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# Simultaneous determination of methadone, buprenorphine and norbuprenorphine in biological fluids for therapeutic drug monitoring purposes

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**Abstract**

Methadone and buprenorphine are two of the drugs most frequently used for abstinence from illicit opioids and in the treatment of pain. A sensitive and selective high-performance liquid chromatographic method with diode array detection for the simultaneous determination of methadone, buprenorphine and norbuprenorphine has been developed. Separation of the three analytes was obtained by using a reversed-phase column (C8, 250 mm × 4.6 mm i.d., 5 μm) and a mobile phase composed of 40% phosphate buffer containing triethylamine, 50% methanol and 10% acetonitrile (final apparent pH 6.0). Loxapine was used as the internal standard. An accurate pre-treatment procedure of biological samples was developed, using solid-phase extraction with C8 cartridges (100 mg, 1 mL) and needing small amounts of plasma or urine (300 μL). The calibration curves were linear over a working range of 10.0–1500.0 ng/mL for methadone and of 5.0–500.0 ng/mL for buprenorphine and norbuprenorphine in both matrices. The limit of quantitation (LOQ) and the limit of detection (LOD) were 1.0 and 0.4 ng/mL for methadone and 0.5 and 0.2 ng/mL for both buprenorphine and norbuprenorphine, respectively. The method was successfully applied to the analysis of plasma and urine samples from patients undergoing treatment with these drugs. Precision and accuracy results were satisfactory and no interference from endogenous or exogenous compounds was found. The method is suitable for the simultaneous determination of methadone and buprenorphine in human plasma and urine for therapeutic drug monitoring purposes.


*Keywords:* Methadone; Buprenorphine; Norbuprenorphine; High-performance liquid chromatography; DAD detection; Biological fluids; Solid-phase extraction

## 1. Introduction

Since 1964, scientific studies have demonstrated the usefulness of the opioid agonist methadone (6-(dimethylamino)-4,4-diphenyl-3-heptanone, MTD, Fig. 1a) in the treatment of heroin addiction by counteracting the withdrawal syndrome and reducing drug craving [1]. It has also been observed that heroin addicts undergoing maintenance treatment with MTD have significant lower mortality than those who do not [2]. MTD competes with other opioids for binding with the $\mu$ receptor [3]: thus, it satisfies acute craving while gradually averting the subject from his/her dependence from heroin. MTD has a long duration of activity (15–60 h) and analgesic potency similar to that of morphine if administered intramuscularly (i.m.) during pain relief therapy [4]. The initial dose in addiction treatment is usually 10–20 mg to suppress the craving and the withdrawal syndrome; this dose can be increased up to 60–120 mg/day or more [5] according to the needs of the patient. MTD is mainly metabolised in the liver by mono- and di-*N*-demethylation; the metabolites thus formed are pharmacologically inactive [6]. After having stabilised the daily dose, MTD plasma levels are usually in the 50–1000 ng/mL range [7], with wide interindividual variability. This is probably due to metabolic differences, with "ultrarapid metabolisers" having very low plasma levels

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of the drug and "poor metabolisers" having very high plasma levels even when taking very low doses of the drug [6]. The main side effects of MTD are: sedation, euphoria, bradycardia, hypotension, loss of libido, nausea and constipation [5]. Furthermore, the sudden suspension of MTD administration can generate a withdrawal syndrome. Nowadays, there are many other drugs available for the maintenance treatment of opioid addiction, having different pharmacologic and pharmacokinetic properties. Among these, one of the most widely used is buprenorphine ((αS,5α,7α)-17-(cyclopropylmethyl)-α-(1,1-dimethyletyl)-4,5-epoxy-18,19-dihydro-3-hydroxy-6-methoxy-α-methyl-6,14-ethenomorphinan-7-methanol, BPR, Fig. 1b) [8]. BPR is considered both a partial agonist at $\mu$ receptors and an antagonist at $k$ receptors [9]. Thus, it has analgesic activity in non-addicted subjects and is used for pain relief therapy [10]. However, it can also counteract the activity of heroin and other opioids and for this reason is used in the treatment of opioid addiction [11]. Moreover, BPR seems to be more effective in opioid-dependent patients affected by depression [12]. The usual dose of BPR during opioid addiction therapy is 8 mg the first day and 16–24 mg the following ones; its long-lasting activity (half life of 37 h and more) also allows to administer a dose of 32 mg every other day [13]. BPR is biotransformed in the liver, primarily through cytochrome P450 (CYP) 3A4, to the active metabolite norbuprenorphine (NBP, Fig. 1c), which is pharmacologically active, and to other conjugated metabolites. However, there is a wide interindividual variability in BPR metabolism, which prevents from using the same dosing schedule for all patients: in fact, some patients do not respond well to alternate-day administration [14], while others have very high plasma levels of the drug even at low doses. Thus, only an accurate monitoring of BPR and its main metabolite NBP levels in biological fluids allows to effectively personalise the therapy. At steady-state, plasma levels of both BPR and NBP are generally within the 0.5–30.0 ng/mL range

