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Journal of Chromatography B, 729 (1999) 19–31

JOURNAL OF CHROMATOGRAPHY B

Novel high-performance liquid chromatographic and solid-phase extraction methods for quantitating methadone and its metabolite in spiked human urine

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Received 2 December 1998; received in revised form 10 March 1999; accepted 15 March 1999

Abstract

A novel solid-phase extraction (SPE) method and HPLC method were developed for the determination of methadone and its metabolite from spiked human urine. For sample cleanup, a spiked urine sample was pretreated with phosphoric acid followed by a well-thought-out SPE method using a 10-mg Oasis HLB 96-well extraction plate. In this SPE method, the concentration of methanol as well as the pH are optimized to preferentially isolate the analytes of interest from the sample matrix. Low elution volumes (200 μl) are achieved; this eliminates evaporation and reconstitution of the sample solution. Recoveries from human urine matrix were greater than 91% with RSD values less than 4.5%. For the HPLC analysis, the separation was obtained using a SymmetryShield RP_{18} column with a mobile phase of 0.1% TFA–methanol (60:40, v/v). Good peak shapes were obtained without the need of addition of any competing reagent to the mobile phase. Additionally, significant signal-to-noise enrichment was achieved by diluting the final SPE eluates four-fold with water.

Keywords: Methadone

1. Introduction

Methadone is a synthetic central-acting analgesic agent with high affinity for μ-opioid receptors. It has been used widely in the treatment of severe pain (such as cancer pain), and in maintenance treatment of opioid addicts [1–3]. Due to the differences in the pharmacokinetics of methadone among different individuals, it is necessary that doses are individualized to achieve optimum treatment. Because of this, many analytical methods have been applied to the quantitation of methadone and its metabolite. These include gas chromatography [4,5], liquid chromatography [6–11], capillary electrophoresis [12], and radioimmunoassay [13]. For HPLC assays, the addition of competing reagents (such as diethylamine, triethylamine, dimethyloctylamine) was necessary in order to obtain good peak shapes for these basic analytes [6,8,9]. For sample preparation prior to the quantitation of methadone and its metabolite in biological fluids or tissues (e.g. urine, serum, plasma, bile, and brain), either liquid–liquid extraction [8,10–12] or solid-phase extraction (SPE) [6,14] has been used. As pointed out by Pierce et al. [6], liquid–liquid extraction methods are time-con-

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00116-4

suming and tedious; recoveries ranging from 90% to as low as 63.5% for methadone, and 42% for metabolites were reported in plasma matrix [15]. The SPE method described by Pierce et al. [6] provided a higher recovery and cleaner extracts than conventional liquid–liquid extraction.

The aim of this study is to report a simple and straightforward SPE method as well as a simplified HPLC method for the determination of methadone and its main metabolite from urine matrices. The SPE method utilizes both the pH and the concentration of the organic modifier to selectively isolate the analytes of interest. No evaporation and reconstitution is needed for the sample clean-up. The final eluate is diluted with water and then quantitated with a simple HPLC method. The HPLC method utilizes a SymmetryShield RP_{18} reversed-phase column and a simple mobile phase without the need of additives for improving peak shape. The effect of the SPE elution solvent and the mobile phase on the HPLC peak will be demonstrated. Strategies for increasing signal-to-noise ratios and simultaneously improving the peak shapes will be discussed as well.

2. Experimental

2.1. Reagents and materials

Methadone hydrochloride, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine perchlorate salt (EDDP), and diphenhydramine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Methanol, trifluoroacetic acid (TFA), phosphoric acid, acetic acid, and ammonium hydroxide were HPLC-grade quality from J.T. Baker (Phillipsburg, NJ, USA). Human urine were obtained from laboratory personnel including two Americans (male and female) and one Asian (male). An Oasis HLB 96-well extraction plate (10 mg/well) and a 96-well extraction vacuum manifold were procured from Waters (Milford, MA, USA). For wash–elute study experiment (Section 2.3), methadone, EDDP, and diphenhydramine were prepared in a pH 7 phosphate-buffered saline (PBS) solution at final concentrations of 30, 20, and 10 μg/ml, respectively. This PBS solution was prepared according to the following: 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. HPLC calibration curves were generated over a concentration range from 0.5 to 10.0 μg/ml for methadone, and from 0.2 to 4.0 μg/ml for EDDP. Each standard solution contained 1.0 μg/ml diphenhydramine as the internal standard. These standard solutions were prepared in 20% methanol containing 0.5% acetic acid.

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 210 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_{18} column or Symmetry C_{18} column (150×3.9 mm, 5 μm particle size) preceded by a Sentry guard column (20×3.9 mm, 5 μm particle size), packed with SymmetryShield RP_{18} or Symmetry C_{18}. The elution was carried out at 30°C. The mobile phase consisted of 0.1% TFA–methanol (60:40, v/v). The flow-rate was set at 1.0 ml/min. For the concentration determination of the analytes, 100 μl each of the sample and the standard solution were injected.

For signal-to-noise enrichment experiments, the sample was diluted with water in ratios of 1:1 and 1:3. Various volumes (25, 50 and 100 μl) of sample were introduced to the column. This sample solution, prior to dilution, consisted of 4 μg/ml of EDDP, 5 μg/ml of diphenhydramine, 10 μg/ml of methadone in 80% methanol containing 2% acetic acid.

2.3. Wash–elute study

A preliminary study which we called the wash–elute study was used to determine the volume as well as the percentage of the methanol concentration needed in the wash step(s) and in the elution step for the subsequent SPE procedure. Eleven 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 of sample solution, prepared in PBS, was loaded onto each well. The analytes were then 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, 90 and 100%). Elutions were performed with 150, 300, 600, 800 and 1000 μl volumes for the wash study. For the elution study, 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, 60, 70, 80, 90 and 100%). Elutions were performed with 150- and 300-μl volumes. All wash and elution eluates were collected separately and brought to a final volume of 1 ml with water for washes and 500 μl for elutes. These final solutions were then analyzed under the HPLC conditions described above.

