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Straightforward solid-phase extraction method for the determination of verapamil and its metabolite in plasma in a 96-well extraction plate

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Abstract

Straightforward solid-phase extraction (SPE) methods were developed for the determination of verapamil and its metabolite in a plasma matrix. The spiked plasma sample was pretreated with 2% phosphoric acid followed by two different SPE methods using a Waters Oasis™ HLB 96-well extraction plate. Recoveries greater than 90% were obtained using both a generic and a selective SPE methods. The generic method is a good starting protocol, and it is applicable to a wide range of compounds. This generic method consists of using 5% methanol as the wash solvent, and 100% methanol for the elution. The limitation of the non-specific method is that it does not remove all plasma constituents that interfere with the quantitation of the metabolite, norverapamil. A second, more specific method was developed using the same Oasis™ HLB sorbent which removes more plasma interferences and provides cleaner extracts for the HPLC–UV analysis. This selective method uses both the methanol concentration and the pH advantageously to preferentially isolate the analytes of interest from a complex sample matrix. Recoveries of greater than 90% with R.S.D.s less than 3.8% were obtained with this selective method.

Keywords: Solid-phase extraction; Extraction methods; Verapamil; Norverapamil

1. Introduction

Bioanalytical methods are required for pharmacokinectic studies in drug discovery and for drug metabolism during the development stages. Historically, liquid–liquid extraction and protein precipitation have been the bottleneck in laboratory productivity since they are not easy to automate. Presently, solid-phase extraction (SPE) has been demonstrated to be more readily automated for high sample throughput [1]. Silica-based reversed-phase sorbents, such as C_{18} or C_8, are the most widely used packings for SPE. However, they have four main limitations.

First, the sorbent must remain wet prior to sample loading. If the sorbent runs dry anytime before the sample loading step in either cartridges or wells in 96-well extraction plates, the consequence is low and variable recovery [2–5].

Second, for polar drugs and metabolites, retention is weak and often results in breakthrough during the loading step, yielding unpredictably low recoveries and poor reproducibility.

Third, basic analytes interact strongly with residual silanols, which, in turn, leads to low recovery with simple elution solvents [2,6,7].

Fourth, the SPE method development is time-consuming and tedious. Often, it takes a long time to develop a method, and this process can take up to two-thirds of the entire analysis [1].

Recently, Cheng and co-workers [8–10] have

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demonstrated the SPE cleanup of a wide range of compounds using a hydrophilic–lipophilic balanced (HLB) sorbent. With one generic SPE method, good recovery (greater than 85.7%) and excellent reproducibility (less than 5.5% R.S.D.s) were obtained for a wide range of compounds including acids, neutrals, bases, parent compounds, and polar metabolites. Additionally, this HLB sorbent is fully wettable with water; therefore, there is no impact of sorbent drying. In this generic method, only the concentration of the organic solvent was manipulated throughout the entire SPE procedure: conditioning with 1 ml of methanol, equilibrating with 1 ml of water, loading with 1 ml of sample solution, washing with 1 ml of 5% methanol in water, and finally eluting with 1 ml of methanol. However, this generic method also extracts other constituents present in the sample matrix (such as serum, plasma, or urine) which, in turn, may interfere with subsequent analysis, especially at low analyte concentrations. Such a method can get rapid results, if selective and sensitive detection methods, such as HPLC–MS–MS, are used.

In this paper, we would like to expand the generic SPE method, in which 5% methanol was used as a wash solvent, and 100% methanol as the elution solvent (the one-dimensional SPE method), into a more selective SPE method, in which both organic concentration and pH are advantageously applied to the SPE steps (the two-dimensional SPE method). This two-dimensional (2D) method is complementary to the one-dimensional (1D) SPE method, and it preferentially isolates the analytes of interest from a complex sample. The principle of this 2D-SPE method will be discussed. The application of the 1D- and 2D-SPE methods for the determination of verapamil and its metabolite will be demonstrated as well.

