dips in the constant background signal of analyte.

Although solvent additives and mobile phase components can cause ion suppression, their impact is relatively insignificant when compared to ion suppression generated by the presence of endogenous materials in biological samples;

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doi:10.1016/j.jpba.2004.10.035

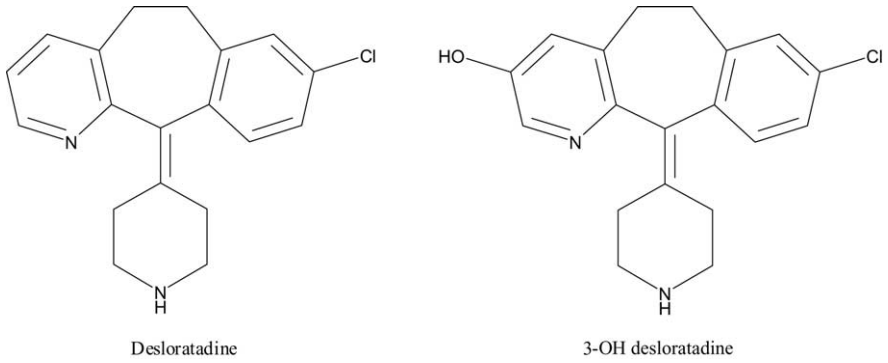

Fig. 1. Structure of desloratadine and 3-hydroxy desloratadine.

i.e., matrix effects [6]. Indeed, matrix effects predominate as the cause of ion suppression. Of all the components in plasma, lipids are the most variable. Both the type and quantity of lipids found in plasma fluctuate within individuals and differ from person to person are primarily dependent on diet and metabolic rate. However, despite significant lipid variation, phosphatidylcholine (also know as 'lecithin') is the principle phospholipid circulating in the plasma [9]. As a zwitterion, phosphatidylcholine can cause ion suppression in both positive ESI and negative ESI modes because of its ability to ionize in both environments. In a recent study, serum albumin and phosphatidylcholine were determined to be the major causes of ionization suppression in analysis of verapamil following protein precipitation and reverse phase solid-phase extraction sample cleanup [10]. Therefore, the removal of phospholipids as a principle agent of ion suppression is an extremely important component of any extraction process. Several techniques have been applied toward the removal of phospholipids in order to obtain cleaner sample extracts [16]. In one such study, a modified lanthanide column was used successfully to remove the bulk of the phospholipids through a two-step cleanup process involving liquid/liquid extraction with methyl *tert*-butyl ether (MTBE) followed by solid-phase extraction on a proprietary column [11]. Although effective, this two-step cleanup process is time consuming and difficult to automate. The goal of our study was to evaluate popular and commercially available ion exchange and mixed mode solid-phase sorbents and propose a simpler procedure for removing phospholipids without impacting the recovery of compounds of interest. To this end, we evaluated three leading solid-phase extraction sorbents available in a 96-well format for their ability to extract the model compounds such as desloratadine and 3-hydroxy desloratadine while removing phospholipids.

2. Experimental

2.1. Materials

Desloratadine with a purity of 98.3%, and 3-hydroxy desloratadine with a purity of 99.3% were synthesized at Schering-Plough Research Institute (Kenilworth, NJ) (Fig. 1). Human plasma with EDTA as the anticoagulant was purchased from Bioreclamation Inc. (Hicksville, NY). All other chemical reagents were purchased from either Fisher Scientific (Fair Lawn, NJ) or Sigma–Aldrich Co. (St. Louis, MO). All chemical reagents were either OPTIMA® Grade or Certified ACS Grade unless otherwise noted.

2.2. Instrumentation

A Sciex API 3000 (Applied Biosystems, Ont., Canada) mass spectrometric system equipped with a TurboIonspray™ interface was used as the detector. Unless otherwise noted, this mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using positive ion electrospray. Desloratadine was monitored with a MRM transition of *m/z* 311 → *m/z* 259 while 3-hydroxy desloratadine was monitored with a MRM transition of *m/z* 327 → *m/z* 275. The following are the API 3000 instrument parameters used for all experiments: nebulizer = 10, curtain gas = 10, collision energy = 30 eV, ion spray voltage = 4000 V, turbo heater temperature = 400 °C. The chromatography system consisted of a pair of Shimadzu (Shimadzu Corporation, Columbia, MD, USA) 10ADvp LC pumps controlled via a Shimadzu SCL-10A system controller. A Shimadzu DGU-14A degasser is standard on this system. Sample injection was automated via a CTC PAL autosampler. Data collection and peak integration were performed using Analyst™ 1.3.1 software.

