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Use of large particles support for fast analysis of methadone and its primary metabolite in human plasma by liquid chromatography–mass spectrometry

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Abstract

A bioanalytical method was developed for the quantitation of methadone (MTD) and its primary metabolite, (EDDP) in plasma. The extraction step was performed within a capillary column packed with large particles (35×0.3 mm I.D.; d_p 30 μm) at high flow-rate conditions (450 μl/min). The separation was performed on a microbore analytical column (55×2 mm I.D.; d_p 3 μm) coupled to a mass spectrometer (MS). This procedure was based on a column-switching unit. Analytes of interest were retained on the precolumn by hydrophobic interactions and backflushed from the precolumn to the analytical column. The detection was carried out with a MS single quadrupole equipped with an electrospray interface. The total analysis time was 6 min. The limits of quantification were evaluated at 10 and 25 ng/ml for MTD and EDDP, respectively. At this level, good accuracies were obtained for both analytes with repeatability values less than 18%.

Keywords: Column switching; Large particle support; Fast analysis; Methadone; 2-Ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine

1. Introduction

In pharmaceutical analysis, considerable effort is devoted to the development of fast, selective and sensitive analytical methods for the determination of drugs and metabolites in complex matrices. Therefore, liquid chromatography coupled with mass spectrometry (LC–MS) is often considered as the method of choice. In the case of biofluids such as serum, plasma and urine analysed by LC–MS, sample preparation is mandatory to remove endogenous material such as proteins, lipids or salts. This step prevents the clogging of the analytical column and reduces MS signal suppression due to the presence of salts and other endogenous materials. However, commonly used preparation methods such as solid-phase extraction (SPE), liquid–liquid extraction (LLE), membrane extraction (i.e. ultrafiltration, dialysis) or protein precipitation [1–3] are recognised as rate-limiting steps in the analytical process. Nevertheless, SPE, which has induced a great deal of interest in the last years as a powerful tool before LC analysis, can contribute to the simpli-

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fication and the speeding up of the sample preparation step [4–6]. The on-line SPE based on new extraction supports, such as restricted access materials (RAM) [7–10] and large particles supports [11–17], which allow the direct injection of biological fluids, can be considered as an attractive approach. The latter allow the application of high flow-rates and can, therefore, considerably reduce extraction time.

When a biological sample is injected on the large particle support (e.g. d_p 30–50 μm), endogenous materials such as proteins are rapidly eluted while small organic analytes are generally retained by hydrophobic interactions. Subsequently, analyte elution can be performed with an eluent containing a high percentage of organic solvent (i.e. acetonitrile, methanol). Several articles in the literature present the use of these extraction supports in single column [11–13] and in column-switching approaches [13–16]. Ayrton and co-workers [11,12] established that a high LC flow-rate is suitable for the rapid determination (less than 1.5 min) of a novel isoquinoline in biological fluids. Jemal et al. [13] developed two analytical methods for the quantitative analysis of drugs and their isomeric compounds (i.e. pravastatin and its isomeric metabolite) in plasma and serum, respectively.

With high flow-rates (4–5 ml/min) applied to 1 mm I.D. columns, a post-column splitter is necessary before the mass spectrometer. With columns of smaller I.D., such as capillary columns possessing inner diameters of 200–300 μm, the flow-rate can be decreased by a factor of 10–25-fold [18] (≈400 μl/min), which is fully compatible with conventional electrospray ionisation–mass spectrometry (ESI–MS) interfaces [19]. Furthermore, the mobile phase consumption is significantly reduced, as well as the injected sample amount. Finally, and as reported in the literature [20,21], the gain of sensitivity can also be important.

In this paper, a straightforward and fast bioanalytical method was developed for the quantitation of methadone (MTD) and its primary metabolite (EDDP) (Fig. 1) in human plasma. MTD is a synthetic opiate used to treat heroin addicts and is an analgesic drug in the treatment of severe pain. The interindividual MTD half-life is extremely variable (15–55 h) [22,23]. Therefore, the development of analytical methods for the quantification of MTD and its major metabolite in biological fluids is required in therapeutic drug monitoring [24]. For this purpose, different matrices can be used such as plasma [25,26], serum [27,28], urine [29] and saliva [30]. In this paper, the extraction method for human plasma was based on the on-line coupling of a precolumn packed with large size particle support to a microcolumn packed with 3 μm C_{18} material.