2. Experimental

2.1. Reagents and materials

Verapamil, norverapamil, and methoxyverapamil were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, phosphoric acid, acetic acid, and ammonium hydroxide were HPLC grade from J.T. Baker (Phillipsburg, NJ, USA). Porcine plasma (with EDTA) was obtained from Equitech-Bio (West Ingram, TX, USA). An Oasis™ HLB 96-well extraction plate (30 mg/well) and a 96-well extraction vacuum manifold were procured from Waters (Milford, MA, USA). The calibration curves were generated over a concentration range from 0.34 to 6.75 μg/ml for verapamil, and from 0.23 to 4.6 μg/ml for norverapamil. Each standard solution contained 2.0 μg/ml methoxyverapamil as the internal standard.

2.2. HPLC apparatus and operating conditions

Isocratic elution was used throughout the entire study. The HPLC system consisted of a Waters Alliance system equipped with a 2690 module and a column heater unit. A Waters 996 photodiode detector was used for detection at 230 nm. The Millennium 2010 Chromatography Manager, version 2.15, was used to control the HPLC system and to perform data acquisition and manipulation. The column used was a Waters SymmetryShield™ RP_8 column (150×3.9 mm, 5 μm particle size) preceded by a Sentry™ RP_8 guard column (20×3.9 mm, 5 μm particle size). The chromatography was carried out at 30°C. The mobile phase was 50 m*M* potassium phosphate, pH 7–acetonitrile–methanol (41:37:22, v/v/v). The flow-rate was set at 1.0 ml/min. For the concentration determination, 40 μl each of the sample and the standard solution were injected.

2.3. Wash–elute study

The wash–elute study is used to determine the percentage of the methanol concentration needed in the wash step(s) and in the elution steps for the subsequent SPE procedure. Details are found in Section 2.4. Verapamil, norverapamil, and methoxyverapamil standard solutions were prepared in a pH 7 phosphate-buffered saline (PBS) solution. This PBS solution was prepared as follows: 200 mg KCl, 8000 mg NaCl, 200 mg KH_2PO_4, and 1150 mg of Na_2HPO_4 were dissolved in a 1-l flask and adjusted to pH 7.0 with 10% phosphoric acid to a final volume of 1 l. Twenty wells in a 96-well extraction plate were conditioned with 1 ml of methanol and equilibrated with 1 ml of water. An aliquot of 1 ml

sample solution was loaded onto each well. For the first 10 wells, the analytes were eluted from each well with methanol–water mixtures, containing 2% ammonium hydroxide, of increasing methanol concentration (0, 10, 20, 30, 40, 50, 60, 70, 80, and 90% methanol). For the next seven wells, the analytes were eluted from each well with methanol–water mixtures, containing 2% acetic acid, of increasing methanol concentration (0, 10, 20, 30, 40, 50, and 65% methanol). All eluates were collected separately and directly analyzed by the above HPLC conditions.

2.4. Solid-phase extraction procedure

Aliquots of freshly thawed drug-free porcine plasma were spiked with drug solutions to produce the desired concentrations. Two levels of sample concentrations were prepared. At the high level, the concentration for verapamil and norverapamil were 0.68 and 0.46 μg/ml, respectively. At the low level, the respective concentrations were 0.14 and 0.092 μg/ml. Each sample contained 0.40 μg/ml of methoxyverapamil as the internal standard. These spiked plasma samples were then acidified with concentrated phosphoric acid to bring the final phosphoric acid concentration to 2%. The acidified sample solutions were then loaded onto extraction plate wells, which had been conditioned with 1 ml of methanol, followed by 1 ml of water. After loading 1 ml of acidified plasma sample solution onto each well, three different SPE methods were carried out as follows.

For the 1D-SPE method, each well was washed with 1 ml of 5% methanol in water, and then eluted with 1 ml of methanol.

For the 2D-SPE method with two washes, each well was washed with 1 ml of 5% methanol, followed by 1 ml of 65% methanol containing 2% ammonium hydroxide, and then eluted with 1 ml of 65% methanol containing 2% acetic acid.

For the 2D-SPE method with three washes, each well was washed with 1 ml of 5% methanol containing 2% acetic acid, followed by 1 ml of 5% methanol containing 2% ammonium hydroxide, followed by 1 ml of 65% methanol containing 2% ammonium hydroxide, and then eluted with 1 ml of 65% methanol containing 2% acetic acid.

All the above eluates were evaporated to dryness in a heating block at 40°C under a gentle stream of nitrogen and reconstituted with 200 μl of water.