2.4. Solid-phase extraction procedure

Aliquots of human urine were spiked with drug solutions to produce the desired concentrations. Two levels of sample concentrations were prepared. At the high level, the concentrations for methadone and EDDP were 2.65 and 1.06 μg/ml, respectively. At the low level, the respective concentrations were 0.53 and 0.21 μg/ml. The choice of the concentration was based upon the methadone critical value (the cut-off concentration) in human urine, 0.3 μg/ml, listed in the Laboratory Test Handbook. Each sample solution contained 0.53 μg/ml of diphenhydramine as the internal standard. These spiked urine samples were then acidified with concentrated phosphoric acid to bring the final phosphoric acid concentration to 2%. Then the acidified sample solutions were loaded onto extraction plate wells, which had been conditioned with 1 ml of methanol, followed by 1 ml of water. After loading 1.5 ml of acidified urine sample solution onto each well, it was washed with 300 μl of 5% methanol containing 2% ammonium hydroxide, followed by 300 μl of 50% methanol containing 2% ammonium hydroxide. Finally, the analytes were eluted with 200 μl of 80% methanol containing 2% acetic acid. No evaporation and reconstitution was performed. The final eluates were diluted with water in a ratio of 1:3 (adding 600 μl of water to 200 μl of final eluate). The final diluted sample solutions were then analyzed by HPLC as described above.

3. Results and discussion

3.1. Chromatographic analysis

3.1.1. Development of the HPLC separation using a simple mobile phase

As shown in Fig. 1, methadone and its metabolite (EDDP) are basic compounds containing a tertiary amine group. The internal standard, diphenhydramine, also contains a tertiary amine. These basic analytes interact with the residual silanol sites present on silica-based reversed-phase sorbents, which causes peak tailing in the HPLC separation. To overcome this problem, it has been shown that the addition of a competing reagent is necessary in order to achieve satisfactory peak shapes for these basic analytes [6,8,9]. In this study, we were able to obtain good peak shapes with a simple mobile phase [0.1% TFA–methanol (60:40, v/v)] without the addition of any competing reagent. Good peak shapes were obtained from both Symmetry C_{18} and SymmetryShield RP_{18} columns (Fig. 2), and USP tailing factors were all between 1.10 and 1.20.

A significant reduction in retention is observed for these basic analytes on the SymmetryShield RP_{18} column (Fig. 2B), as compared to the standard

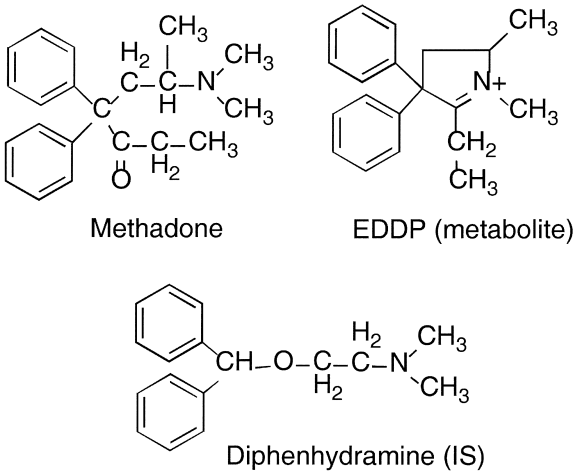

Fig. 1. Structures of methadone and its metabolite.

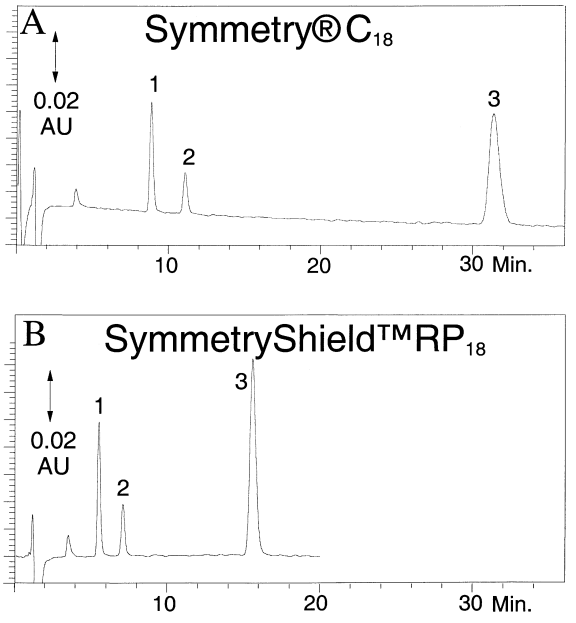

Fig. 2. HPLC separation of methadone and its metabolite using both Symmetry C_{18} and SymmetryShield RP_{18} columns. Peaks: 1=EDDP, the metabolite; 2=diphenhydramine, the internal standard; 3=methadone. A significant reduction in retention time is observed for these basic analytes on the SymmetryShield RP_{18} column (B) as compared to the standard Symmetry C_{18} column (A).

Symmetry C_{18} column (Fig. 2A). This reduction in retention is due to a decrease in the interactions of these basic analytes with the SymmetryShield RP_{18} column compared to the Symmetry C_{18} column. The SymmetryShield RP_{18} column contains an embedded polar functional group (carbamate group). It is believed that the reason for the reduction of the analyte retention is due to the fact that water strongly binds to the polar carbamate group [16]. This tightly bound layer of water prevents interactions between the basic analytes and the residual silanol groups present on the silica surface. Consequently, surface silanols do not play as much of a role in the retention of these basic analytes on the SymmetryShield RP_{18} column as they do in a standard C_{18} column.

HPLC calibration curves were based on peak-area ratio to the internal standard, diphenhydramine. Within the concentration range described in Section 2, linear plots were obtained for methadone as well as the metabolite, EDDP. The correlation coefficients, from ten injections of standard solutions, were 0.999612 and 0.999509 for methadone and EDDP, respectively.

