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Journal of Chromatography B, 834 (2006) 188–194
JOURNAL OF CHROMATOGRAPHY B
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Short communication

Determination of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and alprazolam in human plasma by liquid chromatography–electrospray ionization mass spectrometry

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Received 27 October 2005; accepted 2 February 2006
Available online 6 March 2006

Abstract

A fast liquid chromatographic assay with mass spectrometric detection (LC/MS) has been developed and validated for the simultaneous determination of methadone (MT), its primary metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and alprazolam, in human plasma. The extraction procedure was performed with automatic solid phase extraction, and the compounds were separated with a Sunfire® column using a gradient mode. Deuterated analogues for all of the analytes of interest were used for quantitation. Limits of detection (LOD) were established between 0.5 and 1 ng/ml. Linearity was obtained over a range of 2–2000 ng/ml with an average correlation coefficient (R^2) of >0.99. Intra- and inter-batch coefficients of variation and relative mean errors were less than 10% for all analytes and concentrations. The recoveries were higher than 50.0% in all cases. The method proved to be suitable for evaluation of plasma obtained from patients enrolled in a MT-maintenance program who are frequently treated with alprazolam as a sedative.

Keywords: Methadone; Metabolite; Alprazolam; LC–MS plasma

1. Introduction

Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone, MT) is a synthetic opioid that is primarily used therapeutically in the management of withdrawal symptoms in heroin-dependent users during maintenance therapy. It has μ-opioid receptor agonist activity similar to that observed for morphine [1]. Racemic MT is administered to heroin users undergoing MT maintenance therapy (MMT) in Spain. The pharmacokinetics of MT has been found to be stereoselective, with large inter-individual variability [2,3]. The (*R*)- and (*S*)-enantiomers of MT possess different properties with respect to receptor affinity [4], metabolism [5] and protein binding [6]. There have been many publications describing the determination of MT enantiomers by liquid chromatography with UV detection [7–11], gas chromatography/mass spectrometry [12,13], liquid chromatography/mass spectrometry [14–16] and by capillary electrophoresis [17–19].

Alprazolam is one of the most prescribed benzodiazepines in our country, and despite that it is a safe anxiolytic agent, combined with CNS depressor like MT could be dangerous, even fatal [20].

The aim of the present study was to develop and validate a rapid, specific, sensitive, robust and reliable method for the simultaneous quantitative determination of the MT, its primary metabolite and alprazolam in human plasma by liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS).

2. Experimental

2.1. Chemicals and reagents

(±)MT HCl, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP) perchlorate, alprazolam, (±)MT-d_3, EDDP-

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doi:10.1016/j.jchromb.2006.02.013

d_3 perchlorate, and alprazolam-d_5 were purchased from Cerilliant™ (Austin, TX, USA). Formic acid (99% pure) was obtained from Merck® (Barcelona. Spain). Chromasolv® grade Acetonitrile (99.98% pure) was from Riedel de Häen Sigma-Aldrich Chemie (Schnelldorf, Germany). Purified water was obtained in the laboratory using a Milli-Q water system (Le Mont-sur-Lausanne, Switzerland). R.P. Normapur 2-propanol (99.9% pure), dichloromethane (99.5% pure) and ammonia 28% were purchased from Prolabo (Paris, France). A 0.1 M ammonium carbonate pH 9.30 buffer was prepared by adding a 1 M ammonium hydroxide solution to 900 ml of ammonium carbonate solution (9.6 g/l) to pH 9.3 (determined using a pH-meter) and making up the solution to 1000 ml with ammonium carbonate. Outdated drug-free human plasma was pooled from several donors.

Stock solutions of MT, EDDP and alprazolam and internal standard (I.S.) were prepared at 1 g/l separately in methanol and were kept at −20 °C in the dark for a maximum of 6 months. Each day, working solutions containing a mixture of MT, EDDP and alprazolam were prepared by appropriate dilution of stock solutions with Milli-Q water. The working solutions of respective internal standards at 1 mg/l were prepared by appropriate dilution with methanol and were kept at −20 °C in the dark.

Solid phase extraction (SPE) cartridges HLB (3 cc/60 mg) were supplied by Waters (Barcelona, Spain).