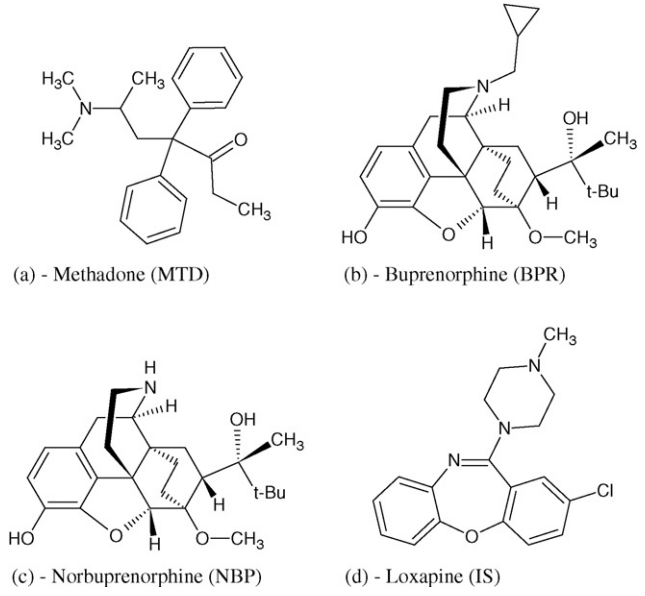


Fig. 1. Chemical structures of (a) methadone, (b) buprenorphine, (c) norbuprenorphine and (d) loxapine (IS).

[15]. The side effects and withdrawal syndrome associated with BPR therapy are similar to those already reported for MTD, however they usually are less severe [16]. For this reason in particular, BPR is also used to facilitate the transition from MTD therapy to opioid antagonist therapy or to reduce withdrawal. In fact, the peculiar receptorial selectivity profile of BPR offers increased safety with respect to traditional full opioid agonists [17]. It is apparent that therapeutic drug monitoring (TDM) with the determination of drug and metabolite plasma levels of patients is very important, especially in the first days of therapy and in case of switching from MTD to BPR. In fact, in the former case, the TDM can establish an objective parameter for the titration of both drugs; in the latter case, it can help deciding the exact schedule of MTD suspension and BPR escalation. The determination of plasma levels of active metabolites (such as NBP) also allows to obtain additional information regarding the efficacy and safety of the treatment. To the best of our knowledge, no analytical methods are currently available which simultaneously determine MTD, BPR and NBP in biological fluids for TDM purposes, even though several methods have been recently published for the quantitative analysis of either MTD in human plasma [18–22] and/or urine [19,23], or BPR (with or without its main metabolite) in plasma [24–29] and/or urine [30]. These methods are based on HPLC with electrochemical [24], mass spectrometry [18,20,21,26–30] or UV-diode array [19,23] detection, on gaschromatography with mass spectrometry detection [29] or finally on capillary electrophoresis [22]; this last technique has until now only been used for MTD. Some papers report the analysis of MTD, BPR and many other drugs in human plasma [31–33] by HPLC with coulometric electrode array detection [31] and by HPLC with mass spectrometric detection [32,33]. However, these methods are not all fully validated and none of them is really suitable for the TDM of patients: they do not include NBP and thus the TDM information that they provide is incomplete. Aim of this study is the development of an accurate analytical method based on liquid chromatography with diode array detection (DAD) for the reliable determination of MTD, BPR and NBP in biological fluids for TDM purposes. The method is fast and feasible and employs a selective SPE procedure for purification of the biological matrix. The photodiode array detection allows to obtain full UV spectral data for the analytes and this in turn allows to identify chromatographic peaks with a higher degree of confidence with respect to normal spectrophotometric detection. This is particularly important when analysing drugs which have a high potential for abuse and when monitoring patients who have a history of drug abuse.