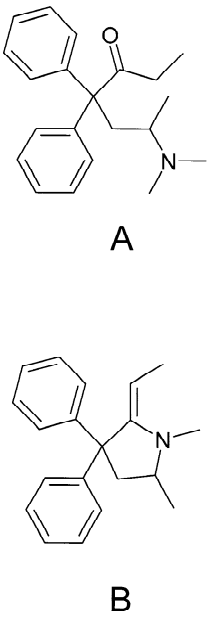

Fig. 1. Chemical structures of methadone and its metabolite. (A) Methadone (MTD); (B) 2-ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidone (EDDP).

2. Experimental

2.1. Chemicals

Methadone (MTD) was purchased from Hanseler (Herisau, Switzerland). 2-Ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine (EDDP) and deuterated methadone (MTD-D_9) were obtained from Radian International (Austin, TX, USA). All reagents were of analytical grade: acetonitrile and formic acid were from SDS (Peypin, France) and water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA).

2.2. Solutions

2.2.1. Aqueous stock solution of MTD and EDDP

Aqueous stock solutions of 10, 1 and 0.1 μg/ml of MTD and EDDP were prepared by appropriate dilutions of a 1000 μg/ml EDDP methanolic solution and of a 1000 μg/ml MTD aqueous solution (i.e. 56 mg of MTD–HCl dissolved in 50 ml water).

2.2.2. Internal standard solution

Deuterated methadone was used as internal standard (I.S.). An aqueous solution of 500 ng/ml was prepared by successive dilution of a 1000 μg/ml MTD-D_9 methanolic solution.

2.3. Sample preparation

Blank plasma was spiked using appropriate amounts of aqueous stock solution to reach a concentration range of 10–500 μg/ml. Spiked plasma was vortex mixed and diluted 1:1 (v/v) with a solution of 500 ng/ml of I.S. (final concentration of 250 ng/ml). After vortex mixing, samples were centrifuged for 5 min at 6000 *g*. Finally, 100 μl of supernatant was injected in the LC–MS system.

2.4. Liquid chromatography

On-line sample preparation and enrichment were performed using a column-switching configuration. The system comprised an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a binary pump used for conditioning and washing the precolumn, a six-port switching valve, a single wavelength UV detector and a mass spectrometer. An additional Agilent Series 1100 LC isocratic pump was included in the system to deliver the mobile phase for the analytical separation. The Chemstation software suite (Agilent Technologies) was used for instrument control, data acquisition and data handling. The switching valve was equipped with a BioStar precolumn (35×0.3 mm I.D., d_p 30 μm) from LC Packings (Amsterdam, The Netherlands). This precolumn was used as a sample extraction column. The separation was performed on a Purospher STAR RP-18e column (55×2 mm I.D. d_p 3 μm) from Merck (Darmstadt, Germany).

Each analysis involved different steps: sample extraction, analyte elution and separation, precolumn wash and re-equilibration. Fig. 2 shows the fluid flow pathways and switching valve configurations. Table 1 reports a complete analysis cycle time. First, 100 μl of sample was injected onto the extraction support with an aqueous mobile phase containing 0.1% (v/v) formic acid at a flow-rate of 450 μl/min (pathway A). Proteins and unretained endogenous hydrophilic material were diverted to the waste while analytes (MTD and EDDP) were retained on the precolumn. Simultaneously, the analytical column was conditioned with a mobile phase constituted of 0.1% (v/v) formic acid in water–acetonitrile mixture (65:35, v/v). After 1.5 min, the valve was switched