2.3. Solid-phase extraction disks

Three 96-well solid-phase extraction disks were evaluated. The first is commercially available from Waters Corporation under the trade name Oasis® HLB MCX. Based on a water wetable polymeric backbone, the 96-well Oasis® HLB MCX plate has dual mode functionality using a sulfonic acid moiety as its strong anionic functional group and a copolymeric backbone for retention by hydrophobic and hydrophilic mechanisms. The 96-well solid-phase extraction plate used for this experiment had a total of 10 mg of sorbent.

The second two disks evaluated were produced by Varian Corporation and sold under the trade name SPEC®. Also available in a 96-well format, these solid-phase extraction disks were packed with 15 mg of sorbent. SCX and MP1 were the two chemistries evaluated for this article. Based on a monolithic silica backbone, the SPEC® SCX utilizes bonded benzenesulfonic acid as its anionic functional group while SPEC® MP1 is a mixed mode sorbent utilizing the same cationic functional group with a C8 aliphatic bonded phase.

3. Results and discussion

Two previous analytical methods were published for detection of desloratadine and 3-hydroxy desloratadine using LC–MS/MS. One method from our laboratory, presented an automated 96-well solid-phase extraction method that utilized a 500 μL aliquot of human heparin plasma [14]. Because this method was based on a C18 extraction system coupled to a reverse phase HPLC system, the non-orthogonal nature of the method required a 6 min run time for resolution of all interferences. More recently, we improved upon this analytical method by coupling reverse phase HPLC with ionic exchange solid-phase extraction. The advantage of such an approach is intuitive given that the extraction is ionic in nature; residual endogenous interferences resulting from extraction are unlikely to present a selectivity problem for the reverse phase chromatography system. The validation of this new method uses only 250 μL plasma and has a runtime of 4 min is [12].

In addition to desloratadine and 3-hydroxy desloratadine, three additional mono-hydroxylated metabolites of desloratadine may also be present in a biological sample from a subject dosed with Clarinex™ [12]. The mono-hydroxylated metabolites cannot be mass separated based on their MRM transitions and therefore chromatographic separation is required. This is accomplished by using a gradient elution profile (Table 1) on a Waters Atlantis™ C18 2 mm × 50 mm column. Mobile phase A consists of 10 mM ammonium formate with 0.2% formic acid, while mobile phase B consists of 10 mM ammonium formate in methanol with 0.2% formic acid. At a flow rate of 0.25 mL/min, 3-hydroxy desloratadine and desloratadine are eluted with retention times of 3.1 and 3.2 min, respectively (Fig. 8). A detailed extraction recovery experiment for desloratadine and 3-hydroxy desloratadine were undertaken using MCX extraction plates at the concentration of 7.5 ng/mL [12]. Two types of extraction recoveries was studied, the first type was determined by comparing the mean peak response from processed samples to the mean peak response from unprocessed (neat) standards. In addition, the mean peak response of the extracted blank matrix samples that were spiked (post-extraction) with QC stock solution was compared to the mean peak response processed QC samples to demonstrate extraction efficiency. For $n = 6$ trials, desloratadine and 3-hydroxy desloratadine showed a average recovery of 31.7% and 46.4%, respectively, compared to neat standards prepared in mobile phase. Comparing to post-spiked samples ($n = 6$), desloratadine and 3-hydroxy desloratadine demonstrated extraction efficiencies of 76.3% and 84%, respectively. It should be noted that under these chromatographic conditions, the phospholipids of interest are not eluted after the initial injection cycle but rather appear as late eluters in second, third, and subsequent injections. Because of the ion-suppressing nature of the phospholipids, recoveries would be significantly impacted if phospholipids were to co-elute with the compounds of interest. In order to monitor the late eluting phospholipids, the third step of the gradient elution was lengthened from 3.8 to 6.8 min. This change allowed the concurrent monitoring of all phospholipids in the same injection cycle.