## 2. Experimental

### 2.1. Chemicals

Methanolic stock solutions of MTD (1 mg/mL), BPR (8 mg/mL) and NBP (3 mg/mL) were kindly provided by the Toxicological Analysis Laboratory directed by Prof. Cesare Baccini at the "S. Maria delle Croci" Hospital (Ravenna, Italy).

All these reference standard had purity ≥98% according to the manufacturer's claim.

Methanol and acetonitrile HPLC grade, 85% (w/w) orthophosphoric acid, 37% (w/w) hydrochloric acid, 2N sodium hydroxide, triethylamine and monobasic potassium phosphate, all analytical grade, were purchased from Carlo Erba (Milan, Italy).

Loxapine (purity >98%) used as the Internal Standard (IS, Fig. 1d) was kindly provided by Lederle Laboratories (Gosport, Hampshire, UK).

Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus from Millipore (Milford, MA, USA).

### 2.2. Apparatus and chromatographic conditions

The chromatographic system was composed of an Agilent (Palo Alto, CA, USA) model 1100 chromatographic pump and photodiode array detector (DAD); the absorbance signal was monitored at 214 nm.

Separations were obtained on a Varian (Harbor City, CA, USA) Microsorb-MV C8 reversed-phase column (250 mm × 4.6 mm i.d., 5 μm). The mobile phase was composed of a mixture of methanol (50%, v/v), acetonitrile (10%, v/v) and a 60 mM phosphate buffer containing 0.5% triethylamine (40%, v/v); the final apparent pH of the mobile phase was brought to 6.0 with 85% (w/w) orthophosphoric acid. The mobile phase was filtered through a Phenomenex (Torrance, CA, USA) membrane filter (47 mm membrane, 0.2 μm, NY) and degassed in an ultrasonic bath. The flow rate was set at 1.0 mL/min and the injections were carried out through a 50 μL loop. Solid-phase extraction (SPE) was carried out on IST (Hengoed, Mid Glamorgan, UK) Isolute C8 cartridges (100 mg, 1 mL) by means of a Vac Elut (Varian) apparatus.

A Crison (Barcelona, Spain) Basic 20 pHmeter, a Büchi (Essen, Germany) Mod. 461 rotary evaporator and an ALC (Milan, Italy) 4225 centrifuge were used.

Agilent Chemstation (Rev. A.09.01) software was used for data handling.

### 2.3. Solutions

The stock solution of the IS (1 mg/mL) was prepared by dissolving 20 mg of pure compound in 20 mL of methanol. All working solutions were prepared by mixing stock solutions of the analytes and the IS and diluting them with the mobile phase to the desired concentrations. Stock solutions are stable for at least 3 months at −20 °C (HPLC assay); standard solutions were prepared afresh daily.

### 2.4. Sample collection

Blood and urine samples were collected at the Drug Addiction Service, ASL of Rome (Italy), and at the Toxicological Analysis Laboratory of the "S. Maria delle Croci" Hospital, Ravenna (Italy), from subjects undergoing therapy with MTD or BPR for at least 2 weeks and were taken 12 h after the last drug administration.

Blood was stored in glass tubes containing EDTA as the anticoagulant, then centrifuged (within 2 h from collection) at 1400 × *g* for 15 min; the supernatant (plasma) was transferred to polypropylene tubes and stored at −80 °C.

Urine samples were collected into polypropylene tubes and stored at −80 °C. Before the SPE pre-treatment, urine samples were subjected to acidic hydrolysis: 1.5 mL of 37% (w/w) HCl were added to 300 μL of urine, the mixture was vortexed for 10 min and left to rest for 20 min at 120 °C. Then, the samples were cooled to room temperature, brought to pH 7.0 with 2N NaOH and filtered (nylon filters, pore size 20 μm, Phenomenex).

"Blank" plasma and urine samples were obtained from healthy volunteers not subjected to any pharmacological treatment.

### 2.5. Sample pre-treatment: SPE procedure

The C8 cartridges were activated by passing 1 mL of methanol through the cartridge five times and then conditioned by passing 1 mL of ultrapure water five times (flow rate: 0.5 mL/min). To 300 μL of plasma or to hydrolysed urine, 600 μL of ultrapure water and 50 μL of IS working solution were added and the resulting mixture was loaded onto a previously conditioned cartridge (flow rate: 0.4 mL/min). The cartridge was then washed twice with 1 mL of ultrapure water and twice with 1 mL of water/methanol (80/20, v/v) mixture (flow rate: 0.5 mL/min). After washing, the cartridge was dried under vacuum (−50 kPa) for 1 min. The analytes were subsequently eluted with 1 mL of methanol (flow rate: 0.4 mL/min), the eluate was dried under vacuum (rotary evaporator), redissolved with 150 μL of mobile phase, then injected into the HPLC system.