3.1.2. Influence of SPE elution solvent and mobile phase on the peak shape of chromatographic elution profiles

A typical SPE sample preparation consists of six steps. The first step is to prepare the sample solution. The second step is to condition and equilibrate the SPE sorbent either in cartridges or in 96-well extraction plates. The third step is to load the sample solution. The fourth step is to wash off interferences present in the sample matrix. The fifth step is to elute the analytes of interest. The sixth and final step is to evaporate and reconstitute the extract prior to chromatographic analysis. Cheng et al. [17] discussed the advantages of performing the evaporation and reconstitution step. There are two distinct advantages: firstly, it can increase the assay sensitivity since the final SPE eluates can be reconstituted to a small volume, and secondly, it can improve the HPLC peak shapes since the final eluate can be reconstituted in the HPLC mobile phase. However, this evaporation and reconstitution step is time-consuming and tedious. Additionally, volatile analytes might be lost during the evaporation step; some basic analytes might be lost due to adsorption to glass vials. Therefore, it would be desirable to have a simplified SPE protocol in which no evaporation and reconstitution step is needed. To achieve this, we employed a low elution volume (200 μl) using a 10 mg/well plate packed Oasis HLB sorbent. The elution solvent used was 80% methanol containing 2% acetic acid (details discussed in Section 3.2.2). The final eluate was then injected, 30 μl, onto the HPLC column without evaporation and reconstitution.

Not unexpectedly, we observed distorted peak shapes (Fig. 3A) due to the mismatch of the sample solvent (in 80% methanol, 2% acetic acid) and the HPLC mobile phase [0.1% TFA–methanol (60:40, v/v)]. Castells et al. [18] have reported that the injection of samples dissolved in solvents different from the mobile phase can result in severe peak distortions because of the difference in viscosity between the solvents. The extraction of the analytes of interest from the SPE sorbent requires the elution

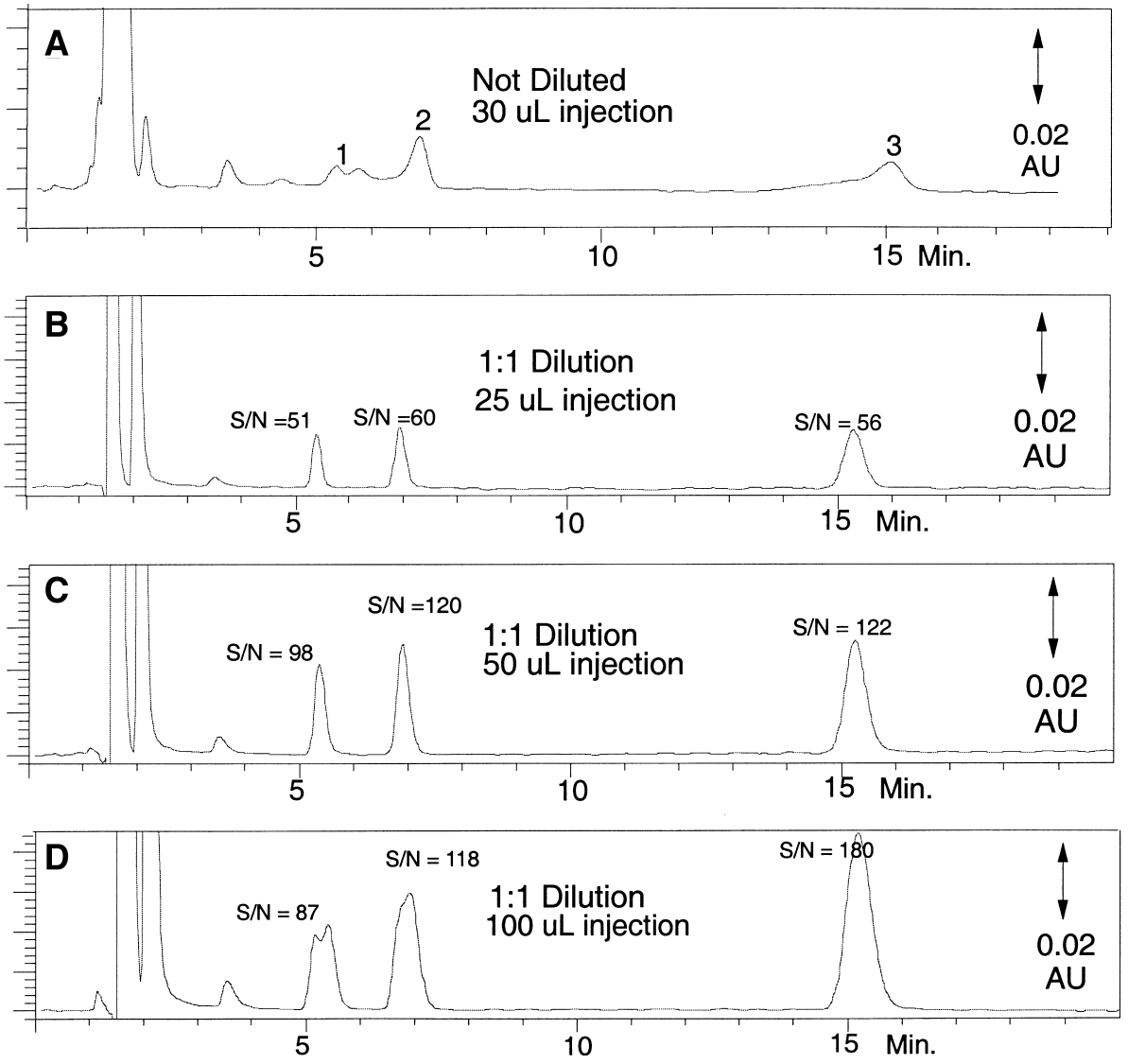

Fig. 3. Comparison of the signal-to-noise ratio for a dilution of the sample solution (80% methanol, 2% acetic acid) 1:1 with water, resulting in a 40% methanol–1% acetic acid solution. The column used was a SymmetryShield RP_{18} column. The elution order is identical to Fig. 2B. (A) Injection (30 μl) of the original sample solution, in 80% methanol, 2% acetic acid; (B–D) injection of the diluted sample solution (1:1 with water) at 25, 50 and 100 μl.