Table 1
Gradient elution chromatography program from the validated bioanalytical method for quantitation of desloratadine and 3-hydroxy desloratadine

Step	Total time (min)	B (%)	A (%)	Flow rate (μL/min)
1	0.50	20	80	250
2	3.30	90	10	250
3	3.80	90	10	250
4	3.81	20	80	250
5	4.20	20	80	250

After 2D optimization [13], the following extraction protocols were selected for all three extraction disks: solid-phase extraction plate is pre-conditioned with 400 μL of methanol followed by 400 μL of 2% formic acid. A 250 μL sample aliquot diluted in 500 μL of 2% formic acid solution is applied to the pre-conditioned solid-phase extraction plate. The sample is loaded under vacuum (<5 psi negative pressure) and the extraction plate is then washed sequentially with 400 μL of 2% formic acid solution followed by 400 μL of acetonitrile: methanol (70:30%, v/v). Analytes are eluted by using two 200 μL aliquots of methanol:acetonitrile:water:ammonia (45:45:10:4%, v/v/v/v). The eluent is then dried under a stream of nitrogen and reconstituted in 150 μL of mobile phase for subsequent LC–MS/MS analysis.

The analytical setup for evaluating ion suppression was as follows: desloratadine and 3-hydroxy desloratadine were infused post-column via a mixing tee at a concentration of 500 ng/mL. Ion suppression resulting from matrix components in the eluent was monitored during gradient elution by continuously acquiring MRM spectra for both desloratadine and 3-hydroxy desloratadine over a 7.5 min period. Control plasma blanks were extracted by using the solid-phase extraction procedure described above and injected into the LC–MS/MS system. A dip in the monitored mass trace was considered to be an effect of ion suppression. In addition to monitoring the MRM channels of desloratadine and 3-hydroxy desloratadine, extracted phospholipids were also monitored by using their corresponding MRM transitions. Example chromatograms for each of the three extraction plates are shown in Figs. 2–4.

From these experiments it is evident that at least two major ion suppression regions are common to all three extraction