2.2. Sample preparation

Plasma samples (0.4–0.6 ml) were centrifuged 10 min at 14,000 rpm. A 250 μl sample of supernatant was collected and combined with 1 ml of carbonate buffer pH 9.3 and the appropriate amounts of working solutions of the compounds, in order to obtain the following calibrating levels: 0, 2, 4, 20, 80, 400, 1000 and 2000 ng/ml. After addition of 100 μl of the internal standard working solution, samples were vortex mixing (10 s) and centrifuged for 5 min at 14,000 rpm. Finally, the supernatant was transferred into a clean glass tube.

Automated solid-phase extraction was performed with a Gilson Aspect XL (Middleton, WI, USA). SPE cartridges were conditioned with 2 ml methanol and 2 ml deionised water. The sample solution (1350 μl) was applied on the SPE cartridge and passed slowly (1 ml/min) under positive pressure. Cartridges were washed successively with 2 ml deionised water and 2 ml of 0.5% ammonia in methanol/water (40:60, v/v), and dried under nitrogen (supplied by the apparatus) 10 min. Analytes of interest were eluted with 2 ml of mixture dichloromethane/2-propanol (75:25, v/v). The eluate was evaporated to dryness under nitrogen at room temperature. The dried sample was reconstituted in 80 μl of mobile phase and vortexed before injection of 10 μl into the LC–MS system.

2.3. Liquid chromatography–mass spectrometry

The LC system was a Waters Alliance 2795 (Waters, Watford, UK) separation module. Chromatographic separations were performed with a Sunfire® 3.5 μm (30 mm × 2.1 mm I.D.) reversed-phase column (Waters, Milford, USA). The mobile phase, delivered at a flow-rate of 0.6 ml/min at room temperature, was a gradient of acetonitrile in 0.1% formic acid programmed as follows: 18% acetonitrile during 0.5 min, increased to 60% in 2 min and decreased to 18%, i.e., original conditions, in 0.3 min and maintained until reaching 3 min.

Table 1
Retention times, selected ions and cone voltages of methadone, EDDP, alprazolam and the corresponding internal standards

Compound	Retention time (min)	Selected *m/z* ratios[a]	Cone voltage (V)
EDDP	0.8	**278.4**	40
		186.3	60
EDDP-d_3	0.8	**281.4**	40
Methadone	1.7	**310.4**	25
		265.3	35
Methadone-d_3	1.7	**313.4**	25
Alprazolam	2.2	**309.1**	30
		281.2	60
Alprazolam-d_5	2.2	**314.1**	30

[a] Quantifying ions are in bold characters.

The detection was performed using a Micromass ZMD 2000 mass spectrometer (Micromass, Manchester, UK) fitted with a Z-spray ion interface. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following ESI-MS parameter settings were applied: capillary voltage, 3 kV; nebulisation gas flow, 750 l/h; cone gas flow, 85 l/h drying gas temperature 375 °C and source heater temperature, 115 °C. High-purity nitrogen was used as nebulisation and desolvatation gas. For optimising ionization and ion transmission conditions of all the compounds, 10 μl of a 5 μg/ml solution of each substance in the mobile phase were injected without chromatographic separation into the mass spectrometer. In order to obtain the highest possible intensity for quantitation and confirmation ions, fragmentation energy ("cone voltage") was optimised for each one. During this experiment, a mass range from *m/z* 150 to 400 was monitored. Acquisition was made in the selected ion monitoring mode of positive ions, with a dwell time of 0.12 s. For quantitation purposes, the corresponding protonated molecule $[M+H]^+$ of each compound was selected as the quantification ion and one main fragment was selected as confirmation ion. Data acquisition, peak integration and calculation were interfaced to a computer workstation running MassLynx NT 3.5 and QuanLynx 3.5 software.

Table 1 shows retention times, selected ions and cone voltages for the analytes of interest and their corresponding internal standards.

2.4. Validation

The analytical validation was performed according the guiding principles of the FDA [21]. The following criteria were used to evaluate the method: sensitivity, linearity, intra- and inter-batch precision, accuracy and recovery.

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and lower limit of quantitation

(LLOQ). LOD was defined as the concentration with a signal-to-noise (S/N) ratio of at least 3, while LLOQ was the lowest standard with an S/N ratio of at least 10 and acceptable precision and accuracy. Both parameters were determined empirically by analysis of a series of decreasing concentrations of the drug-fortified plasma in five replicates.