with a high concentration of organic solvent (e.g. 100% methanol). Typically, the elution solvent is a stronger eluting solvent than the HPLC mobile phase, so only a small amount of sample solution can be injected until peak distortions are observed. In one of our experiments, a 15-μl (instead of 30-μl) volume of the sample solution (in 80% methanol, 2% acetic acid) was injected onto the HPLC column; better peak shapes were observed. However, this approach also decreases the sensitivity.

Another common approach used in SPE is to evaporate the final eluate to dryness and then re-dissolve the sample in a mobile phase. This approach is time-consuming and tedious. Here, we are using a

faster and more efficient method: the dilution of the sample with water.

The principal behind the signal-to-noise enrichment by dilution of the sample is rather simple. Due to the fact that after dilution with water the sample is dissolved in a solvent more polar than the mobile phase, the sample is concentrated on the top of the column and peak distortion is avoided. In addition, the addition of an aqueous solution to the sample can also be used to adjust the pH of the sample and to make it compatible with the mobile phase.

In the following, we will briefly outline the principle of the dilution procedure by calculating the enrichment factor that is achieved by sample dilution. In general, the logarithm of the retention factor k can be approximated to be a linear function of the concentration of organic solvent in the mobile phase c with a slope to be m:

$$\ln k = \ln k_0 - mc$$

We can also define the enrichment factor e_f as being the ratio of the retention factor in the sample solvent k_{SS} to the retention factor in the mobile phase k_M:

$$e_f = \frac{k_{SS}}{k_M}$$

The retention factor in the mobile phase k_M is:

$$k_M = k_0 \, e^{-mc_M}$$

And the retention factor in the sample solvent k_{SS} is:

$$k_{SS} = k_0 \, e^{-mc_{SS}}$$

Therefore, the enrichment factor is simply:

$$e_f = e^{m(c_M - c_{SS})}$$

Consequently, the enrichment simply depends on the difference in the concentration of the organic modifier between the sample solvent and the HPLC mobile phase.

While the factor m is usually not known for our analytes, we can assume some typical values and analyze the relationship in the above equation (the enrichment factor equation). A typical value of m is around 10 for small molecules, and around 20 for peptides [19,20]. The case for methadone or other common pharmaceuticals can be treated by assuming a value of 10. The enrichment for this typical case of small molecules (shown in Fig. 4) is a function of the difference between the composition of the sample solvent and the mobile phase (Δc). One can use this graph as a guidance in the enrichment procedures. For instance, if there is no difference in composition between the sample solvent and the mobile phase ($\Delta c = 0\%$), the enrichment is equal to 1. This means that there is no enrichment when the sample solvent is the same as the mobile phase composition. If a composition difference between the sample solvent and the mobile phase is at 20% ($\Delta c = 20\%$), one can expect to achieve a factor of 7.4 enrichment.

Therefore, to maximize sensitivity and also to improve the peak shapes without performing the evaporation and reconstitution step after the SPE step, we simply diluted the final SPE eluate with water and injected a larger sample volume onto the HPLC column. We diluted the sample solution, in a 80% methanol–2% acetic acid solution, 1:1 with water, which results in a 40% methanol–1% acetic acid solution. This results in a solvent composition of the sample which matches the mobile phase composition [0.1% TFA–methanol (60:40, v/v)]. At this dilution, we were able to improve peak shapes as well as signal-to-noise ratio (S/N) for the peaks at 25- and 50-μl injection volumes (Fig. 3B and C). At 100-μl injection volume (Fig. 3D), the S/N increases further for the last eluting peak (methadone, peak 3), but for the earlier eluting peaks, peaks 1 and 2 (EDDP and diphenhydramine), peak distortion is observed, especially for the earliest eluting peak. The S/N ratio for the first peak decreases from 98 to 87, while the second peak maintains approximately the same S/N ratio as for 50-μl injection volume.

Fig. 5 shows the results of a further dilution of the sample solution at 1:3 with water. At this dilution, the concentration of the organic solvent is reduced to 20% methanol–0.5% acetic acid solution, which is more polar than the HPLC mobile phase [0.1% TFA–methanol (60:40, v/v)]. Under these conditions, the sample is enriched and concentrated on the top of the column, without distortion of peaks even at a very large injection volume. At this dilution (1:3), we were able to obtain good peak shapes and an improved S/N ratio at injection volumes of 25, 50 and 100 μl (Fig. 5A, B and C, respectively). It is noteworthy that the S/N ratio increases at the higher dilution factor at the same mass injected onto the