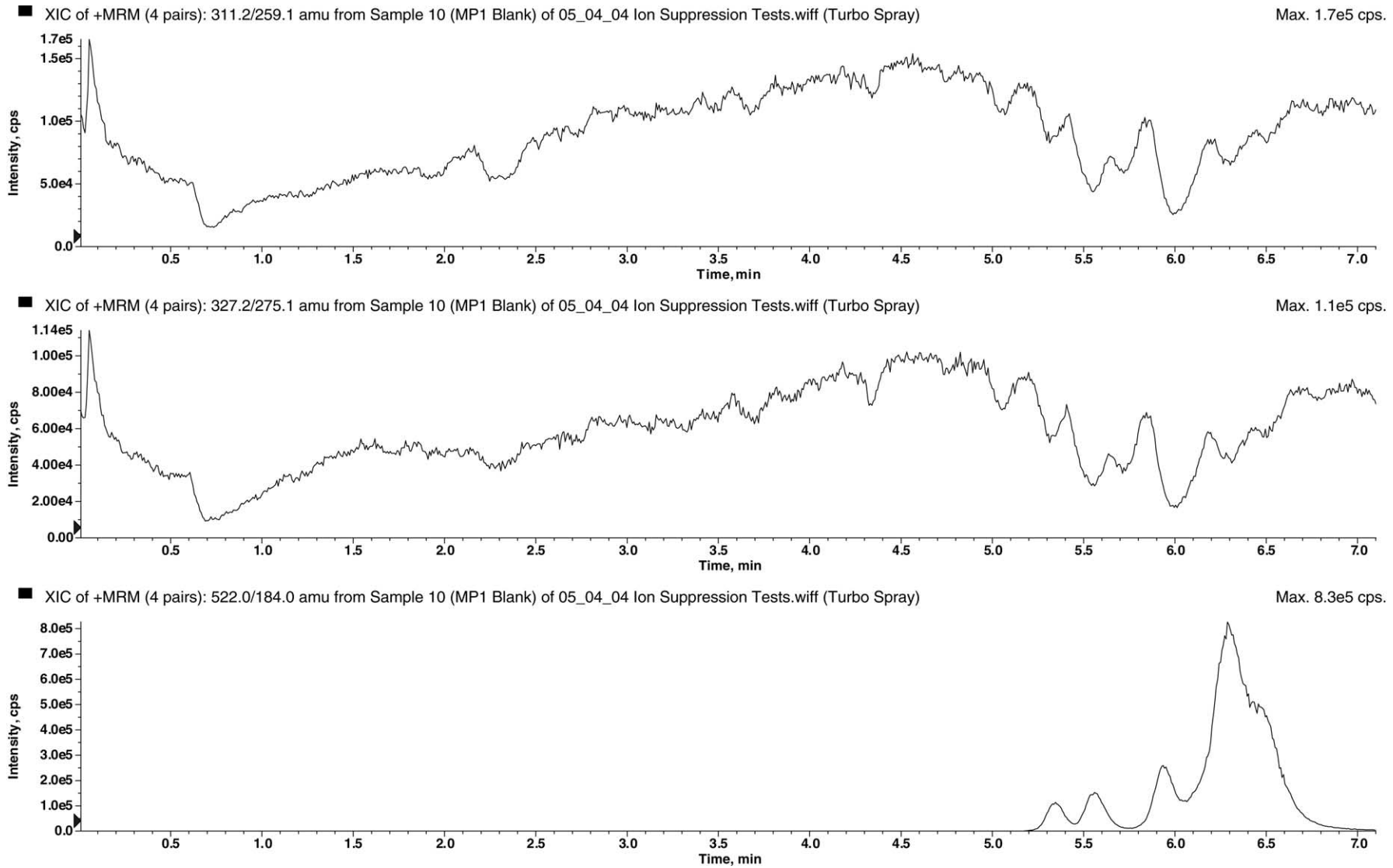

Fig. 2. Multiple reaction monitored ion chromatograms for desloratadine (top trace), 3-hydroxy desloratadine (middle trace) and phosphatidylcholine monoester (bottom trace) during post-column infusion and subsequent injection of a SPEC® MP1 extracted control blank plasma sample.

