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Short communication

Solid-phase extraction of methadone enantiomers and benzodiazepines in biological fluids by two polymeric cartridges for liquid chromatographic analysis

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Abstract

The aim of this work was to present the advantages of two polymeric cartridges (Oasis HLB from Waters and Abselut Nexus from Varian) for the solid-phase extraction of methadone enantiomers and its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and of some benzodiazepines (diazepam, flunitrazepam, nitrazepam, oxazepam) in serum and urine in comparison with classical C18-bonded-silica cartridges or liquid extraction. After addition of serum or urine samples, these two cartridges were washed with a water–methanol mixture (95:5, v/v) and eluted with diethylether. After rapid evaporation, the residue was regenerated with mobile phase and injected either in a chiral column (Cyclobond I-2000 RSP) for methadone enantiomers and its metabolite or in a reversed-phase column (Symmetry Shield RP8) for benzodiazepines. The results showed that the chromatograms of blank serum and urine were cleaner than those obtained from classical solid-phase extraction or liquid extraction. The recoveries from these two polymeric cartridges were higher (95–102%) than those obtained by the two previous classical methods and the total time for extraction and solvent evaporation was also shorter (about 6–7 min). For methadone and benzodiazepine extraction, the use of acidic or alkaline buffer was not necessary.

Keywords: Polymeric extraction cartridges; Methadone; EDDP; Diazepam; Flunitrazepam; Nitrazepam; Oxazepam; Enantiomer separation

1. Introduction

The extraction of drugs and toxics in biological matrices for physical chemical analysis (HPLC, GC, MS), a key step of bioanalysis, is used to clean the samples by removing proteins and other interfering biological compounds before analysis. However, classical extraction techniques, such as liquid extraction and solid-phase extraction using reversed-phase silica sorbents have many problems when there are the differences in chemical nature (polarity, affinity, pH, etc.,) between extracted compounds and extraction solvents or solid-phase extraction sorbents. The classical commonly used sorbents are porous silica particles surface-bonded with C18 or other hydrophobic alkyl groups. The limitations of today's sorbents require the analyst to watch carefully and control closely the extraction procedure. Therefore, it is difficult and time-consuming to achieve high, reproducible recoveries for analysis of numerous drugs, especially a mixture of apolar compounds and polar ones, such as drugs and their polar metabolites. Recently, some polymeric extraction cartridges are commercialized and can be used at pH from 1 to 14

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and with many different polar and apolar organic solvents (methanol, chloroform, diethylether, etc.) contrary to classical reversed-phase silica extraction columns. Therefore, it is easier to find an appropriate extraction condition for a specific compound and especially for a mixture of analytes with different chemical properties, such as polarity, pH, affinity, etc. [1–4].

The aim of this work was to present the advantages of two polymeric cartridges (Oasis HLB from Waters and Abselut Nexus from Varian) for the solid-phase extraction of methadone and its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and of some benzodiazepines (diazepam, flunitrazepam, nitrazepam, oxazepam) in serum and urine in comparison with liquid extraction for methadone or with C18-bonded-silica cartridge for benzodiazepines. These extracted compounds will be analyzed either by chiral HPLC for methadone enantiomers and its metabolite or by reversed-phase mode for benzodiazepines. These methods were applied to the determination of methadone enantiomers and its metabolite in some treated patient serum and also of oxazepam in some treated rabbit serum.

2. Materials and methods

2.1. Chemicals and reagents

All drugs, chemicals and solvents were of analytical purity and purchased from Sigma (St. Louis, MO, USA). Deionized water was purified by Milli Q.UV Plus system (Millipore, Milford, MA, USA).

2.2. Materials

The liquid chromatographic system consisted of a Waters pump Model 600E (Milford, MA, USA), a Waters UV–vis photodiode array detector Model 994, a Waters printer plotter Model 5200, a Shimadzu integrator Model CR-6A (Kyoto, Japan) and a Rheodyne injector Model 7125, fitted with a 50 μl loop. A column chiller system Model 7955 with a temperature range between 0 and 50 °C ± 0.2 °C was purchased from Jones Chromatography Inc. (Lakewood, Colorado, USA). An extraction vacuum apparatus for solid-phase extraction was purchased from Supelco (Bellefonte, PA, USA).