3. Results and discussion

3.1. Chromatographic analysis

As shown in Fig. 1, verapamil and its metabolite, norverapamil, are basic compounds containing amino groups. Verapamil has a tertiary amino group, and norverapamil, the metabolite of verapamil, has a secondary amino group. The internal standard, methoxyverapamil, also is a tertiary amine. These basic analytes interact with the residual silanol sites present on silica-based reversed-phase sorbents, which causes low recovery in the SPE and peak tailing in the HPLC separation. To overcome this problem, it has been shown that the addition of a competing reagent, such as diethylamine or 2-aminoheptane, is necessary in order to achieve good peak shapes for these basic analytes [11–13]. In this study, we were able to obtain good peak shapes with a simple mobile phase, 50 m*M* pH 7 potassium phosphate–acetonitrile–methanol (41:37:22, v/v/v), without adding any competing reagent. Good peak shapes were obtained (Fig. 2), and USP tailing factors were all between 1.18 and 1.07. HPLC calibration curves were based on peak-area ratio to the internal standard, methoxyverapamil, and was performed according to a least-squares fit of data to a straight line. Within the concentration range described in the experimental section, linear plots were obtained for verapamil as well as norverapamil. The correlation coefficients were 0.999887 and 0.999831 for verapamil and norverapamil, respectively.

3.2. Principle of the 2D-SPE method

There are two main factors which control the analyte retention in reversed-phase chromatography (HPLC and SPE). One is the concentration of the organic modifier, and the other is the pH. The analyte retention decreases with an increase of the concentration of the organic modifier. Bolliet and Poole [14] evaluated the influence of acetonitrile, methanol and isopropanol as retention selectivity modifiers in reversed-phase liquid chromatography

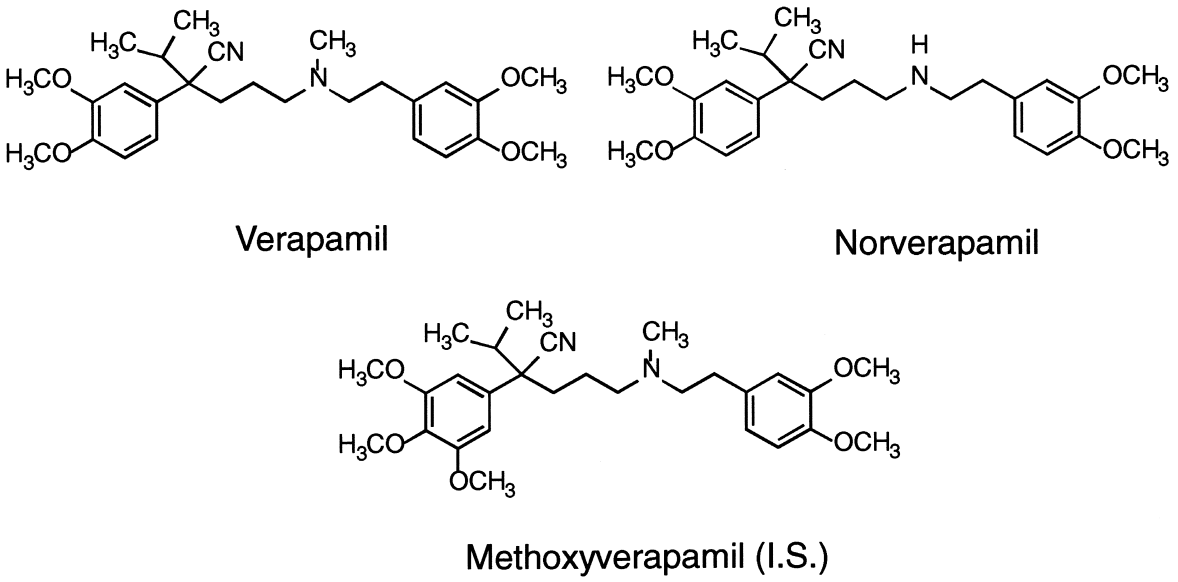

Fig. 1. Structures of verapamil and its metabolite. They all have very similar structures and contain amino groups. Verapamil is a tertiary amine, norverapamil, the metabolite of the verapamil, is a secondary amine, and methoxyverapamil, the internal standard, is a tertiary amine.

on a poly(styrene–divinylbenzene) macroporous sorbent. For the analytes they studied, the plots of log k against mobile phase composition for methanol are approximately linear or slightly convex, while the plots are virtually all concave and quite steep for isopropanol and acetonitrile. This observation is similar to the behavior of silica-based reversed-phase packings.