### 2.6. Method validation

#### 2.6.1. Calibration curves

Aliquots of 50 μL of analyte standard solutions at seven different concentrations containing the IS at a constant concentration were added to 300 μL of blank plasma or to hydrolysed urine. The resulting concentration ranges in plasma or urine were 10.0–1500.0 ng/mL for MTD; 5.0–500.0 ng/mL for BPR and NBP; 50 ng/mL (constant) for the IS. These mixtures were subjected to the previously described SPE procedure and injected into the HPLC system. At the end of the SPE procedure, the concentration ranges of the compounds of interest became the following: 20.0–3000.0 ng/mL for MTD; 10.0–1000.0 ng/mL for BPR and NBP; 100 ng/mL for the IS. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analytes (expressed as ng/mL) and the calibration curves constructed by means of the least-squares method. One stock solution was used for each replicate.

The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated according to USP XXVII [26] and "Crystal City" [27] guidelines as the analyte concentrations which give rise to peaks whose heights were 10 and 3 times the baseline noise, respectively.

*2.6.2. Extraction yield (absolute recovery)*

The procedure was the same as that described under "Calibration Curve", above, except the points were at three different concentrations, corresponding to the upper limit, middle point and lower limit of each calibration curve (i.e., plasma or urine concentrations of 10.0, 750.0 and 1500.0 ng/mL for MTD, 5.0, 250.0 and 500.0 ng/mL for BPR and NBP). The analyte/IS peak area ratios were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

*2.6.3. Precision*

The assays described under "Extraction yield" were repeated six times within the same day to obtain repeatability (intraday precision) and six times over 6 different days to obtain intermediate precision (interday precision), both expressed as Percentage Relative Standard Deviation (R.S.D.%) values.

*2.6.4. Selectivity*

Blank plasma and urine samples from six different volunteers were subjected to the SPE procedure and injected into the HPLC; the resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was: no interfering peak higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different drugs active on the Central Nervous System were injected at concentrations higher than the respective therapeutic levels; if the resulting chromatograms contain any interfering peak, the potentially interfering compounds are subjected to the SPE and injected to see if they are extracted.

*2.6.5. Accuracy*

Accuracy was evaluated by means of recovery assays. The assays described under "Extraction yield" were carried out adding standard solutions of the analytes and the IS to real plasma or urine samples (already analysed) taken from patients subjected to treatment with MTD or BPR. The assays were repeated three times during the same day to obtain mean recovery data.

## 3. Results and discussion

*3.1. Chromatographic conditions*

Preliminary experiments were carried out using a C8 reversed-phase column (150 mm × 4.6 mm i.d., 5 μm) and a mobile phase composed of a pH 6.4 phosphate buffer and acetonitrile (50/50, v/v) was used, with a flow rate of 1.6 mL/min [26]. Under these conditions, the peak of MTD was partially overlapping that of BPR, thus a longer column (250 mm × 4.6 mm i.d., 5 μm) was tested with a flow rate of 1.0 mL/min. These modifications allowed to obtain baseline resolution of the analytes, however the run times were quite long (about 16 min). Thus, the mobile phase was made more lipophilic by adding methanol; this solvent resulted to selectively influence the retention of the analytes: run times were shortened while retaining good resolution. Best resolution was observed when the apparent pH was adjusted to 6.0. One can note that the apparent pH of the mobile phase resulted to be a critical parameter, since small changes of its value (±0.2) led to a sharp loss of resolution.

Loxapine was found to be suitable for use as the Internal Standard (IS). Under the final conditions, the peaks are neat and well separated and a chromatographic run lasts less than 8 min.

*3.2. Solid-phase extraction procedure*

In order to apply the method to complex biological matrices such as human plasma and urine, a reliable sample pre-treatment procedure is needed to eliminate potentially interfering endogenous and exogenous compounds, thus enhancing method selectivity and column lifetime. For this purpose it was decided to use SPE, which allows to obtain highly reproducible and reliable results in relatively short times, using low volumes of organic solvents with good purification of the biological samples.