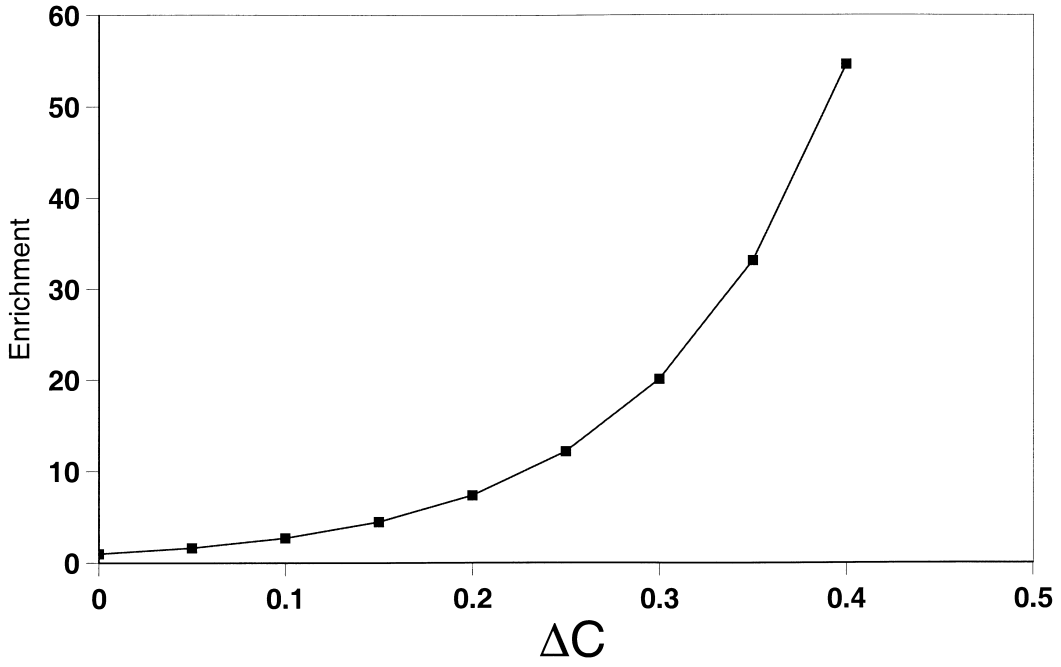

Fig. 4. Typical enrichment for small molecular analytes. The enrichment is a function of the difference between the composition of the sample solvent and the HPLC mobile phase (Δc).

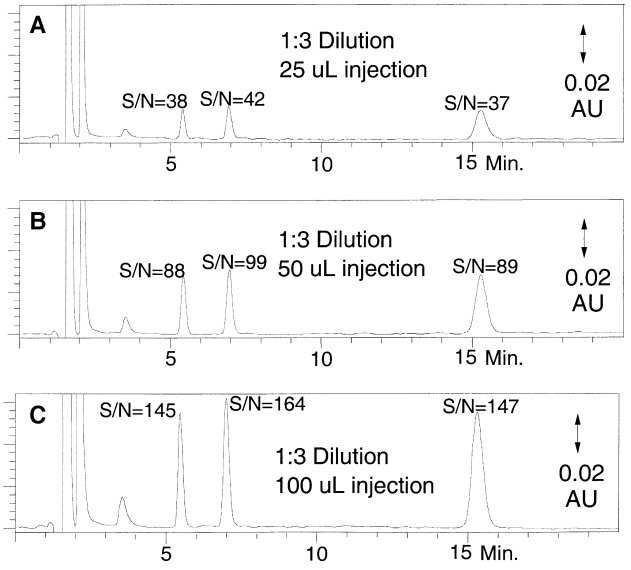

Fig. 5. Comparison of the S/N ratio for a dilution of the sample solution (80% methanol, 2% acetic acid) 1:3 with water, resulting in a 20% methanol–0.5% acetic acid solution. The column used and the elution order are identical to Fig. 3. With this dilution (1:3), good peak shapes and improved S/N ratio at 25-, 50- and 100-μl injection volumes (A, B and C, respectively) were obtained.

HPLC column (Figs. 3B and 5B as well as Figs. 3C and 5C). The S/N enhancement results from the interplay between the dilution of the sample solution by water and the enrichment of the sample on the top of the HPLC packing material due to the fact that the sample solvent is more polar than the mobile phase.

It is also interesting to note that the difference in the concentration of the organic modifier between the sample solvent and the mobile phase (Δc) is 20% (chromatograms shown in Fig. 5). According to the prediction of the enrichment (Fig. 4), one should achieve a factor of 7.4 enrichment. For this particular experiment, we achieved a factor of three for the enrichment (compare Figs. 3B and 5C). No peak distortion was observed at the 100 μl injection at the 1:3 dilution (Fig. 5C); this indicates that a even larger volume (at 1:3 dilution) could be injected onto the column, and one should achieve further enrichment. Based upon the results obtained from the Fig. 5C, the detection limits at three times of background noise were 8.3, 9.1 and 20 ng/ml for EDDP, diphenhydramine, and methadone, respectively.

3.2. SPE method

3.2.1. Sample pretreatment

The suitability of sample pretreatment for the spiked urine was investigated in this study. We added either phosphoric acid or ammonium hydroxide into the spiked urine sample solution containing 3 μg/ml of methadone, 1.2 μg/ml of EDDP, and 1.5 μg/ml of diphenhydramine. The results with and without adding acid or alkaline prior to loading the sample onto a 10 mg/well Oasis HLB 96-well extraction plate are listed in Table 1. For three replicate analyses, recoveries ranging between 70 and 85% were observed when the sample solution was not pretreated. Recoveries >90% were observed when the sample solution was acidified; poor recoveries (<40%) were observed at alkaline pH. In fact, methadone and EDDP are basic analytes. One would expect better recovery of these basic analytes under alkaline than under acidic conditions. Presumably, under acidic conditions, these basic and hydrophobic analytes are less likely to bind to the walls of sample vials or transfer tubes. Due to the fact that the acidification provides better recovery, the following studies were performed using sample solution acidified with phosphoric acid (final solution containing 2% phosphoric acid).

3.2.2. Determination of volume and percentage of methanol in the wash and elution steps

Two variables which affect the retention of analytes in reversed-phase HPLC and SPE are the organic solvent concentration and the pH. The retention of the analytes decreases with an increase in the organic concentration. When the pH is varied, the analyte retention depends on the nature of the compounds. Basic analytes are not ionized, and they exhibit high retention on a reversed-phase retention sorbent. This is the case for methadone and its metabolite. The Oasis HLB sorbent is a hydrophobic divinylbenzene-based polymer, which provides excellent pH stability across the entire pH range (0 to 14) [21–23]. This broader pH range allows the flexibility of SPE methods development.