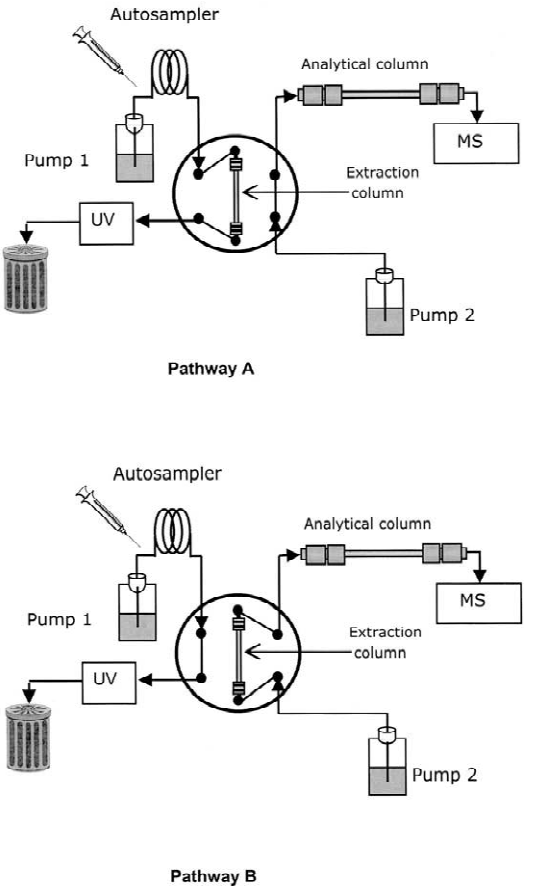

Fig. 2. Column switching configuration. (A) Sample loading, precolumn wash and re-equilibration position; (B) elution, separation. See Table 1 for switching valve position.

Table 1
Complete cycle analysis time with valve positions

Time (min)	Valve position (Pathway)	Comments
0.0	Loading (A)	Sample loading (100 μl) at 450 μl/min[a]
1.5	Elution (B)	Precolumn backflush onto the analytical column followed by an isocratic separation at 250 μl/min[b]
2.5	Loading (A)	Precolumn wash with high organic percentage at 450 μl/min[c]
4.5	Loading (A)	Precolumn re-equilibration with loading solvent at 450 μl/min[a]
6.0	Loading (A)	End of isocratic separation, ready for next injection

[a] Loading solvent: 0.1% (v/v) formic acid in water.
[b] Isocratic mobile phase: 0.1% (v/v) formic acid in water–acetonitrile (65:35, v/v).
[c] Washing solvent: 0.1% (v/v) formic acid in water–acetonitrile (20:80, v/v).

and analytes were eluted in the back flush mode with the mobile phase at a flow-rate of 250 μl/min and transferred to the analytical column (pathway B). After 1 min (t=2.5 min) the valve was switched to its original position for precolumn washing and reconditioning (pathway A). The precolumn washing was performed with a mobile phase constituted of 0.1% (v/v) formic acid in water–acetonitrile mixture (20:80, v/v) for 2 min. Meanwhile, analytes were separated on the analytical column and detected by mass spectrometry. Both, column and precolumn were thermostated at 25 °C.

2.5. Mass spectrometry

The LC system was coupled to an Agilent Series 1100 MSD single quadrupole (Agilent Technologies) equipped with an orthogonal electrospray interface. Nitrogen was used both as a nebulizing gas at a pressure of 25 p.s.i. (1 p.s.i.=6894.76 Pa), and as a drying gas at a temperature of 300 °C with a flow-rate of 10 l/min. Electrospray voltage was set to 3500 V (positive ionisation mode). Selected ion monitoring (SIM) mode was chosen for the analyzer with a skimmer voltage optimized for each ion: 65 V for ions 310 u (MTD+H^+) and 319 u (MTD-D_9+H^+), and 110 V for ion 278 u (EDDP+H^+). Dwell time was 392 ms for each ion.

3. Results and discussion

3.1. Method development

Large particle supports (i.e. d_p of 30 to 50 μm) allow injection of biological fluids directly with an aqueous mobile phase at a high flow-rate without generating a high back pressure [17]. This high flow-rate combined with the large size particle result in the rapid passage of proteins and other endogenous materials through the support while the analytes are generally retained by means of hydrophobic interactions. Many publications describe the use of single column approach in which the extraction column is directly coupled to the mass spectrometer [11–13]. However, these supports present low efficiency due to the size of the particles leading to a relatively poor resolution between analytes [13,16]. Therefore, incomplete separation may hamper the quantitative determination of drugs by MS signal suppression due to the co-elution of compounds and by interferences arising from biotransformation [13,31,32]. Moreover, isobaric compounds cannot be analysed. To overcome these limitations, the coupling of an extraction and a separation column is recommended. Additionally, this dual column setup offers better sensitivity than the single column approach since a preconcentration step can be performed.