With respect to the influence of the pH, the analyte retention depends on the nature of the compounds. At low pH (usually at two pH units lower than the pK_a of the analyte), acidic analytes are present in their unionized form, and they exhibit high retention on a reversed-phase column. At high pH (usually at two pH units higher than the pK_a of the analyte), the analytes are ionized, and they have low retention. For a basic analyte, the retention is opposite to that of the acidic analyte. At low pH, the analyte is ionized, and it has low retention; at high pH, the analyte is in its unionized form, and it has high retention. For a neutral analyte, the analyte retention remains unchanged throughout the entire pH range. Divinylbenzene-based polymers, such as the Oasis™ HLB sorbent, provide excellent pH stability ranging between pH 0 and 14. This broad pH range allows for increased flexibility in SPE methods development.

The full range of pH stability (pH 0–14) is not available for siliceous sorbents. At alkaline pH, hydroxyl ions (OH^-) can attack and dissolve the silica; at low pH, the alkyl bonded phase is susceptible to hydrolysis [15,16]. Additionally, strong secondary silanol interactions between the residual silanol groups present on the silica-based sorbents and the basic analytes complicate the reversed-phase retention mechanism. The degree of silanol interaction depends upon the ionic strength of the sample matrix as well as the pH [17]. At acidic pH, the ionization of the silanols is suppressed, while at neutral pH, the silanols are fully ionized. This further complicates the elution protocol, and often a large elution volume is needed in order to obtain good recovery [6,7].

3.3. 2D-SPE method for basic analytes

There are different approaches to selectively isolate the analytes of interest from a complex sample solution. In a typical two-dimensional SPE method, both the pH and the organic concentration are