Calibration, using internal standardization, was done by linear regression analysis over a maximum concentration range from 2 to 2000 ng/ml. For each standard curve, seven different concentrations were used, not including the blank matrix. Regression line was calculated by the method of least squares and expressed by the correlation coefficient (R^2). Precision and accuracy were evaluated over the linear dynamic range, at three different concentration levels. Intra-batch precision and accuracy was assessed by five determinations per concentration in 1 day, while inter-batch precision and accuracy was evaluated by six determinations per concentration in different batches. Precision (% coefficient of variation, %CV) was calculated as the percentage of the average divided by the standard deviation in peak area ratio of the replicates, while accuracy was expressed as the percentage of the ratio of calculated concentration to nominal concentration.

Recovery or extraction efficiency (%) for each analytes was determined (three times) at low and high concentration levels, and it was calculated by comparison of the peak-area ratios of the compounds spiked to blank samples and extracted as previously described with those of unextracted standards at the same concentration.

2.4.1. Ion suppression study

The absence of ion supression was demonstrated according the next procedure: five different sources of drug free plasma were extracted as described previously. The extracts were then fortified with all drugs at concentration of 50 ng/ml. A reference solution containing formic acid:acetonitrile (82:18) was also fortified with all drugs to the same nominal concentration. The reconstituted extracts and reference solutions were injected into the LC–MS system. Peak areas obtained from the extracts were compared with the corresponding peak areas produced by the reference solutions. The mean area ratios (reconstituted extract in plasma/reference solution) are presented in Table 2. Thus, no excessive ion-supression was observed for each drugs.

2.5. Application of the method

The validated method was applied to plasma samples obtained from four MMT patients. Each plasma sample was prepared based on the procedure detailed.

Table 2
Results of supression studies

Compound	Mean area ratios (reconstituted extract in plasma/reference solution)	CV
Methadone	0.994	12.3
EDDP	0.899	6.84
Alprazolam	1.02	12.6
Methadone-d_3	1.01	10.7
EDDP-d_3	0.907	6.50
Alprazolam-d_5	1.01	12.1

3. Results and discussion

3.1. LC–MS method development

The separation of three compounds was achieved in less than 2.5 min. The stability of the LC method was evaluated by calculation of the variation of retention times. R.S.D., calculated from retention times obtained over 150 injections, proved to be less than 3.0% for all compounds, indicating good chromatographic stability. Fig. 1 shows chromatograms of spiked plasma at 20 and 1000 ng/ml.

3.2. Calibration and validation

Calibration using internal standardization with deuterated analogues of the drugs was performed. Stable isotope internal standards were employed in order to minimize the effects on the ionisation potential of the compounds by interferences from the biological matrix or the LC method.

To prevent cross-talk interference caused by isotopic contributions to the target ion or by contribution of non-deuterated impurities in the standard, high-purity analogues with the highest possible extent of deuteration and highest purity were selected. For MT and 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP) only the d_3 analogue was commercially available. Alprazolam had a deuteration of at least 5. EDDP had a purity of at least 99.0% as reported by the manufacturer.

The completed method was evaluated according to the criteria described in Section 2. Representative linearity results are detailed in Table 3.

LOD was established between 0.5 and 1 ng/ml and LLOQ was 2 ng/ml for all cases (Fig. 2). Linearity was obtained with an average correlation coefficient of >0.99, over a dynamic range from the LOQ value up to 2000 ng/ml for each of the analytes.

Table 4 provides the results from the validation studies for all the analytes. The intra- and inter-precision ranged from

Table 3
Limit of detection (LOD), lower limit of quantitation (LLOQ), and calibration results for methadone, EDDP and alprazolam in plasma by LC/ESI/MS

Compound	Internal standard	LOD (ng/ml)	LOQ (ng/ml)	Equation	$R^2 \pm$ S.D.	Linear dymamic range (ng/ml)
Methadone	Methadone-d_3	1	2	$Y = -0.0085 + 0.0080X$	0.999 ± 0.00059	2–2000
EDDP	EDDP-d_3	0.5	2	$Y = 0.0017 + 0.0024X$	0.999 ± 0.00014	2–2000
Alprazolam	Alprazolam-d_5	1	2	$Y = 0.0004 + 0.0028X$	0.999 ± 0.00026	2–2000

S.D. on solpe: MTD: 0.0031, EDDP: 0.0015, ALP: 0.0021. S.D. on intercept: MTD: 0.0059, EDDP: 0.0125, ALP: 0.0056.