Methadone and its metabolite are basic analytes (Fig. 1). Their retention on the sorbent should be significantly different in an acidic or basic environment. These basic analytes will be more retained at a high pH value and less retained at a lower pH. The

Table 1
Urine sample pretreatment: final spiked urine sample solution containing either 2% phosphoric acid or 2% ammonium hydroxide[a]

Compound	No treatment	Acidified (2% H_3PO_4)	Alkaline (2% NH_4OH)
Methadone (1.0 μg/ml)	72.3	91.9	25.7
EDDP (0.4 μg/ml)	75.4	93.0	31.8
Diphenhydramine (I.S.) (0.5 μg/ml)	83.0	95.1	35.3

[a] Results were obtained from three replicate analyses.

judicious selection of an appropriate pH and methanol concentration for the wash and elution steps should enable us to obtain significantly cleaner extracts. A wash step using aqueous methanol at high pH would remove acidic and neutral interferences while ensuring that the basic analytes remain adsorbed. Elution with acid at the same methanol concentration should then extract only the basic analytes. Additionally, to conserve the solvent consumption and to eliminate the evaporation and reconstitution step, we would like to use a smaller volume in both wash and elution steps.

To simplify the protocol for the determination of the volume, pH and percentage of methanol in the wash and elution steps, the analytes were prepared in a saline solution instead of in a urine matrix. The profile for the wash–elute study exhibited similar results for methadone, EDDP (metabolite), and diphenhydramine (internal standard). The representative wash–elute profiles for methadone are shown in Figs. 6 and 7. Fig. 6 shows the results for the wash study, which contains 2% ammonium hydroxide at five different wash volumes (150, 300, 600, 800 and 1000 μl). The effect of methanol concentration and wash volume under this basic condition was determined. More methadone is washed off as the wash volume increases. Also, at 50% methanol concentration, the analyte starts to manifest for all wash volumes. At 150 and 300 μl wash volumes, a negligible amount of methadone, at 0.21% for 150 μl and 0.25% for 300 μl, is washed off. Therefore, we chose 300 μl of 50% methanol containing 2% ammonium hydroxide as the final wash solvent before the elution step. The purpose of this wash study is to determine the highest concentration of organic which can be used without washing off the analytes of interest. The results obtained in Fig. 6 fulfilled this purpose. The possible reason that some recoveries were higher than 100%, especially at higher concentration of organic washes, was that the HPLC peaks were significantly broadened at the higher organic contents; therefore, the quantitation of these peaks was higher than the actual area.

Fig. 7 shows the results of methadone elution study which contains 2% acetic acid at increasing methanol concentration (0 to 100%). The effect of methanol concentration and elution volume under this acidic condition was determined. Fig. 7A shows seven consecutive elutions of 150 μl elution volume at different percentages of methanol. In order to

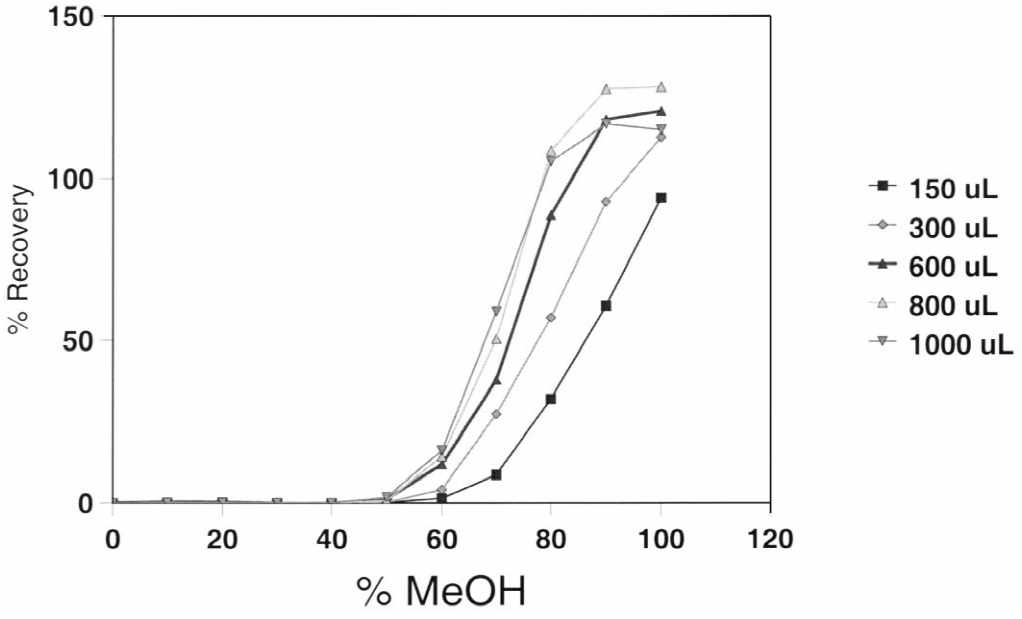

Fig. 6. Multi-wash study for the determination of the volume as well as the percentage of methanol containing 2% ammonium hydroxide for the wash protocol. This figure shows the results from five different wash volumes.