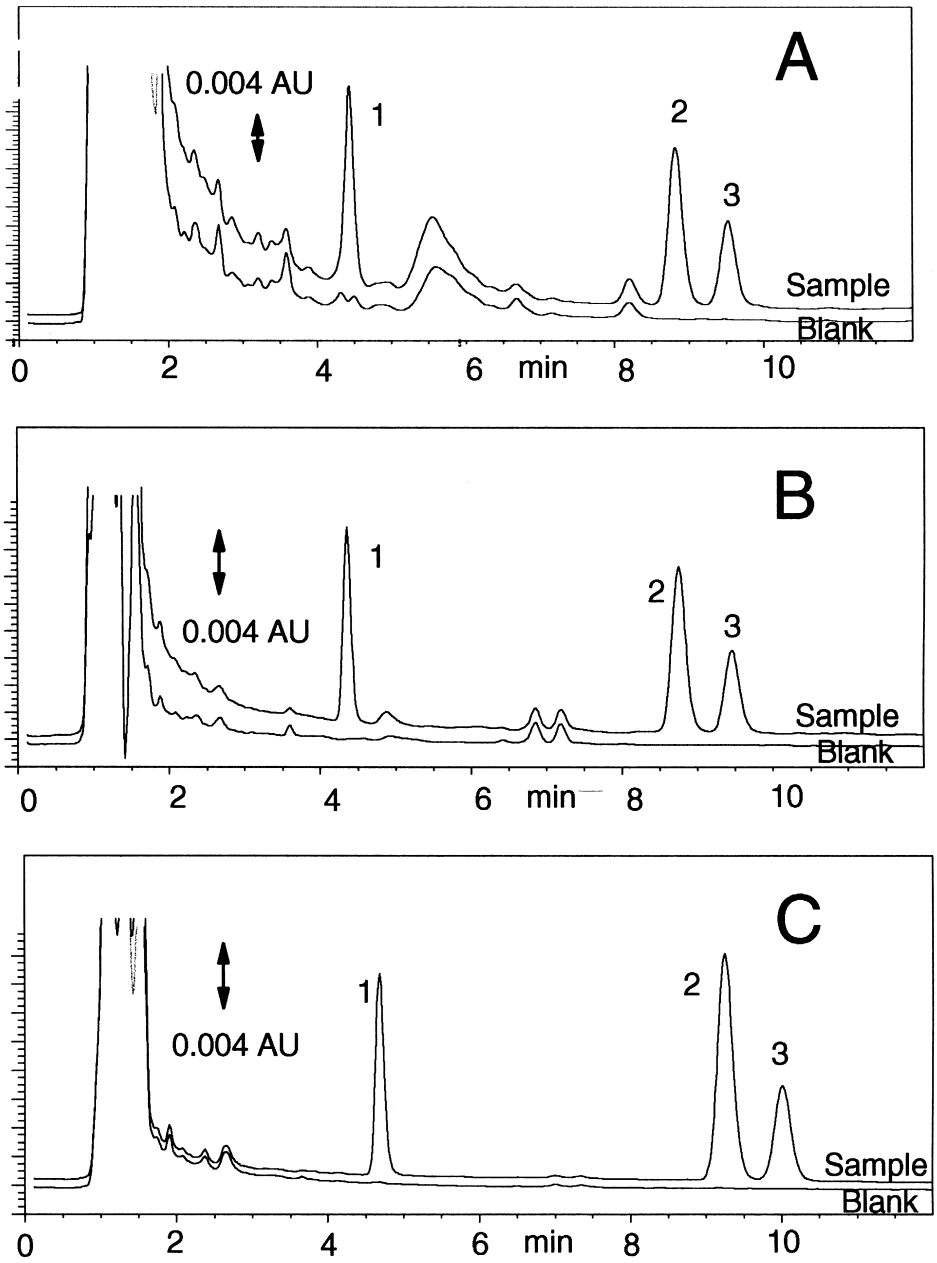

Fig. 2. Comparison of three different SPE methods for the determination of verapamil and its metabolite in a plasma matrix. (A) Representative chromatograms (sample solution and plasma blank) from the 1D-SPE method (only one washed with 1 ml of 5% methanol in water, and then eluted with 1 ml of methanol). (B) Representative chromatograms (sample solution and plasma blank) from the 2D-SPE method with two washes (first washed with 1 ml of 5% methanol, second washed with 1 ml of 65% methanol containing 2% ammonium hydroxide, and then eluted with 1 ml of 65% methanol containing 2% acetic acid). (C) Representative chromatograms (sample solution and plasma blank) from the 2D-SPE method with three washes (first washed with 1 ml of 5% methanol containing 2% acetic acid, second washed with 1 ml of 5% methanol containing 2% ammonium hydroxide, third washed with 1 ml of 65% methanol containing 2% ammonium hydroxide, and then eluted with 1 ml of 65% methanol containing 2% acetic acid. All the experiments were performed simultaneously in an Oasis™ HLB 96-well extraction plate. Peaks: (1) norverapamil (the metabolite); (2) verapamil; and (3) methoxy-verapamil (the internal standard). The 2D-SPE method with three washes provided the cleanest background.

simultaneously manipulated to selectively isolate these basic analytes from a complex sample matrix. After initial conditioning with methanol followed by equilibration with water, the sample solution is loaded onto the hydrophilic–lipophilic cartridges/wells at low pH without any organic solvent (the load step). The reason for using a low pH value is to release bound drugs from proteins present in the plasma matrix, and the purpose of using no organic solvent is to retain the analytes. The second step (the first wash step) is to wash with 5% methanol at high pH, typically containing 2% ammonium hydroxide. 5% methanol is employed to remove proteins present in the sample matrix, and the purpose of the high pH is to retain the basic analytes since they are unionized at high pH and therefore have high retention. The third step (the second wash step) is to wash with a high methanol concentration at high pH. Again, the high pH is used to increase the retention of the basic analytes onto the SPE sorbent, and the high organic concentration is used to eliminate endogenous interferences present in the sample matrix. The fourth step (the elution step) is to elute the analytes of interest at low pH simply because basic analytes are ionized and therefore retained less by the sorbent.