Two kinds of polymeric sorbents were used: 1 ml Oasis HLB extraction cartridge [poly(divinylbenzene-co-*N*-vinylpyrrolidone)] from Waters (Milford, MA, USA) and 1 ml Abselut Nexus extraction cartridge (chemical structure unknown) from Varian (Harbor, CA, USA), both for the extraction of these previous drugs in spiked serum and urine.

Two kinds of HPLC analytical columns (250 mm × 4.6 mm i.d., 5 μm) were used: a chiral column Cyclobond I-2000 RSP (β-cyclodextrin derivatized with R,S-hydroxypropyl ether-bonded phase, 5-μm) (Astex, Whippany, NJ, USA) for the analysis of methadone enantiomers and its major metabolite (EDDP) and a reversed-phase column SymmetryShield RP-8 (Waters) for the separation of four benzodiazepines. Each analytical column was connected with a guard column (10 mm × 3.2 mm i.d.) packed with the same bonded phase.

2.3. Extraction procedures

After addition of serum or urine samples (100–200 μl), these two polymeric cartridges were washed with 1 ml of water–methanol mixture (95:5, v/v) and eluted with diethylether (2 × 1 ml). About 200 mg anhydrous Na_2SO_4 were added in this extracted solvent by slightly shaking the collected glass tube (5 ml). After transferring the supernatant extracted solvent to a clean glass tube and rapid evaporation under nitrogen stream at about 40 °C, the residue was regenerated with 100 μl of mobile phase, then 50 μl aliquot was injected either into a chiral column for methadone enantiomers and its metabolite (EDDP) analysis or into a reversed-phase column for the separation of four benzodiazepines. For Oasis HLB extraction cartridge, the column was conditioned with 1 ml methanol then 1 ml deionized water before the addition of biological samples. This precondition was not necessary for Abselut Nexus extraction cartridge. For the analysis of methadone enantiomers and EDDP in spiked serum and urine, 100 μl of papaverine hydrochoride (2 μg/ml in water) used as an internal standard were added in the same time with the sample into the polymeric cartridges.

For the solid-phase extraction of four benzodiazepines using classical reversed-phase-bonded-silica technique, a Bond Elut C18 extraction column (1 ml) (Varian) was chosen for this study of comparison. This column must be preconditioned with a column volume of methanol and then with water before adding serum or urine samples (100–200 μl) mixed with the same volume of 0.1 M sodium borate buffer (pH 9.5). After drawing the column by vacuum, the matrix was washed twice with water, followed by 40 μl of methanol, then eluted with 2 × 1 ml methanol. The total eluent was dried under a stream of nitrogen and the residue was reconstituted in 100 μl of mobile phase. A 50 μl aliquot of this extract solution was injected into the analytical column.

A liquid extraction technique previously described [5] with slight modification was also chosen for the comparison study of methadone enantiomers and its metabolite EDDP. Briefly, samples were added with 100 μl of internal standard (papaverine hydrochloride), 300 μl of 10% anhydrous sodium carbonate in water and 4 ml of hexane. After vortexing then centrifuging, the upper layer was transferred, then dried and regenerated with 100 μl of mobile phase.

The recovery was calculated by the areas of peaks after extraction compared to those of peaks obtained from direct injection of standard solutions in mobile phase at the same concentrations.

2.4. HPLC analysis

For the HPLC analysis of four benzodiazepines (diazepam, flunitrazepam, nitrazepam, oxazepam), the reversed-mobile phase was obtained by a mixture of acetonitrile and 0.1 M KH_2PO_4 buffer (40:60, v/v) and pumped at a flow rate of 0.90 ml/min. The UV detection was set at 230 nm. A reversed-phase column SymmetryShield RP-8 (Waters) was used for the separation of these four benzodiazepines and its temperature was set at 20 °C.

For the separation of methadone enantiomers and its metabolite EDDP, an enantioselective HPLC previously described [5] with slight modification was used. Briefly, the reversed-mobile phase was obtained by a mixture of acetonitrile, 1% triethylamine acetate buffer (TEAA), pH 4.5, and water (19:9:72, v/v/v) and pumped at a flow rate of 0.50 ml/min. The UV detection was set at 210 nm. A chiral column Cyclobond I-2000 RSP (Astex) was used for this chiral analysis and its temperature was maintained at 18 °C with a column chiller system.