Among available sorbents, the first choice was a mixed cationic exchange/lipophilic resin (BondElut Certify), but severe interference was found. Hydrophilic/lipophilic balance cartridges (OASIS HLB) were then tested; however, extraction yields of the analytes were quite low with this sorbent. Finally, cyclohexyl (CH) and octyl (C8) sorbents were tried. While CH cartridges gave rise to interference and to a low extraction yield of the IS, C8 cartridges gave good results. Thus, they were chosen for subsequent SPE procedure development. Since the elimination of interference resulted to be the critical step of the pre-treatment, to the initial washing step with 2 × 1 mL of water, a further washing step with a more lipophilic solvent (a water/methanol 80/20, v/v, mixture) was added: under these conditions most matrix compounds were eliminated without loss of analytes. Elution with 1 mL of methanol gave high extraction yields of the analytes; the eluate was then dried and redissolved with 150 μL of mobile phase. This procedure concentrates the analytes two times. The last matrix residues were eliminated if the final solution was left to rest at low temperature (−20 °C) for 2 h and then injected. Using the developed procedure good purification of the biological matrices and very satisfactory extraction yield results were obtained for all three analytes (Table 1). The chromatograms of a blank plasma sample from an healthy volunteer and of the same blank plasma sample spiked with 100 ng/mL of the analytes and of the IS (injected concentration) are shown in Fig. 2a and b, respectively. The chromatograms of a blank urine sample from an healthy volunteer and of the same blank urine sample spiked with 100 ng/mL of the analytes and of the IS (injected concentration) are shown in Fig. 2c and d, respectively. As can be seen, no interference from the matrix is present and all peaks are still neat and well resolved. It should be noted that the theoretical concentrations of the analytes in the biological samples are half those reported for the injected solutions since the SPE procedure concentrates the analytes 2:1 with respect to the original samples.

Table 1
Extraction yield and precision results

| Compound | Concentration (ng/mL) | Extraction yield (%)[a] | Repeatability (R.S.D.%)[a] | Intermediate precision (R.S.D.%)[a] | Retention times (min) | Intermediate precision of retention times (R.S.D.%)[a] |
|---|---|---|---|---|---|---|
| MTD | 10.0 | 96 | 5.5 | 6.1 | | |
| | 750.0 | 98 | 3.9 | 4.6 | 5.1 | 2.1 |
| | 1500.0 | 99 | 2.8 | 3.7 | | |
| BPR | 5.0 | 97 | 4.9 | 5.9 | | |
| | 250.0 | 97 | 4.0 | 4.9 | 6.0 | 1.8 |
| | 500.0 | 98 | 3.7 | 4.3 | | |
| NBP | 5.0 | 97 | 4.3 | 5.2 | | |
| | 250.0 | 98 | 3.7 | 4.7 | 4.0 | 2.3 |
| | 500.0 | 99 | 3.2 | 3.9 | | |
| IS | 50.0 | 98 | 3.0 | 4.0 | 7.0 | 1.6 |

[a] $n = 6$.

Table 2
Linearity parameters

| Compound | Linearity (ng/mL)[a] | Equation coefficients, $y = a + bx$[b] | | $r^2$ | LOQ (ng/mL)[a] | LOD (ng/mL)[a] |
|---|---|---|---|---|---|---|
| | | *a* | *b* | | | |
| MTD | 10.0–1500.0 | 0.0029 | 0.0021 | 0.9997 | 1.0 | 0.4 |
| BPR | 5.0–500.0 | 0.0034 | 0.0040 | 0.9994 | 0.5 | 0.2 |
| NBP | 5.0–500.0 | 0.0031 | 0.0039 | 0.9993 | 0.5 | 0.2 |

[a] Plasma or urine concentration. The concentrations in the injected solutions can be found multiplying the reported values by two.
[b] $y$ = analyte/IS peak area ratio; $x$ = analyte concentration, ng/mL.