In this work, a precolumn packed with a hydrophobic stationary phase was used for MTD and EDDP extraction. An aqueous loading mobile phase (formic acid 0.1%) allowed high extraction recovery and avoided protein precipitation into the system. This step was performed at a high flow-rate (450 μl/min) to avoid column clogging by the biomaterial [32]. Under these conditions, endogenous material was eluted from the extraction support in 1.5 min. This phenomenon was monitored with UV at 280 nm (Fig. 3). Simultaneously, the analytes were strongly retained by the extraction support without excessive backpressure. Placing a filter prior to the precolumn extended its lifetime by removing any particulates, which might remain in the sample after centrifugation. A C_{18} analytical column (Purospher STAR RP-18e) was chosen for the separation of MTD and EDDP [30]. The eluting mobile phase consisted of 0.1% (v/v) formic acid in water–acetonitrile mixture (65:35, v/v) to obtain a complete desorption of analytes from the precolumn and provide sufficient selectivity in the analytical column for the separation of MTD and EDDP. Retention times for MTD and EDDP were 3.4 and 2.9 min, respectively, with an overall analysis time of 6 min (sample preparation–separation). Fig. 4 shows chromatograms for a plasma spiked with 250 ng/ml of MTD and EDDP, and 250 ng/ml of I.S.

In preliminary tests, a carry-over between runs was observed (≈3%) for MTD and EDDP. In order to suppress this, a precolumn washing step was applied with 0.1% formic acid in water–acetonitrile (20:80, v/v). This step was performed simultaneously with MTD and EDDP separations to reduce the overall analysis time. Because the washing solvent contained already 20% of loading mobile phase, the precolumn re-equilibration time was shorter.

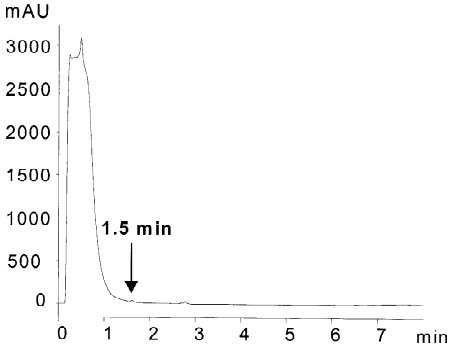

Fig. 3. Profile of endogenous material for spiked plasma at 250 ng/ml. LC–UV parameters: injection volume 100 μl. Extraction column: BioStar (35×0.3 mm I.D., 30 μm particle size). Loading mobile phase: 0.1% formic acid in water at a flow-rate of 450 μl/min. UV detection 280 nm.

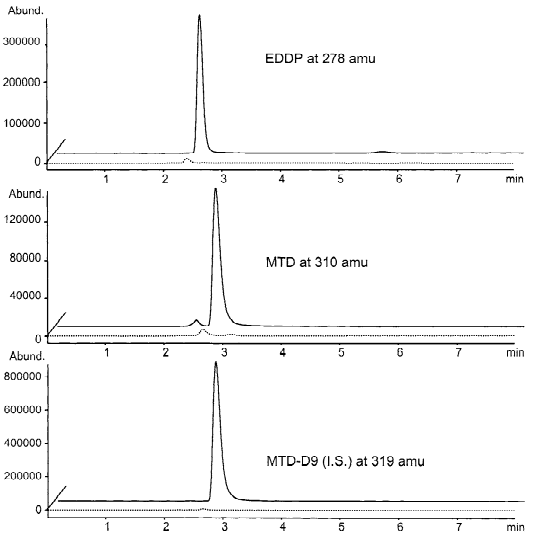

Fig. 4. Chromatograms of blank (- - -) and spiked (—) plasma at 250 ng/ml. LC–MS parameters: injection volume 100 μl. Extraction column: BioStar (35×0.3 mm I.D., 30 μm particle size). Loading mobile phase: 0.1% formic acid in water at a flow-rate of 450 μl/min. Analytical column: Purospher STAR RP-18e (55×2 mm I.D., 3 μm particle size). Elution mobile phase: 0.1% formic acid in water–acetonitrile (65:35, v/v) at a flow-rate of 250 μl/min in the back flush mode. MS detection: SIM at 310 u for MTD, 278 u for EDDP and 319 u for MTD-D_9.