2.5. Application of the method

Serum samples of three patients under maintenance treatment for opiate dependence were taken 3 h after a single dose of racemic Mtd solution (45–90 mg/dose) and used to test the applicability of the described method for methadone. For the applicability of benzodiazepine method, serum samples from three rabbits treated orally with 25 mg/kg oxazepam were taken at $T = 2$ h. One hundred microliters of an internal standard solution of flunitrazepam (2 μg/ml) were added in the same time with samples or standards before extraction for the determination of this benzodiazepine.

3. Results and discussion

3.1. Chromatograms

Typical chromatograms of blank human serum and serum spiked with methadone racemic and EDDP metabolite obtained by the extraction of Oasis HLB polymeric cartridge in the presence of papaverine as an internal standard and by the enantioselective HPLC are shown in Fig. 1. Typical chromatograms of blank human urine and urine spiked with four benzodiazepines (diazepam, flunitrazepam, nitrazepam, oxazepam) obtained by the extraction of Abselute Nexus polymeric cartridge and by the reversed-phase HPLC are shown in Fig. 2. No endogenous interfering peaks were observed with drug-free human serum and urine at the retention time of analyzed compounds (methadone enantiomers, EDDP, I.S. and 4 benzodiazepines). However, by the liquid extraction previously described for methadone enantiomers and EDDP, an interfering peak was observed at the position of internal standard (papaverine) after enatioselective HPLC analysis. Therefore, the chromatogram time of this modified technique was shorter than that previously described thanks to the removing of all interfering biological compounds in serum and

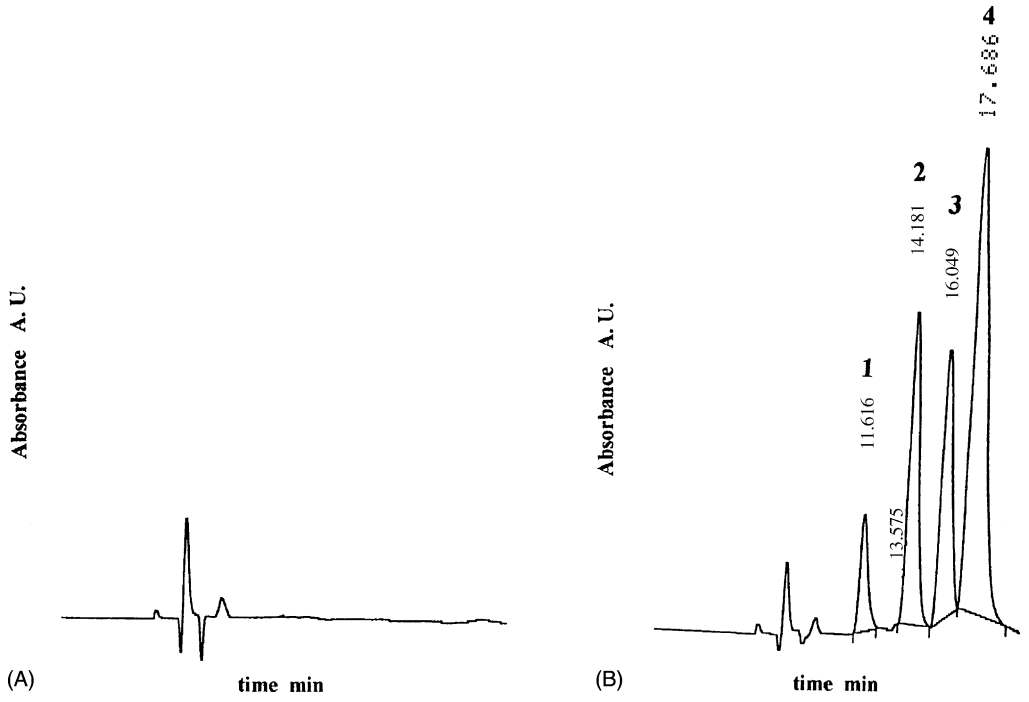

Fig. 1. Chromatograms of serum spiked with (R,S)-methadone and EDDP metabolite extracted by Oasis HLB cartridge (1 ml) and separated by chiral HPLC column (Cyclobond RS-P 2000). *Legends*: (A) blank human serum and (B) spiked human serum: (1) internal standard (papaverine: 2 μg/ml); (2) R-methadone: 2.5 μg/ml; (3) S-methadone: 2.5 μg/ml; (4) EDDP metabolite: 5 μg/ml.