### 3.3. Method validation

#### 3.3.1. Linearity

Having assured the suitability of the SPE procedure, calibration curves were set up on blank plasma and urine by adding to the samples standard solutions of the analytes at different concentrations and of the IS at constant concentration (100 ng/mL) and subjecting the resulting mixture to the SPE procedure. Good linearity ($r^2 > 0.9992$) was obtained over the following concentration ranges in both matrices: 10.0–1500.0 ng/mL for MTD, 5.0–500.0 ng/mL for BPR and NBP (corresponding to the following concentration ranges in the injected solutions: 20.0–3000.0 ng/mL for MTD, 10.0–1000.0 ng/mL for BPR and NBP). The LOQ was 1.0 ng/mL for MTD and 0.5 ng/mL for BPR and NBP, while the LOD was 0.4 ng/mL for MTD and 0.2 ng/mL for BPR and NBP (corresponding to the following concentrations in the injected solutions: LOQ, 2.0 ng/mL for MTD and 1.0 ng/mL for BPR and NBP; LOD, 0.8 ng/mL for MTD and 0.4 ng/mL for BPR and NB). Both values were calculated according to the United States Pharmacopoeia [34] and "Crystal City" guidelines [35]. Linearity parameters are reported in detail in Table 2.

#### 3.3.2. Precision and extraction yield

Extraction yield (absolute recovery) and precision assays were carried out on blank plasma and hydrolysed urine spiked with analyte concentrations corresponding to the upper limit, middle point and lower limit of the calibration curves. The results of these assays are reported in Table 1.

Table 3
Drugs tested for possible interference

| Therapeutic class | Compounds | $t_R$ (min) |
|---|---|---|
| Analytes and IS | MTD | 5.1 |
| | BPR | 6.0 |
| | NBP | 4.0 |
| | IS | 7.0 |
| Antidepressants | Amitriptyline | 6.6 |
| | Citalopram | 4.4 |
| | Fluoxetine | 5.5 |
| | Fluvoxamine | 5.6 |
| | Imipramine | 5.5 |
| | Sertraline | 6.8 |
| | Venlafaxine | 4.4 |
| Anxiolytics–hypnotics | Bromazepam | 3.7 |
| | *Brotizolam* | *5.4* |
| | Clonazepam | 4.6 |
| | *Diazepam* | *6.0* |
| | Flurazepam | 4.6 |
| | Lorazepam | 4.4 |
| Abuse drugs | Codeine | 3.7 |
| | Morphine | 3.5 |
| | Amphetamine | n.d.[a] |
| | Methamphetamine | n.d. |
| | MDMA (Ecstasy) | 2.7 |
| | Δ9-THC (tetrahydrocannabinol) | n.d. |
| | 11-Nor-9-carboxy-Δ9-THC | 11.5 |

[a] n.d. = not detected within a 30-min run.