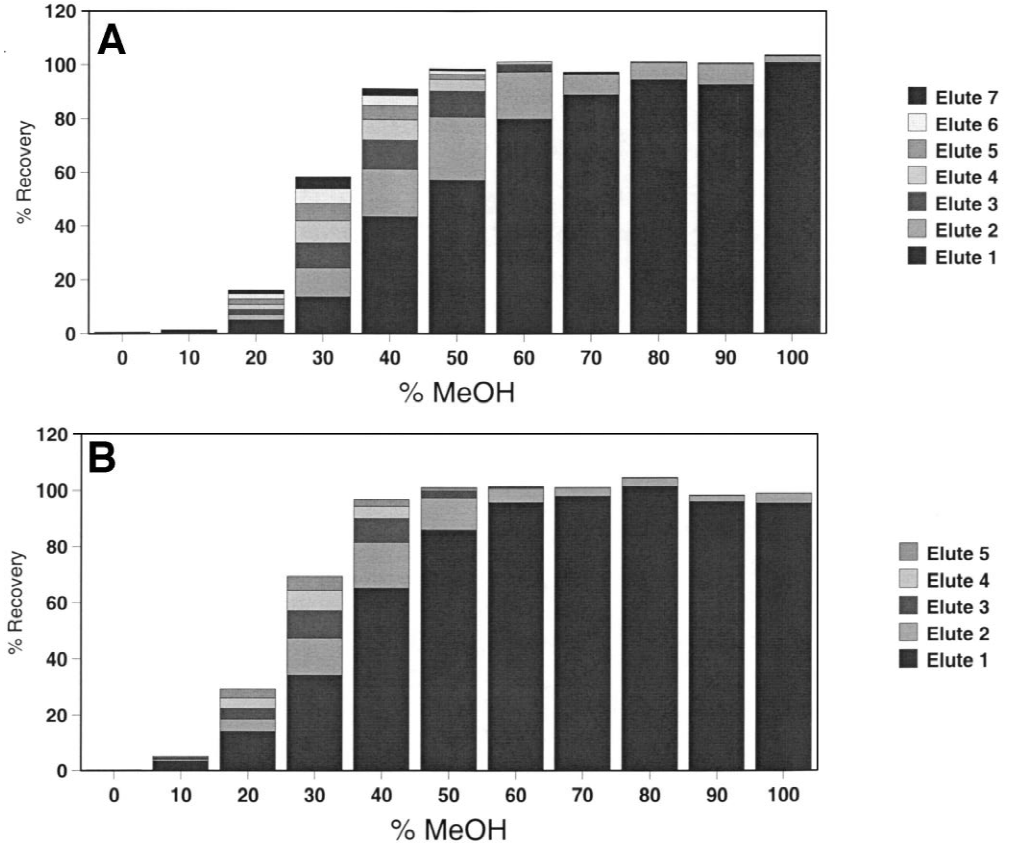

Fig. 7. Study of the determination of the volume as well as the percentage of methanol containing 2% acetic acid for the elution protocol. (A) Seven consecutive elutions of 150 μl at different percentages of methanol; (B) five consecutive elutions of 300 μl at different percentages of methanol.

obtain 100% recovery of methadone for seven consecutive elutions, >50% of methanol is needed. For the first elution (elute 1), methadone is eluted as the percentage of methanol increases; >90% of methadone comes out at a methanol concentration ⩾80%. A similar elution pattern was observed when the elution volume was increased from 150 to 300 μl. Fig. 7B shows five consecutive elutions of 300 μl elution volume at different percentages of methanol. With 300 μl elution volume, only five consecutive elutions of 40% methanol are needed to recover 100% of methadone. For the first elution (elute 1), methadone is eluted out as the percentage of methanol increases; >95% of methadone comes out at a methanol concentration ⩾60%. Based upon these results, we chose 80% methanol containing 2% acetic acid at 200 μl as the elution solvent.

Therefore the complete SPE steps are as follows: load 1.5 ml of acidified urine; first wash with 300 μl of 5% methanol containing 2% ammonium hydroxide; second wash with 300 μl of 50% methanol containing 2% ammonium hydroxide; elute with 200 μl of 80% methanol containing 2% acetic acid; add 600 μl of water to the above eluate (dilute the eluate with water at 1:3). With this protocol, we were able to obtain excellent results for the recoveries of methadone and its metabolite in three different urine matrices. This wash–elute study is a general, convenient and useful tool to determine the volume and percentage of methanol containing acid or base which can be used for the wash and elution protocols for SPE. With this SPE strategy, clean background extracts as well as high and consistent recoveries of analytes are obtained. More applications will be

shown using this strategy in upcoming publications. Additionally, the wash and elute studies will be simplified: only using 300 μl wash solvent and 200 μl elution solvent.

3.2.3. Recovery of methadone and its metabolite

To demonstrate the application of the SPE method developed, three different urine matrices spiked with methadone and its metabolite were investigated. The results, from different genders and races of human urine matrix, for methadone and its metabolite are summarized in Table 2. For the male 1 urine, the recoveries were all >94% with RSD values <4.4%. For the male 2 urine, the recoveries were all >92% with RSD values all <4.4%. Lastly, for the female 1 urine, the recoveries were all >91.9% with RSD values all <3.7%. Overall recoveries were >93% with RSD values <4.0%. The number of replicates at the high level was 12 for each (36 total) and at the low level was 10 for each (30 total). The absolute recovery of the internal standard, diphenhydramine, was determined by comparing the average peak area from replicate analyses to the average peak area of ten injections of standard. The mean recoveries of the internal standard, from all urine matrices, were 93.1% with 3.9% RSD. The ten standard injections had an area RSD of 4.9%. All the experiments discussed above were performed using a 10 mg/well Oasis HLB 96-well extraction plate. It is normally close to impossible to make sure all the wells in a 96-well extraction plate do not run dry under vacuum with classical reversed-phase sorbents. Low and irreproducible results are often obtained if the sorbent is accidentally run dry before loading the sample solution. Therefore, when many samples are processed in parallel, it is important to use a sorbent (such as Oasis) that maintains its binding capacity even if drying occurs.

Representative chromatograms of urine blanks and spiked urine samples from these three different urine matrices are shown in Fig. 8. The representative chromatograms from an American male, an American female, and an Asian male are depicted in Fig. 8A, B and C, respectively. The respective urine blank is always compared to the chromatogram from the same spiked urine sample solution. All three peaks are well separated, and no interfering peaks were observed. The elution sequence is EDDP (peak 1, metabolite, spiked concentration at 1.6 μg/ml), diphenhydramine (peak 2, internal standard, spiked concentration at 0.53 μg/ml), and methadone (peak 3, spiked concentration at 2.65 μg/ml).