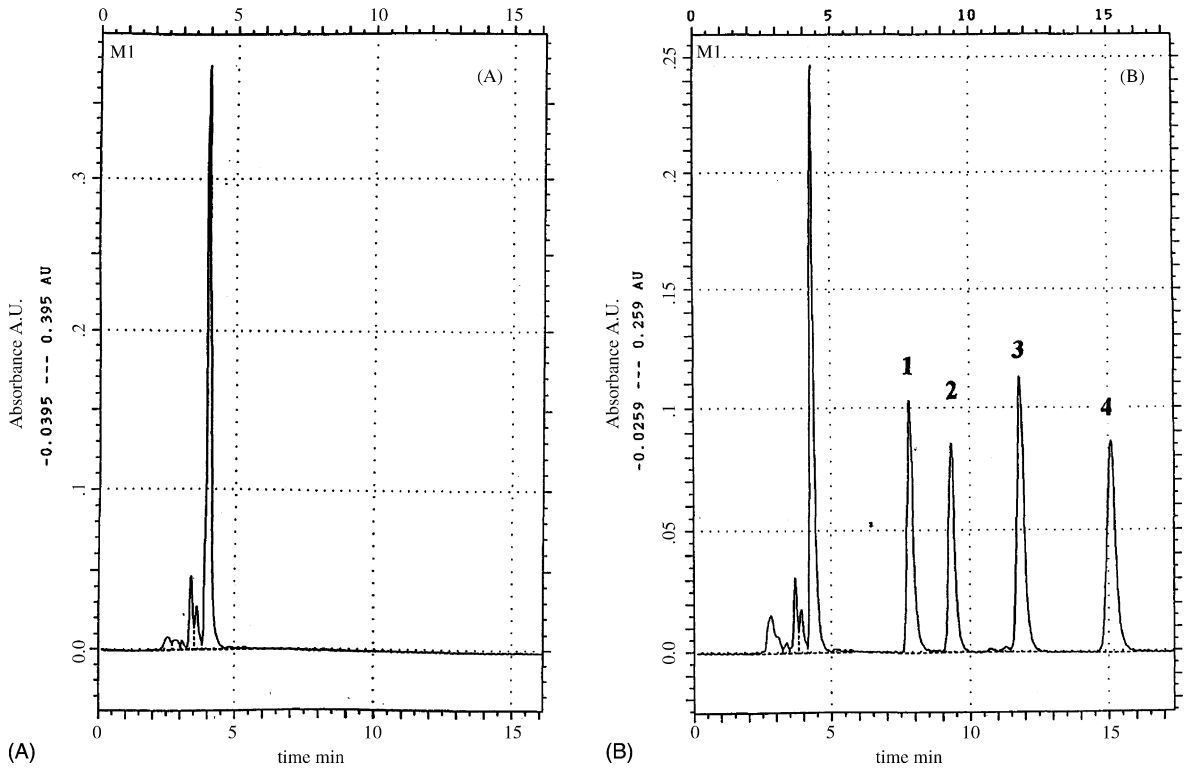

Fig. 2. Chromatograms of urine spiked with four benzodiazepines (oxazepam, nitrazepam, flunitrazepam, diazepam), at 1 μg/ml, extracted by Abselut Nexus cartridge (1 ml) and separated by reversed-phase HPLC column (Symmetry Shield RP8). *Legends*: (A) blank human urine and (B) spiked human urine: (1) oxazepam; (2) nitrazepam; (3) flunitrazepam; (4) diazepam.

urine by these two polymeric extraction cartridges. For the extraction of four benzodiazepines in serum and urine by the classical Bond Elut C18 extraction cartridge, some small interfering peaks were also observed at the position of oxazepam and nitrazepam after reversed-phase HPLC analysis, contrary to the extraction technique obtained by these two polymeric cartridges. No difference in the chromatogram aspects of blank serum and urine obtained by the Oasis HLB cartridge and the Abselute Nexus one was observed.