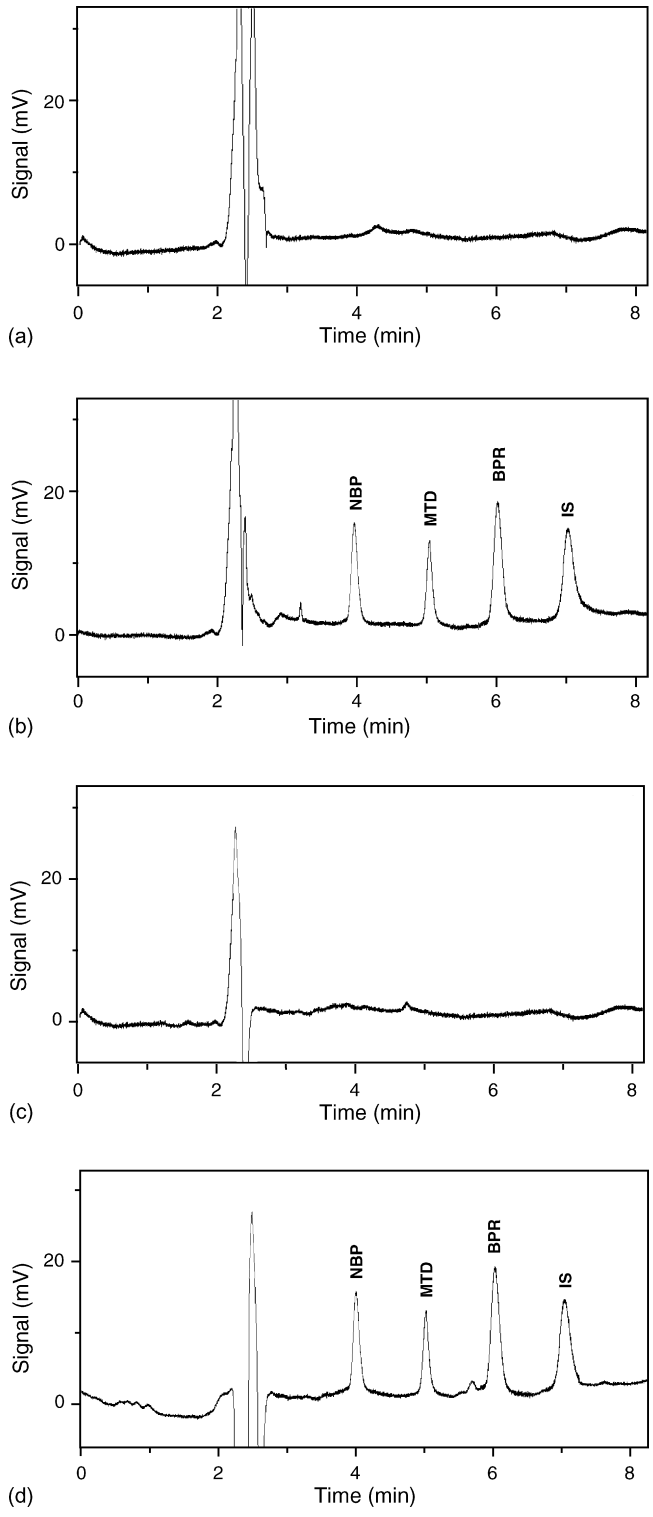


Fig. 2. Chromatogram of (a) a blank plasma sample; (b) a blank plasma sample spiked with 100 ng/mL of each analyte and the IS; (c) a blank urine sample and (d) a blank urine sample spiked with 100 ng/mL of each analyte and the IS. Concentrations in plasma and urine are half those reported, i.e., 50 ng/mL for all analytes and the IS. *Chromatographic conditions*: stationary phase, C8 reversed-phase column (250 mm × 4.6 mm i.d., 5 μm); mobile phase, methanol/acetonitrile/60 mM phosphate buffer containing 0.5% triethylamine (50/10/40, v/v/v), final apparent pH 6.0; flow rate, 1.0 mL/min; injection volume, 50 μL; detection wavelength, 214 nm.

As one can note, mean extraction yields were very good, being always higher than 96% for MTD, 97% for BPR and for NBP (98% for IS). Precision results were also satisfactory: R.S.D. values for repeatability were always lower than or equal to 5.5% for all analytes (3.0% for the IS); R.S.D. values for intermediate precision were lower than or equal to 6.1% for all analytes (4.0% for the IS).

### *3.3.3. Selectivity*

Selectivity was evaluated by injecting into the HPLC standard solutions of several drugs: antidepressants, anxiolytics–hypnotics and abuse drugs. The complete list of these drugs is reported in Table 3. As can be seen, only two of the tested drugs (brotizolam and diazepam) have retention times similar to those of the analytes. However, further assays demonstrated