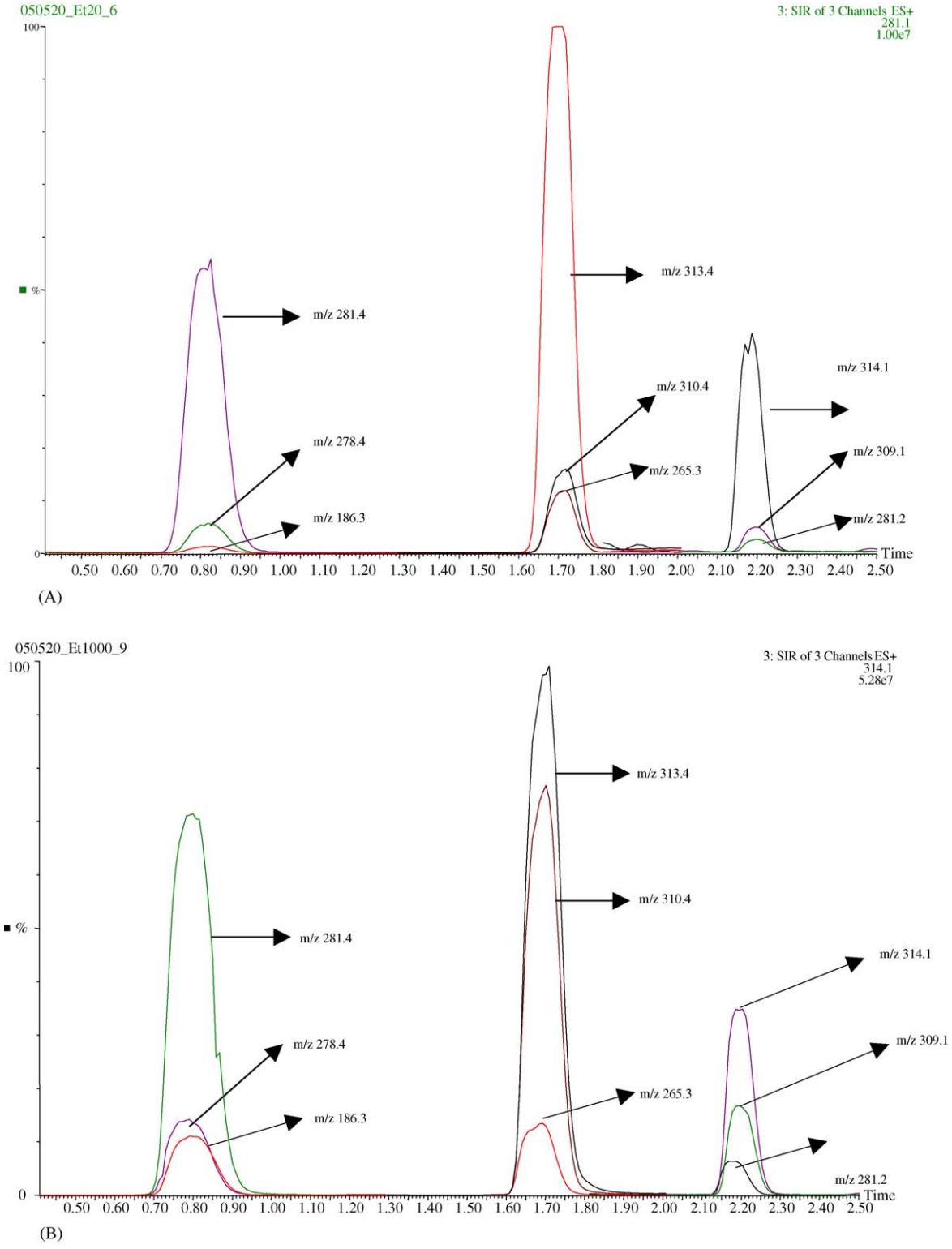

Fig. 1. Chromatograms of spiked plasma 20 ng/ml (A), and 1000 ng/ml (B).

2.42 to 2.91%CV and from 1.17 to 9.28%CV, respectively, while the intra- and inter-accuracy ranged from 1.25 to 6.09 and from 0.40 to 7.20, respectively for all three analytes. The results of the plasma validation study showed that both the intra- and inter-batch precision and accuracy for the method were satisfactory for all analytes. The recovery of the analytes at two concentrations (low and high) ranged from 50.0 to 91.2%.