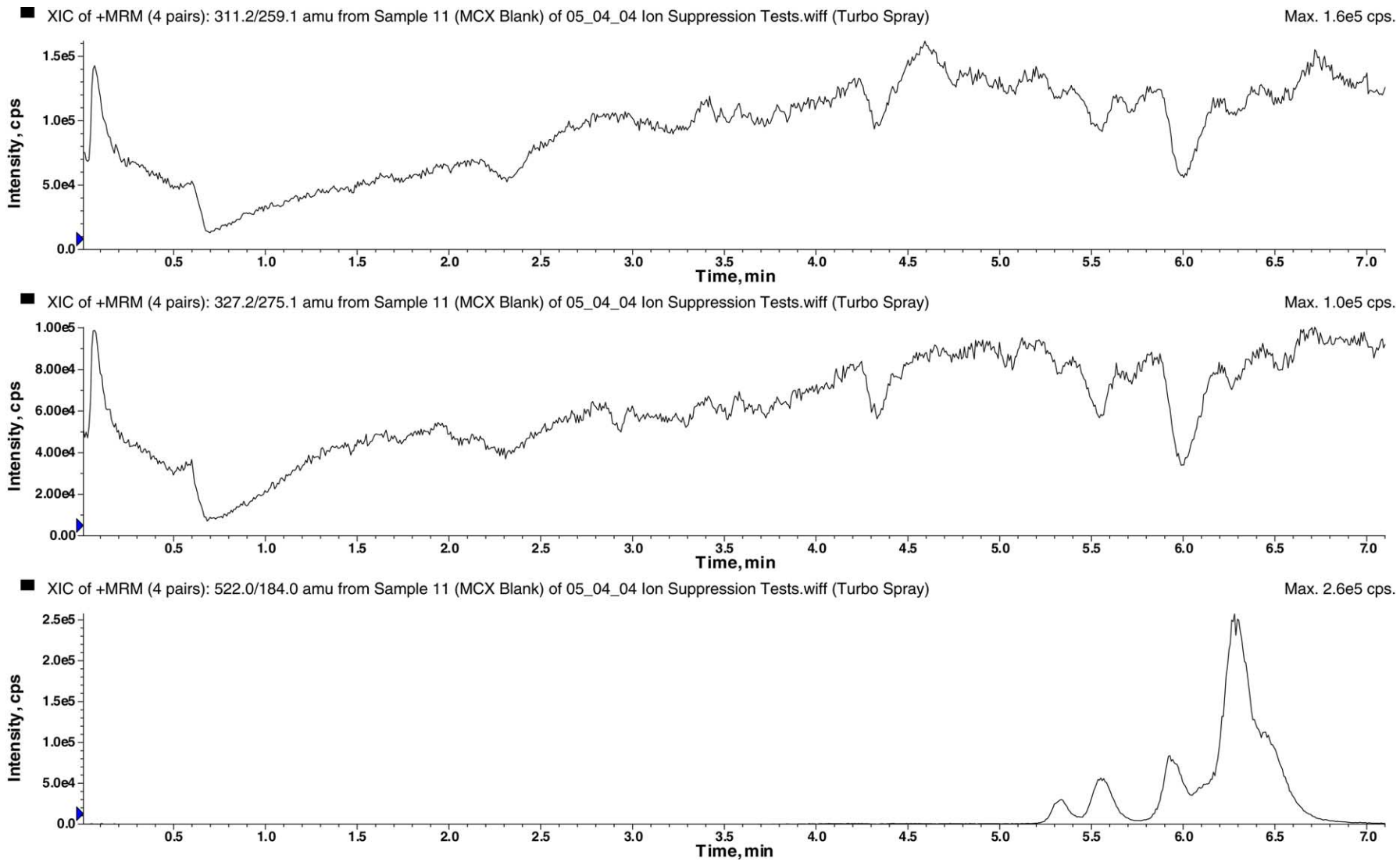

Fig. 3. Multiple reaction monitored ion chromatograms for desloratadine (top trace), 3-hydroxy desloratadine (middle trace) and phosphatidylcholine monoester (bottom trace) during post-column infusion and subsequent injection of an Oasis® HLB MCX extracted control blank plasma sample.