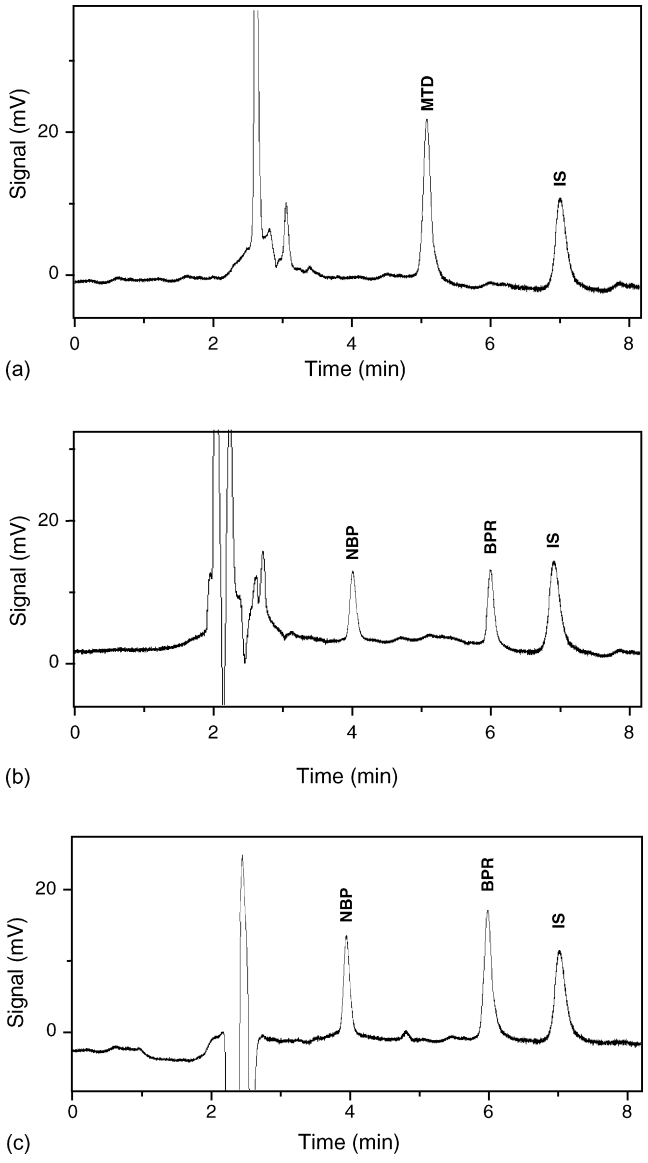


Fig. 3. Chromatograms of: (a) a plasma sample from a patient treated with 20 mg/day of MTD; (b) a plasma sample from a patient treated with 32 mg every other day of BPR; (c) a urine sample from a patient treated with 16 mg/day of BPR. *Chromatographic conditions*: as in Fig. 2.

that these compounds are not extracted with the developed SPE procedure, thus they do not give interference in the determination of MTD, BPR and NBP. Furthermore, six blank plasma samples and six hydrolysed urine samples were injected after SPE and none of them produced peaks from endogenous compounds which could interfere with the determination. Therefore, the method has demonstrated to be very selective.

### 3.4. Application to patient plasma and urine samples

Having thus validated the method, it was applied to the analysis of plasma and urine samples at the Drug Addiction Service, ASL of Rome (Italy), and at the Toxicological Analysis Laboratory of the "S. Maria delle Croci" Hospital, Ravenna (Italy), from subjects undergoing therapy with MTD or BPR. As an example, a chromatogram of a plasma sample from a patient undergoing therapy with 20 mg/day of MTD is shown in Fig. 3a. Again, peak shapes and resolution are very satisfactory and no interference is present. The MTD concentration found in this sample was 95.0 ng/mL. The chromatogram of a plasma sample from a patient undergoing therapy with 32 mg of BPR every other day is shown in Fig. 3b: peak shapes and resolution are very similar to those obtained with spiked blank plasma and no interference is apparent. The BPR concentration found in this real sample was 30.0 ng/mL, while that of NBP was 35.2 ng/mL. The extraction yield of the IS was 98%. An example of the analysis of a urine sample from a patient treated with 16 mg/day of BPR is shown in Fig. 3c: the neat and well resolved peaks are present, without any interference from the matrix. BPR levels were found to be 51.7 ng/mL and NBP levels were 50.4 ng/mL.

Accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations were added to plasma and urine samples containing known amounts of the analytes (i.e., samples which had been already analysed). Then, the recovery of the analytes was calculated, as well as the standard deviation of the assays. Mean recovery values were higher than or equal to 96% for all analytes. Thus, method accuracy is good.

## 4. Conclusion

The HPLC method with DAD detection presented herein for the simultaneous analysis of MTD, BPR and NBP is simple, rapid and sensitive.

The SPE procedure implemented for the sample pretreatment, based on C8 cartridges, allows obtaining very good extraction yield (>96% for all analytes) and precision (R.S.D. $\leq$6.1%) results and optimal purification from endogenous and exogenous interference. Furthermore, the method only needs minute amounts of biological fluids (300 μL). When compared to the other methods found in the literature which use SPE [18,24], the present method has the advantage of simultaneously determining MTD, BPR and NBP for TDM and of having better precision. Moreover, thanks to the SPE procedure, it allows to obtain very high extraction yields and selectivity. Hence, it is suitable for the TDM of patients switching from the former to the latter drug, as well as those who are subjected to monotherapy. It should be noted that this capability is very important since both MTD and BPR doses should be personalised and the most safe way to accurately switch from MTD to BPR is that of constantly monitor the levels of both drugs and the active metabolite in body fluids of the patients. In fact, this allows minimising adverse effects and maximising therapeutic efficacy, thus obtaining better compliance from the patients.

Finally, the method gives reliable and complete results for both drugs even in those cases when a patient undergoing substitutive therapy with BPR takes illicit MTD as an abuse drug.

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