3.2. Analytical parameters

The inter-day precision (R.S.D.) and recovery of methadone enantiomers and EDDP metabolite in spiked serum and urine obtained from Oasis HLB and Abselute Nexus polymeric extraction cartridges and analyzed by chiral HPLC are gathered in Table 1. The standard calibration curves of serum and urine exhibited good linearity with correlation coefficients (r) greater than 0.999 for R-(−)- and S-(+)-Mtd and EDDP metabolite over the range of concentrations tested (0.02–5.0 μg/ml racemic Mtd and EDDP). The inter-day precision ($n = 6$) of three serum samples from three patients treated by methadone varied between 3.1 and 4.8% for methadone enantiomers with their concentrations found about 0.02–0.05 μg/ml and with their ratio R/S between 0.3 and 0.5. The same parameter R.S.D. for EDDP metabolite varied between 2.8 and 3.9% with EDDP concentrations found about 0.015–0.03 μg/ml and with ratio EDDP/rac. Mtd between 0.25 and 0.45. The same parameters (inter-day precision and recovery) for benzodiazepines obtained from Oasis HLB and Abselut Nexus cartridges and analyzed by reversed-phase HPLC are shown in Table 2. The standard calibration curves of serum and urine exhibited good linearity with correlation coefficients (r) greater than 0.999 for four benzodiazepines (diazepam, nitrazepam, flunitrazepam and oxazepam) over the range of concentrations tested (0.05–5.0 μg/ml). The inter-day precision ($n = 6$) of three serum samples from three rabbits treated by Oxazepam varied between 2.6 and 3.9% with their concentrations found about 0.48–0.61 μg/ml. The same parameters (inter-day precision and recovery) for methadone enantiomers and EDDP extraction obtained by liquid technique and for benzodiazepines extraction obtained by Bond Elut C18 column are shown in Tables 3 and 4, respectively. Recovery from these two polymeric cartridges was much higher than that obtained by previous classical methods (about >10%): 95–103% by polymeric cartridges versus 88–95% by liquid extraction for methadone enantiomers and EDDP, and 97–102% by polymeric cartridges versus 84–94% by Bond Elut C18 column for benzodiazepines. The total extraction time (including solvent evaporation) by Oasis Cartridge or Nexus Cartridge for methadone and benzodiazepines in serum and urine was about 7 min for the first column and 6 min for the other. In contrast, that by these two classical techniques described

Table 1
Inter-day precision and recovery of serum and urine spiked with methadone (Mtd) and EDDP metabolite after solid-phase extraction by Oasis HLB or Abselut Nexus polymeric sorbents ($n = 6$)

Compounds	Inter-day precision (R.S.D.%) (μg/ml)			Recovery (%) (μg/ml)		
	0.05	0.1	0.5	0.05	0.1	0.5
Serum by Oasis HLB						
(R)-Mtd	4.15	3.75	3.06	95.7 ± 4.5	97.7 ± 3.4	98.4 ± 2.3
(S)-Mtd	3.75	3.85	3.64	97.8 ± 3.5	98.5 ± 5.1	98.7 ± 1.8
EDDP	3.35	4.16	2.85	98.2 ± 3.3	100.1 ± 4.8	99.2 ± 2.4
Urine by Oasis HLB						
(R)-Mtd	3.12	3.24	2.72	96.7 ± 3.0	97.6 ± 4.1	97.0 ± 2.1
(S)-Mtd	3.95	4.02	3.06	98.8 ± 2.3	98.1 ± 2.8	96.4 ± 2.3
EDDP	3.84	4.28	2.55	96.8 ± 4.2	97.5 ± 4.1	94.8 ± 3.1
Serum by Abselut Nexus						
(R)-Mtd	3.57	4.15	2.95	94.8 ± 3.3	100.4 ± 4.2	97.4 ± 3.3
(S)-Mtd	3.65	3.85	2.93	96.4 ± 4.2	97.1 ± 2.3	95.7 ± 2.1
EDDP	3.86	3.12	4.14	95.9 ± 5.2	99.6 ± 4.3	94.9 ± 2.2
Urine by Abselut Nexus						
(R)-Mtd	3.32	3.18	2.42	97.1 ± 4.2	99.5 ± 4.6	98.1 ± 3.1
(S)-Mtd	4.12	3.08	2.66	99.5 ± 5.0	96.7 ± 2.5	97.5 ± 2.8
EDDP	3.85	4.01	3.53	95.7 ± 4.3	98.9 ± 4.6	94.8 ± 3.1

herein was time-consuming and tedious: about 15 min for methadone and EDDP by liquid extraction technique, and about 35 min for benzodiazepines by Bond Elut C18 extraction cartridge. The last technique using methanol as elution solvent needed more time for solvent evaporation than the technique described herein using diethylether for eluting these two polymeric cartridges. The classical C18-bonded-silica phase cannot endure to strong apolar organic solvents, contrary to the polymeric sorbents.