The aim of this study was develop a very fast LC–ESI-MS method for the determination of the MTD, EDDP and alpra-

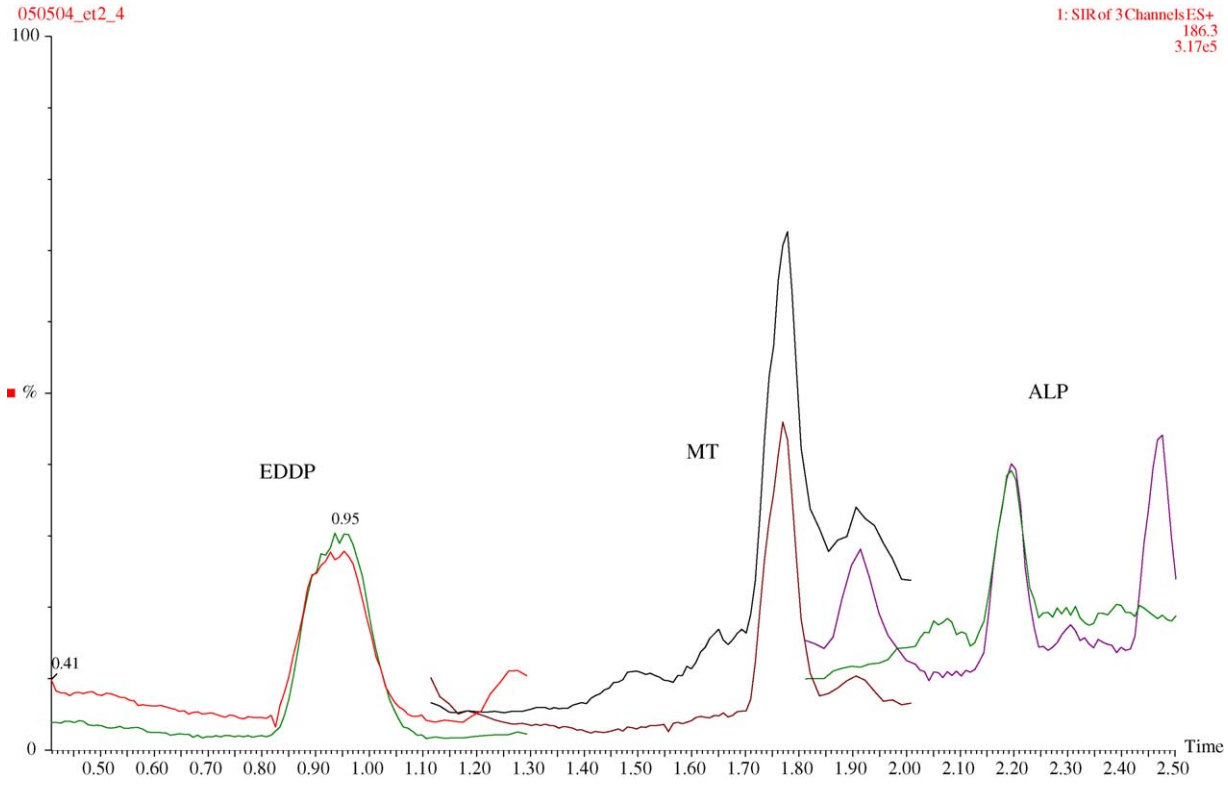

Fig. 2. Chromatograms of spiked plasma 2 ng/ml (LLOQ).

Table 4
Results from the validation studies for interested analytes

Concentration added (ng/ml)	Recovery (%) ($n=5$)	CV (%)	Intra-batch study ($n=5$)		Inter-batch study ($n=6$)	
			CV (%)	Relative mean error (%)	CV (%)	Relative mean error (%)
Methadone						
2			2.9	4.8	10.4	4.8
4					9.5	10.9
20	83.9	9.4			6.7	4.1
80					2.7	1.2
400			2.4	6.1	2.3	0.9
1000	83.6	10.8			2.2	2.1
2000			2.6	1.2	1.1	0.9
EDDP						
2			1.9	1.1	8.5	2.4
4					9.3	2.9
20	50.6	17.9			3.5	1.5
80				5.7	3.6	0.3
400			1.6	3.7	2.0	1.4
1000	58.1	22.3			1.9	0.8
2000			2.4	0.1	1.2	0.1
Alprazolam						
2			6.1	6.2	5.7	2.8
4					5.1	2.0
20	91.2	5.2			2.5	1.2
80					3.9	1.4
400			1.7	2.4	2.7	2.7
1000	89.0	9.8			2.5	0.9
2000			2.8	0.8	1.5	0.1