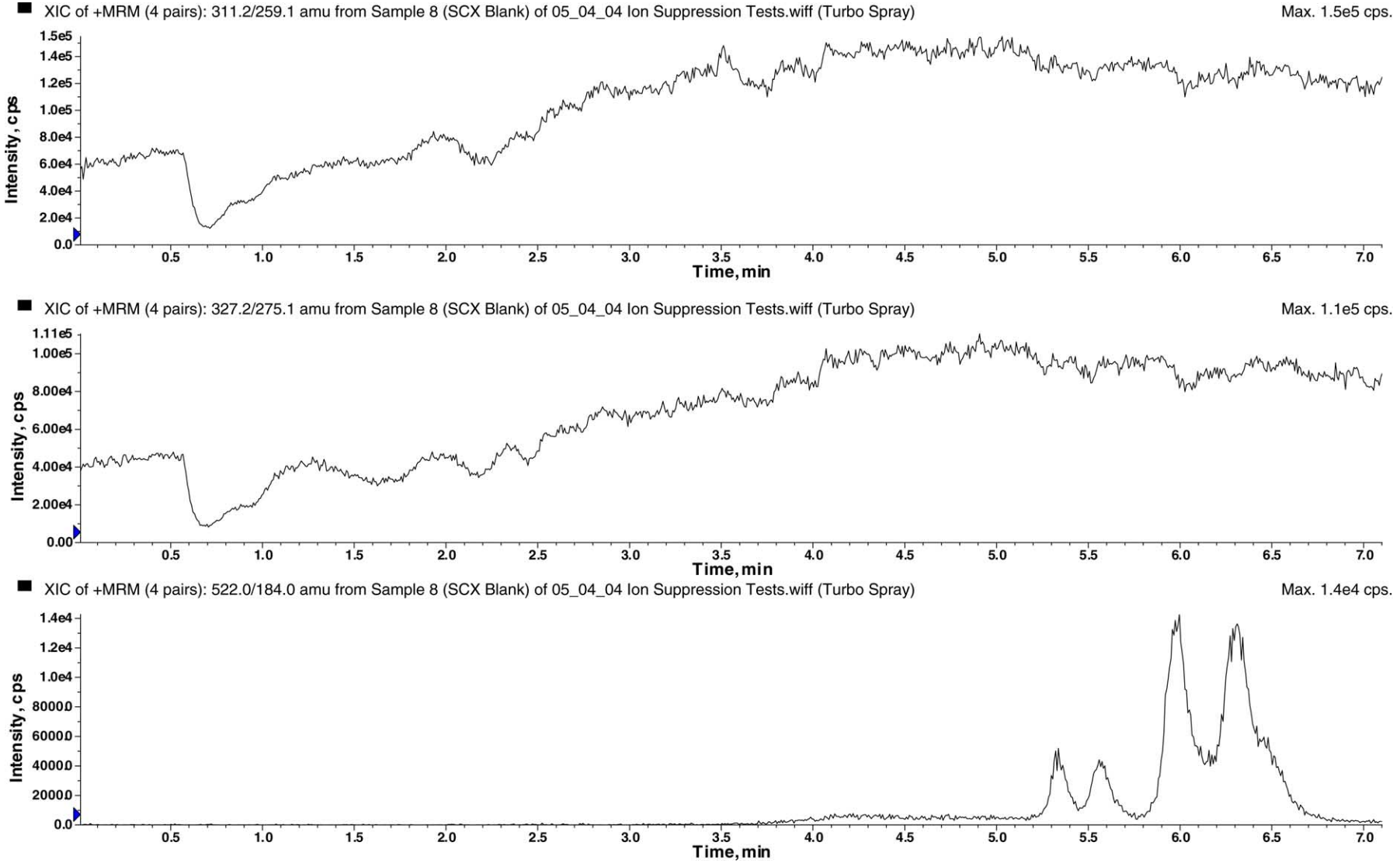

Fig. 4. Multiple reaction monitored ion chromatograms for desloratadine (top trace), 3-hydroxy desloratadine (middle trace) and phosphatidylcholine monoester (bottom trace) during post-column infusion and subsequent injection of a SPEC® SCX extracted control blank plasma sample.

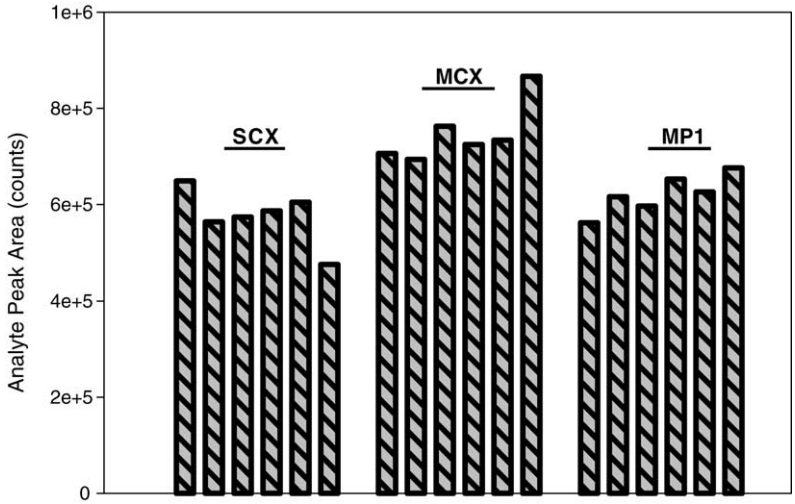

Fig. 5. Comparison of overall extraction recoveries of desloratadine from SPEC® SCX, Oasis® HLB MCX, and SPEC® SCX extraction plates.

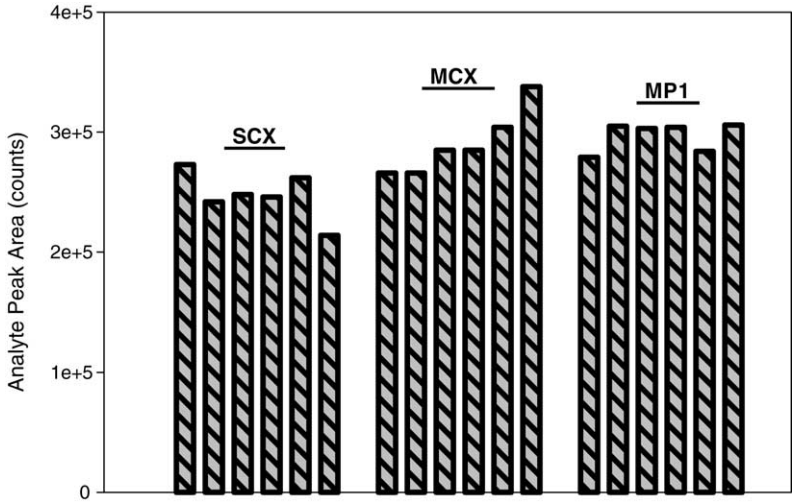

Fig. 6. Comparison of overall extraction recoveries of 3-hydroxy desloratadine from SPEC® SCX, Oasis® HLB MCX, and SPEC® SCX extraction plates.