The detection limits (signal-to-noise ratio >3) of the assay after extraction by these columns were about 1.0 ng/ml for each methadone enantiomer and 2.0 ng/ml for EDDP metabolite and about 1.0 ng/ml for benzodiazepines (diazepam, nitrazepam, flunitrazepam and oxazepam).

No difference between these two polymeric cartridges (Oasis HLB and Abselut Nexus) was observed about extraction performances for the studied compounds with correlation coefficients about 0.9999, except that Abselute Nexus

Table 2
Inter-day precision and recovery of serum and urine spiked with four benzodiazepines after solid-phase extraction by Abselut Nexus or Oasis HLB polymeric sorbents ($n = 6$)

Compounds	Inter-day precision (R.S.D.%) (μg/ml)			Recovery (%) (μg/ml)		
	0.05	0.1	0.5	0.05	0.1	0.5
Serum by Abselut Nexus						
Diazepam	3.75	3.10	2.96	97.8 ± 4.5	99.5 ± 3.4	99.4 ± 2.3
Flunitrazepam	5.12	4.00	3.26	96.1 ± 3.8	96.5 ± 3.1	97.4 ± 2.8
Nitrazepam	4.25	3.97	3.50	94.2 ± 4.3	95.8 ± 4.2	95.2 ± 3.4
Oxazepam	3.74	3.55	2.16	98.5 ± 3.8	100.5 ± 3.5	99.4 ± 2.3
Urine by Abselut Nexus						
Diazepam	4.78	2.76	1.72	96.2 ± 4.5	95.8 ± 4.2	99.0 ± 2.1
Flunitrazepam	4.82	4.75	3.06	95.4 ± 4.2	96.5 ± 3.2	96.4 ± 2.9
Nitrazepam	3.67	3.05	2.55	94.8 ± 3.8	96.2 ± 2.9	95.8 ± 3.1
Oxazepam	3.52	2.87	2.06	99.4 ± 3.3	101.0 ± 4.1	98.4 ± 2.3
Serum by Oasis HLB						
Diazepam	3.95	3.05	2.75	100.7 ± 3.9	98.8 ± 3.7	99.7 ± 1.9
Flunitrazepam	3.72	3.25	2.90	96.4 ± 3.7	97.8 ± 2.7	95.4 ± 1.7
Nitrazepam	4.26	4.08	3.10	95.2 ± 3.5	98.4 ± 3.5	96.2 ± 3.0
Oxazepam	3.20	2.55	2.78	97.5 ± 2.3	98.7 ± 3.2	99.0 ± 3.3
Urine by Oasis HLB						
Diazepam	4.52	3.21	2.15	97.5 ± 4.4	100.7 ± 3.8	99.5 ± 2.4
Flunitrazepam	3.21	2.68	2.80	94.5 ± 3.5	97.8 ± 2.9	99.5 ± 2.4
Nitrazepam	4.10	3.35	3.05	95.1 ± 3.6	97.3 ± 3.9	96.1 ± 1.1
Oxazepam	2.95	3.25	3.16	97.4 ± 3.1	98.5 ± 4.5	95.4 ± 1.8

Table 3
Inter-day precision and recovery of serum and urine spiked with methadone (Mtd) and EDDP metabolite after liquid extraction ($n = 6$)

Compounds	Inter-day precision (R.S.D.%) (μg/ml)			Recovery (%) (μg/ml)		
	0.05	0.1	0.5	0.05	0.1	0.5
Serum						
(R)-Mtd	5.50	4.84	4.26	87.2 ± 3.3	90.5 ± 3.7	93.5 ± 3.3
(S)-Mtd	6.18	5.62	4.32	89.5 ± 4.3	91.5 ± 4.6	92.4 ± 2.6
EDDP	5.15	4.95	4.15	84.8 ± 3.2	87.6 ± 4.9	88.2 ± 2.2
Urine						
(R)-Mtd	4.95	4.52	3.78	88.7 ± 4.3	92.5 ± 4.5	91.0 ± 2.4
(S)-Mtd	5.48	5.14	3.67	89.5 ± 2.1	89.1 ± 5.0	90.5 ± 3.1
EDDP	5.45	5.75	4.57	87.8 ± 5.2	89.2 ± 3.3	88.8 ± 2.1