3.4. Wash–elute study (determination of percentage of methanol in the 2D-SPE method)

Before performing the 2D-SPE method, the organic concentration in the wash step(s) and in the elution step needs to be determined. To simplify the SPE protocol, the sample analytes are prepared in a saline solution instead of in the plasma matrix. The results of wash–elute study for verapamil and its metabolite are shown in Table 1. The wash study contains 2% of ammonium hydroxide in each elution step. No analyte is eluted until the methanol concentration reaches 70% containing 2% ammonium hydroxide. This means that the highest organic concentration, containing 2% ammonium hydroxide, that can be used is 70%. For this particular study, we chose 65% methanol containing 2% ammonium hydroxide as the final wash solvent before the elution step.

The elution study contains 2% acetic acid in each

Table 1
Wash–elute study for the verapamil and its metabolite

	Area (μV s)			
	% MeOH	Norverapamil	Verapamil	Verapamil methoxy
Wash (base)	0	0	0	0
	10	0	0	0
	20	0	0	0
	30	0	0	0
	40	0	0	0
	50	0	0	0
	60	0	0	0
	70	0	0	0
	80	47 842	34 095	63 58
	90	85 704	63 158	178 178
Elute (acid)	0	0	0	0
	10	0	0	0
	20	0	0	0
	30	0	0	0
	40	39 269	73 146	86 789
	50	68 259	121 820	137 899
	65	61 830	109 165	123 229

The wash study contains 2% ammonium hydroxide at increasing methanol concentration from 0 to 90%. The elute study contains 2% acetic acid at increasing methanol concentration from 0 to 65%. From these results, the wash solvent(s) can be either acidic, up to 30% methanol containing 2% acetic acid, or basic, up to 70% methanol containing 2% ammonium hydroxide, since no analyte is eluted out. To obtain complete elution, the elution solvent should be acidic (2% acetic acid) and the methanol concentration should be greater than 60%.

elution step, no analyte is eluted until the methanol concentration reaches 30% containing 2% acetic acid. This means that we can also use up to 30% methanol containing 2% acetic acid as the wash solvent simply because no analyte is eluted. At a methanol concentration higher than 30%, the analytes of interest start to elute out. To achieve complete elution of the analytes of interest, we chose 65% methanol containing 2% acetic acid as the elution solvent.

This wash–elute study is a useful and convenient tool to determine rapidly what percentage of methanol containing either acid or base can be used for wash solvent(s) as well as for elution solvent(s). From Table 1, it becomes clear that the wash solvent(s) can be acidic, with up to 30% methanol containing 2% acetic acid, or basic, with up to 70% methanol containing 2% ammonium hydroxide, since no analyte is eluted out. To achieve complete elution of the analytes, the elution solvent should be acidic (2% acetic acid) and also the methanol concentration should be greater than 60%. In the next section, we will compare the 1D- and 2D-SPE methods (with two and three washes) to each other.

3.5. Comparison of SPE methods

It is interesting to compare the results for verapamil in plasma using three different SPE methods: 1D method, 2D method with two washes, and 2D method with three washes. The chromatogram from the plasma blank is always compared to the chromatogram from the sample solution in these three different methods, and the results are shown in Fig. 2. Fig. 2A shows the results from the 1D-SPE method, which is the simplest method. All these three peaks are well separated, and they all have good peak shapes. The USP tailing factors were 1.18, 1.07 and 1.08 for norverapamil, peak 1, verapamil, peak 2, and methoxyverapamil, peak 3, respectively. Peak 1 coeluted with some interferences present in the plasma matrix. Peaks 2 and 3 were free from any interference from the plasma matrix. This 1D-SPE method, the generic SPE method, extracts not only the analytes of interest but also other plasma constituents that interfere with the quantitation of peak 1. To separate norverapamil from the endogenous interferences present in the plasma matrix, further optimization of the HPLC separation is needed. This might be time-consuming since all the endogenous interferences will move unpredictably, and they might co-elute with the peaks of interest. Another way to eliminate the endogenous interferences is to selectively isolate the analytes of interest from the plasma matrix, and at the same time to wash off the endogenous interferences as much as possible.

This can be accomplished easily using the 2D-SPE method which is a more selective method. Fig. 2B shows the results from the 2D-SPE method with two washes: the first wash with 5% methanol and the second wash with 65% methanol containing 2% ammonium hydroxide. Again the chromatogram from the plasma blank is compared to the chromatogram from the sample solution. All these three peaks are well separated, and they are all free from any interference from the plasma matrix. Clearly, the 2D-SPE method provides a much better background signal. The USP tailing factors were 1.14, 1.09 and 1.12 for norverapamil, verapamil, and methoxyverapamil, respectively.