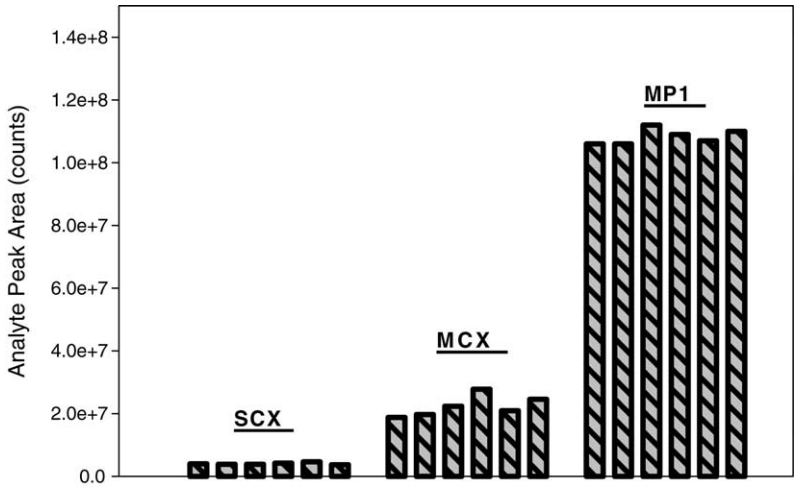

Fig. 7. Comparison of a surrogate phospholipid, phosphatidylcholine octadecyl monoester, from SPEC® SCX, Oasis® HLB MCX, and SPEC® SCX extraction plates.