Table 4
Inter-day precision and recovery of serum and urine spiked with four benzodiazepines after solid-phase extraction by Bond Elut C18 sorbent ($n = 6$)

Compounds	Inter-day precision (R.S.D.%) (μg/ml)			Recovery (%) (μg/ml)		
	0.05	0.1	0.5	0.05	0.1	0.5
Serum						
Diazepam	5.25	5.12	3.86	89.4 ± 3.2	91.4 ± 4.0	91.4 ± 3.2
Flunitrazepam	5.78	4.86	4.76	82.5 ± 2.8	84.6 ± 3.4	89.5 ± 2.8
Nitrazepam	6.12	5.32	4.52	84.2 ± 4.2	85.4 ± 3.2	89.2 ± 3.7
Oxazepam	4.92	4.75	3.36	85.9 ± 3.8	88.5 ± 4.1	84.9 ± 3.1
Urine						
Diazepam	4.85	4.58	3.11	90.1 ± 4.5	92.5 ± 4.1	92.2 ± 3.5
Flunitrazepam	5.25	5.45	4.16	83.5 ± 4.1	85.5 ± 3.1	85.4 ± 3.3
Nitrazepam	5.36	5.25	4.55	83.8 ± 3.1	89.2 ± 3.3	89.8 ± 2.1
Oxazepam	4.52	3.84	3.36	87.8 ± 3.2	94.5 ± 3.5	90.8 ± 1.8

cartridge did not need column activation by methanol and water before use.

For methadone and benzodiazepine extractions by these two polymeric cartridges, the use of acidic or alkaline buffer was not necessary, contrary to the classical methods described herein or elsewhere [5–9]. Moreover, these polymeric cartridges can be used at pH from 1 to 14 and with many apolar and polar organic solvents (chloroform, diethylether, methanol, etc.) as elution liquid, contrary to the C18 or C8 silica-bonded phase columns [1]. These macroporous polymers exhibit both hydrophylic and lipophylic retention characteristics, therefore they can retain a wide spectrum of both polar and non-polar compounds. This characteristic is important for the extraction of drugs and metabolites, such as methadone and its metabolite EDDP, diazepam and its polar metabolite oxazepam cited in this study. These polymeric cartridges are both presented as the universal extraction sorbent, since they are capable to extract acidic, neutral and basic compounds whether polar or non-polar [1]. Therefore, it is easier to find an appropriate extraction condition for a specific compound and especially for a mixture of analytes with different chemical properties, such as polarity, pH, affinity, etc. Recently, many analysts have used these two polymeric sorbents for the extraction of drugs, metabolites or other compounds in biological matrices [10–16]. However, the application of these two polymeric extraction cartridges to the bioanalysis of methadone enantiomers and its metabolite EDDP as well as of the four benzodiazepines in the same time (oxazepam, nitrazepam, flunitrazepam and diazepam) remains very scarce in the literature [17,18]. In addition, some parameters, such as chromatography time, resolution, of two HPLC techniques described hereby are also more improved than those previously cited in the literature [5,18]. The development of this reversed-phase HPLC technique can be applied to the analysis of benzodiazepines in the case of polypoisoning of these drugs for emergency toxicology and to the simultaneous determination of diazepam and its active oxazepam metabolite by using flunitrazepam as internal standard for pharmacokinetic study and therapeutic monitoring.

Briefly, recovery, time extraction and chromatogram aspect obtained in this study were better with these two polymeric cartridges (Oasis HLB and Abselute Nexus) than with C18-bonded-silica cartridges or with liquid extraction. Moreover, these cartridges can be used at pH from 1 to 14 and with many apolar and polar organic solvents (chloroform, diethylether, methanol, etc.) contrary to classical reversed-phase silica extraction columns. Therefore, they are suitable for the extraction of chemical compounds with different polarity in biological matrices and could be used either in pharmacokinetic studies of drugs and their metabolites, such as methadone enantiomers and its EDDP metabolite, diazepam and its active oxazepam metabolite or in polypoisoning situation by different physical and chemical analysis methods, such as the two HPLC techniques described herein.

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