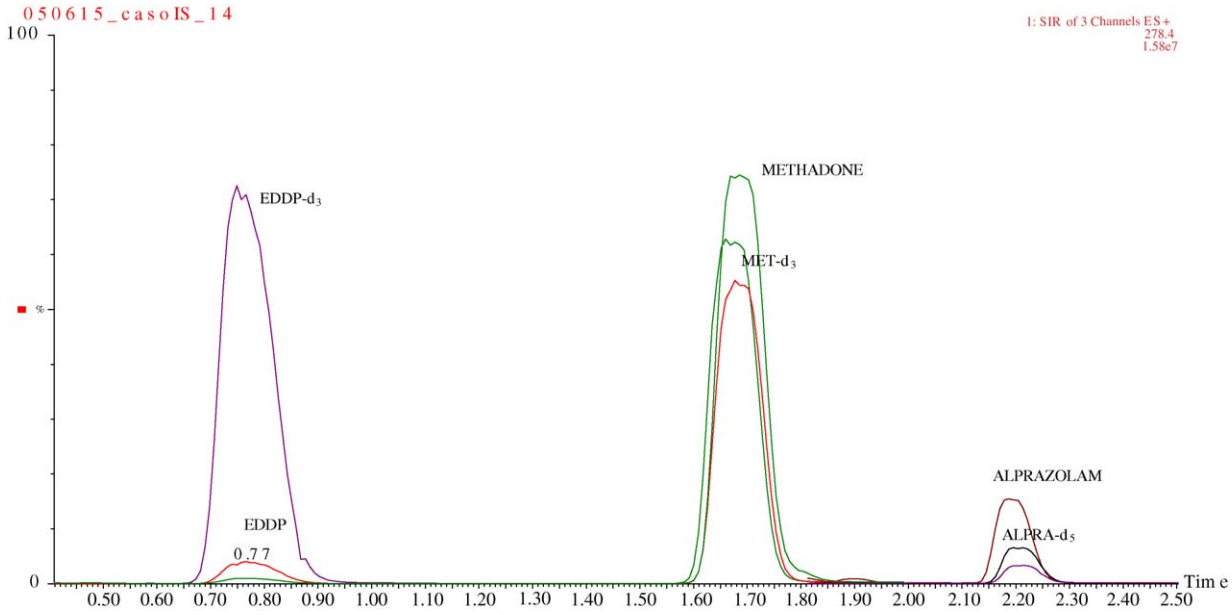

Fig. 3. Representative chromatogram of the analysis of a plasma sample collected from a patient who had received methadone (30 mg/day).

Table 5
Concentration of methadone, EDDP and alprazolam in plasma samples from patients in a methadone maintenance program (ng/ml)

Patient	Methadone dose (mg/day)	Methadone (ng/ml)	EDDP (ng/ml)	Alprazolam dose	Alprazolam (ng/ml)
1	30	375.5	22.4	uk	165.5
2	45	377.8	18.0	uk	35.0
3	90	757.8	46.5	uk	23.8
4	45	374.2	23.4	uk	15.3

uk: unknown.

zolam in a single run. The method achieves the sorter chromatography for these molecules up to now. The LLOQ founded for MTD and/or EDDP were according with other in the refs. [22–25]. We found a wide calibration range that is especially useful in forensic cases.

Finally, the method was applied to plasma specimens of patients undergoing methadone maintenance treatment with know methadone dose and unknown alprazolam dose. A representative chromatogram of a real sample from a patient who had received MT (30 mg/day) is presented in Fig. 3. Preliminary results obtained from four patients receiving 30, 45 or 90 mg/day are presented in Table 5. All of the samples were found to have quantifiable amounts of all analytes, with concentrations ranging from 375.5 to 757.7 ng/ml for MT, from 17.9 to 46.4 ng/ml for EDDP and from 15.2 to 165.5 ng/ml for alprazolam.

The method fulfilled our analytical standard criteria. LC–MS provided high specifity for all compounds, and no cross-talk interference with the deuterated internal standards was observed. The sensitivity and linear dynamic range of the method were clinically relevant to monitor drug use by plasma analysis. The method achieved precise and accurate plasma measurements of the compounds of interest. The recovery was acceptable. Analytes proved to be stable in stock solutions as well as during analysis. LC–MS proved to be a viable analytical tool for plasma analysis of licit and illicit drugs.

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