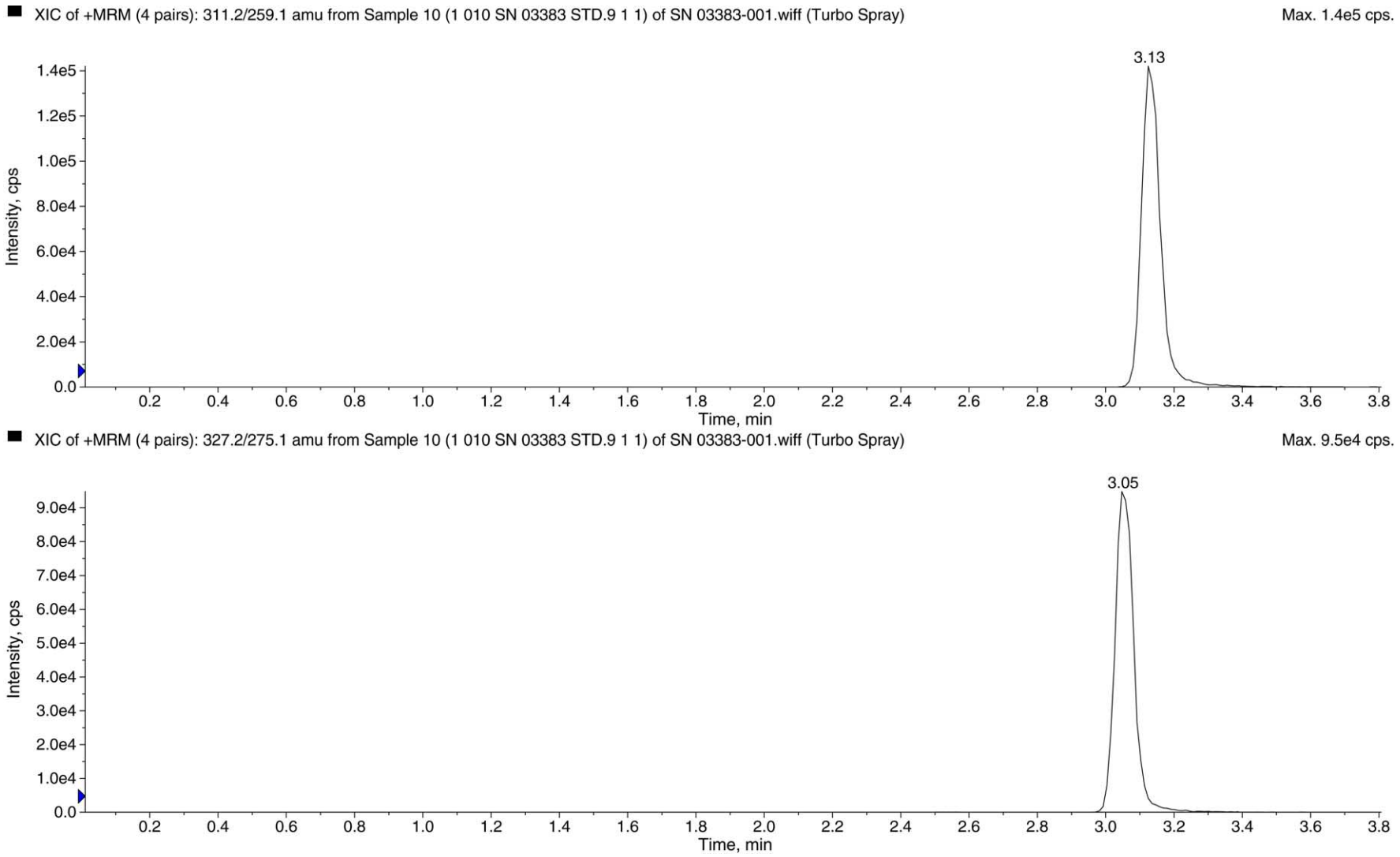

Fig. 8. Typical MRM chromatograms showing elution of a 25 μL injection of extracted 10 ng/mL spiked human plasma sample of desloratadine (top trace) and 3-hydroxy desloratadine (bottom trace) under described HPLC condition.

plates. The first major ion suppression region, most likely caused by the elution of polar proteins and carbohydrates, occurs near the void volume of the column; i.e., at around 0.7 min. The second major ion suppression region, presumably caused by phospholipids, occurs between 5 and 7 min post-column. Of the three solid-phase extraction plates, samples extracted by using the SPEC® SCX had the least amount of ion suppression while the SPEC® MP1 displayed the most ion suppression. Specifically, the amount of phospholipids, as determined by peak height for one surrogate phospholipid, were observed to increase from 1.4e4 counts per second (cps) for SPEC® SCX extracted blanks (Fig. 4) to 8.3e5 cps for SPEC® MP1 extracted blanks (see Fig. 2). Moreover, the corresponding ion suppression dip increased proportionally as the amount of phospholipids increased (Figs. 2–4).

The overall extraction efficiencies of desloratadine and 3-hydroxy desloratadine for all three extraction plates are summarized in Figs. 5 and 6. For these experiments, six replicate extractions were performed on a plasma standard containing 10 ng/mL each of desloratadine and 3-hydroxy desloratadine. As can be seen from the graphical representation, similar recovery efficiencies were observed for desloratadine and 3-hydroxy desloratadine on all three extraction plates. In an attempt to improve recovery, the organic wash step was subsequently modified to include 2% formic acid. Modest improvements were seen only for the SPEC® SCX phase whereby recoveries were increased to similar or slightly higher than that observed for the Oasis® HLB MCX extraction plate. The recovery of the surrogate phospholipid for each of the three extraction plates is summarized in Fig. 7. The capacity of the SPEC® SCX plate to remove phospholipid from the eluent is approximately 30 times greater than that of the SPEC® MP1 plate and approximately seven times greater than that of the Oasis® HLB MCX plate. This result was anticipated given that the tertiary structure of a phospholipid in an aqueous environment would favor interaction on a phase with significant hydrophobic character. Unlike the Oasis® HLB MCX phase or the SPEC® MP1 phase, the silica based SPEC® SCX particle does not provide a mechanism for hydrophobic interactions with phospholipids.

4. Conclusion

Phospholipids are a significant class of endogenous plasma extractable that can lead to ion suppression in bioanalytical determinations. Major ion suppression regions corresponding to the elution of phospholipids during chromatographic separation were observed in this study. The late elution of these compounds can lead to imprecision and inaccuracy in bioanalytical methods. Clearly, these data highlight the importance of investigating chromatographic resolution of analytes from phospholipids. Were it not for chromatographic resolution, the measured recoveries of desloratadine and 3-hydroxy desloratadine would have been significantly lower.

MRM transitions of surrogate phospholipids can be monitored during method development to estimate the potential for ion suppression during methods optimization of extraction. Simply monitoring these MRM transitions represents a less cumbersome alternative to post-column infusion.

Although mixed mode phases can fulfill the requirements of retaining both analytes and diverse metabolites, this study suggests that reverse phase retention mechanisms can be detrimental in eliminating ion suppression caused by late eluting phospholipids. Indeed, if an analyte and its metabolites can be retained using an ion-exchange mechanism alone, mixed mode extraction phases should be avoided.

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