3.2. Quantitative analysis

In order to estimate the potential of the developed method for the quantitative analysis of plasma samples, a preliminary validation was performed. The strategy involved two steps and, according to ICH recommendations [33], two kinds of plasma samples were prepared: calibration samples and quality control (QC) samples. In a first step, the most appropriate calibration curve model was selected. In

Table 2
Accuracy and repeatability values for MTD and EDDP ($k=4$, $n=3$)[a]

Theoretical concentration [ng/ml]	MTD		EDDP	
	Accuracy [%]	Repeatability[b] [%]	Accuracy [%]	Repeatability[b] [%]
10	107.2	17.4	41.4	28.2
25	107.8	9.9	92.0	10.7
250	100.0	1.5	101.3	3.6
400	101.9	2.8	97.5	1.7

[a] k = number of concentrations; n = number of plasma samples from different individuals.
[b] C.V. = coefficient of variation.

a second step, evaluation of limit of quantification (LOQ), method precision (i.e. repeatability) and accuracy were achieved with QC samples.

To evaluate the response function, six concentration levels ($k=6$) were performed over the range 10–500 ng/ml for MTD and EDDP with calibration samples. Peak–area ratios of analytes over I.S. were plotted as a function of theoretical concentrations of MTD and EDDP. As for QC samples, the first concentration level corresponded to the estimated LOQ in the development study. Since the relationship between the area ratio and the analyte concentration was linear in the considered calibration range, the simplest least squares method was selected. By applying this regression model, the response function for each analyte gave the following equations, where y is the analyte/I.S. area ratio and x the analyte concentration, $y = 0.0018x - 0.0025$ and $y = 0.0027x + 0.0223$ for MTD and EDDP, respectively. The linear model gave determination coefficients (R^2) of 0.9998 and 0.9970 for MTD and EDDP, respectively.

After fitting the calibration curves, the precision and accuracy for MTD and EDDP were determined with QC samples at four different concentrations ($k=4$), computed from the analytical responses. Each QC sample was analyzed in triplicate ($n=3$). The first concentration level (10 ng/ml) corresponded to the expected LOQ evaluated as a signal-to-noise ratio of 10 for both analytes in preliminary experiments. The three other concentrations (25, 250 and 400 ng/ml) were selected to represent the entire investigated range.

Method accuracy was evaluated by the recoveries (i.e. the ratio of measured concentration over theoretical concentration expressed in %). Repeatability was determined by the coefficient of variation (C.V., %) of accuracy assays at each tested level. As reported in Table 2, accuracy and precision were found to be satisfactory over the tested concentration range for MTD, in accordance with recommendations for the analysis of biological samples [34]. In fact for MTD, C.V. (repeatability) was lower than 10% with recovery values (accuracy) included between 90 and 110% of the target value. The lowest concentration tested (10 ng/ml) was confirmed as the LOQ for MTD with precision and accuracy values better than 20%. For EDDP, C.V. lower than 11% were obtained with accuracy values included between 90 and 110% of the target value at concentrations of 25, 250 and 400 ng/ml. For this compound, the first concentration level corresponding to 10 ng/ml was eliminated from the range and the LOQ set at 25 ng/ml using the simplest least square linear model.

Selectivity was assessed by processing QC coming from three different batches of plasma; no interfering endogenous compounds as well as no differences in recovery calculation were observed.

4. Conclusion

Direct injection of biological fluids in large size particle stationary phases was evaluated successfully for the analysis of MTD and its primary metabolite EDDP in human plasma. The developed method exhibited very good quantitative performances in terms of accuracy and repeatability with a total analysis time of less than 6 min. With an automated and fast sample preparation, the overall analytical

run time is drastically reduced compared to a conventional off-line extraction (e.g. LLE, SPE) followed by a LC separation (1–2 h). Coupling an extraction support with large particles to an analytical system is a very attractive approach to achieve high-throughput analysis of drugs and metabolites in biofluids and will certainly gain increasing popularity in the bioanalytical field.

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