4. Conclusions

We have developed novel SPE and HPLC methods for the determination of methadone and its metabolite in human urine. With the SPE method, high and consistent recoveries were obtained using a 10 mg/

Table 2
Intra-day recoveries of methadone and its metabolite in spiked urine sample matrices; the number of replicates at the high level is 12 for each (36 total) and at the low level is 10 for each (30 total)

Compound	Conc. (μg/ml)	Male urine 1		Male urine 2		Female urine 1		Mean values	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Methadone	2.65 (H)	102.9	1.6	103.8	2.1	106.3	1.6	104.3	2.2
	0.53 (L)	106.8	4.4	108.4	3.3	103.8	3.7	106.1	4.0
EDDP	1.06 (H)	105.5	1.1	105.4	2.0	106.4	1.4	105.7	1.6
	0.21 (L)	103.6	1.7	98.5	4.4	97.5	3.3	99.8	3.2
Diphenhydramine (I.S.)	0.53	94.7	4.4	92.8	3.1	91.9	3.6	93.1	3.9

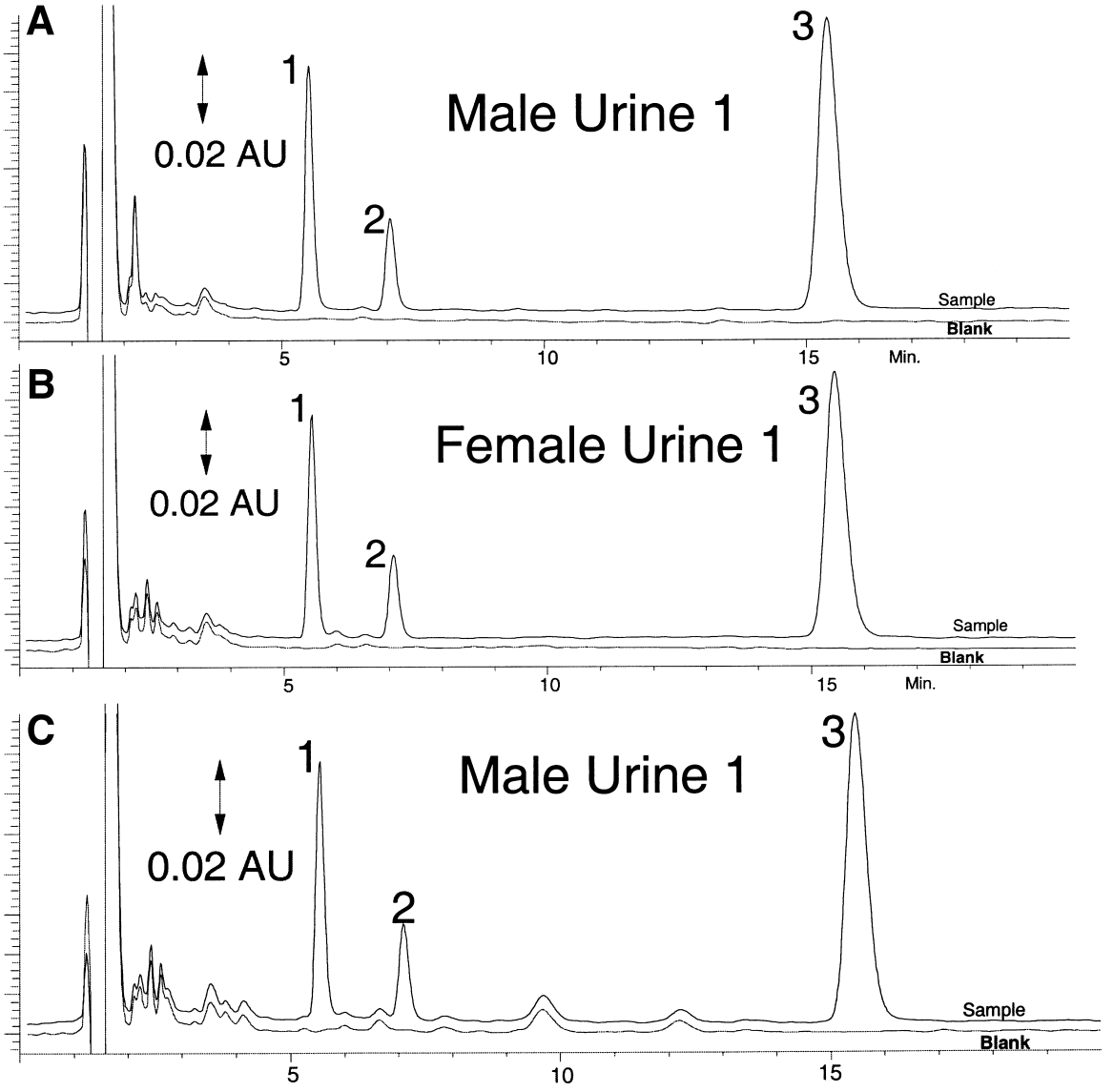

Fig. 8. Representative chromatograms of urine blanks and spiked urine samples from three different urine matrices. The representative chromatograms from male 1, female 1 and male 2 are depicted in A, B and C, respectively. The respective urine blank is always compared to the chromatogram from the spiked urine sample solution. Chromatographic conditions and elution order are identical to Fig. 5C.

well Oasis HLB sorbent. The SPE method utilizes a low elution volume. pH and methanol concentration were optimized simultaneously to selectively isolate the analytes of interest from urine matrices. No evaporation and reconstitution step was needed for the sample clean-up. No adverse impact of sorbent drying on the performance of the SPE was observed. For the HPLC separation, good separation and good peak shapes were obtained with a simple mobile phase (without the addition of silanol suppressing agents) using a SymmetryShield RP_{18} column. Signal-to-noise enrichment was achieved by diluting the final SPE extracts with water, and injecting large volumes that were enriched at the top of the HPLC column. This procedure comprises an improvement on older methods.

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