Fig. 2C shows the results from the 2D-SPE method with three washes: the first wash with 5% methanol containing 2% acetic acid, the second wash with 5% methanol containing 2% ammonium hydroxide, and the third wash with 65% methanol containing 2% ammonium hydroxide. Again, all three analytes are well separated, and they are all free from any endogenous interferences from the sample matrix. Among these three different methods, the 2D-SPE method with three washes provides the cleanest extracts. The USP tailing factors were 1.10, 1.06 and 1.07 for norverapamil, verapamil, and methoxyverapamil, respectively.

The results from these three SPE methods are summarized in the Table 2. All the results reported here were taken out of the Millennium software directly, without any background subtraction. For the 2D-SPE methods, either with two or three washes, the recoveries were all greater than 90%, and the R.S.D.s were all less than 4.0%. For the 1D-SPE method, the recoveries and the R.S.D.s are good except for the norverapamil at low concentration, 0.092 μg/ml. The high recoveries and high R.S.D.s from the 1D-SPE method are due to the interferences from the plasma matrix as shown in Fig. 2A. After

Table 2
Recoveries of verapamil and its metabolite in spiked plasma using three SPE methods

Compound	Conc. (μg/ml)	1-Dimensional		2-Dimensional (two washes)		2-Dimensional (three washes)	
		Recovery (%)	R.S.D. (n=12)	Recovery (%)	R.S.D. (n=12)	Recovery (%)	R.S.D. (n=12)
Verapamil	0.68	100.4	0.95	114	1.3	97.2	0.74
	0.14	101.5	3.39	101	3.1	89.6	0.96
Norerapamil	0.46	117.6	2.56	110	1.5	92.2	1.05
	0.092	164.1	10.8	106	3.8	90.2	2.02

The results shown here were obtained by the Millennium software directly, without any background subtraction. The high recovery and poor R.S.D. for norverapamil from the 1D method were due to the interferences present in the plasma matrix (Fig. 2A). After background subtraction, norverapamil had recoveries at 106 and 107% at a concentration of 0.46 and 0.092 μg/ml, respectively.

background subtraction, the norverapamil recovery was 106% at a concentration of 0.46 μg/ml, and 107% at a concentration of 0.092 μg/ml.

All the experiments discussed above were performed in a 96-well extraction plate. This makes the SPE methods development very convenient. It should be noted that it is very difficult to make sure all the wells in a 96-well extraction plate do not run dry in a 96-well extraction vacuum manifold. Therefore, it is important to use a sorbent that is not affected by drying.

Regardless of what method is chosen for the sample preparation, very good results can be obtained from the above three different SPE methods. So, the choice of the SPE method depends upon the purpose of the experiment. The 1D-SPE method is a fast, generic method. With this simple protocol, high recoveries and consistent results were obtained for a wide range of compounds [5,8–10]. The 2D-SPE method is complementary to the 1D method, and it selectively isolates the analytes of interest from a complex sample solution. This selective method provides a much cleaner background signal, which in turn, provides higher selectivity and better sensitivity.

4. Conclusions

The HLB sorbent is unique and universal for reversed-phase SPE applications. This copolymer has no residual silanols to complicate its interaction with basic analytes, such as verapamil and its metabolite. With the 1D-SPE method, in which only the organic concentration is manipulated to isolate the analytes of interest, good recoveries were obtained for verapamil and its metabolite in a plasma matrix.

Additionally, this polymeric sorbent is stable across a wide pH range (pH 0–14). We can use the increased pH range advantageously in SPE method development to provide cleaner extracts for these basic compounds. With the 2D-SPE method, in which both pH and organic concentration are applied to isolate the analytes of interest, high and consistent recoveries were obtained for the determination of verapamil and its metabolite in plasma. The 2D method is generally more specific, and this feature can be used to improve the performance of the SPE procedure. Both 1D- and 2D-SPE methods are highly precise, easy to perform, and suitable for the determination of verapamil and its metabolite in the biological matrix.

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