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Short communication

Rapid determination of methadone and its major metabolite in biological fluids by gas–liquid chromatography with thermionic detection for maintenance treatment of opiate addicts

Nassima Chikhi-Chorfi[a], Chuong Pham-Huy[a,*], Hervé Galons[b], Nathalie Manuel[c], William Lowenstein[c], Jean-Michel Warnet[a], Jean-Roger Claude[a]

[a]*Laboratoire de Toxicologie (EA 207), Faculté de Pharmacie, Université de Paris V, 4 avenue de l'Observatoire, 75006 Paris, France*
[b]*Laboratoire de Chimie Organique, Faculté de Pharmacie, Université de Paris V, 4 avenue de l'Observatoire, 75006 Paris, France*
[c]*Centre Monte Cristo, Hôpital Laennec, 42, rue de Sèvres, 75007 Paris, France*

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Abstract

A rapid gas–liquid chromatographic assay is developed for the quantification of methadone (Mtd) and its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), in biological fluids of opiate addicts. After alkaline extraction from samples with lidocaine hydrochloride as internal standard, Mtd and EDDP are separated on SP-2250 column at 220°C and detected with a thermionic detector. The chromatographic time is about 6 min. The relative standard deviations (R.S.D.) of Mtd and EDDP standards are between 1.5 and 5.5%. Most drugs of abuse (morphine, codeine, narcotine, cocaine, benzoylecgonine, cocaethylene, dextropropoxyphene etc) are shown not to interfere with this technique. The method has been applied to study the levels of Mtd and EDDP metabolite in serum, saliva and urine of patients under maintenance treatment for opiate dependence. EDDP levels were found higher than those of Mtd in urine samples from four treated patients, but lower in serum and undetectable in saliva. However, Mtd concentrations were higher in saliva than in serum.
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1. Introduction

Methadone (Mtd) is a synthetic analgesic agent widely used in programs for the treatment of narcotic addiction [1]. In humans, Mtd is mainly metabolized by hepatic cytochrome P-450 3A4 and secondary by P-450 2D6 to a major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), resulting from an *N*-demethylation followed by a cyclisation of mother molecule [2]. Because of inter-individual differences in the Mtd pharmacokinetics, it is necessary to control the levels of Mtd and also of its major metabolite in biological fluids in order to obtain maximum treatment efficacy and to prevent toxicity.

For the quantification of Mtd in biological matrices, previously published analytical assays include gas chromatography (GC) using either flame ionisation [3–6] or thermionic detection [7,8], mass spectrometry [9], high-performance liquid chromatography (HPLC) [10,11], fluorescence polarization

*Corresponding author

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immunoassay (FPIA) [11] and radioimmunoassay (RIA) [12]. Recently, some stereoselective analyses of Mtd including enantioselective HPLC using either the α1-acid glycoprotein (AGP) column [13,14] or the β-cyclodextrin RSP-bonded phase [15,16], chiral GC [17] and stereospecific RIA [18] have been reported in the literature. Most of them analyze only Mtd and not its major metabolite EDDP.

The purpose of this report is to develop a rapid and specific method for the simultaneous determination of Mtd and its major metabolite, EDDP, in human biological fluids by gas liquid chromatography utilizing lidocaine hydrochloride as internal standard and SP-2250 column with thermionic detection. The method has been applied to study the levels of Mtd and EDDP in serum, saliva and urine of addicts under maintenance treatment for narcotic dependence.

2. Experimental

2.1. Chemicals

Racemic methadone hydrochloride was kindly provided by Central Pharmacy of Hospitals (Paris, France). Major primary methadone metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine perchlorate (EDDP), lidocaine hydrochloride used as internal standard (I.S.) were purchased from Sigma (St Louis, MO, USA). Other chemicals, drugs and solvents were of analytical purity and all obtained from Sigma.

2.2. Apparatus and chromatographic conditions

A Delsi model 30 gas chromatograph (Perichrom, Paris, France) equipped with a thermionic detector was connected with an Enica 21 integrator (Perichrom, Paris, France). The chromatographic separation was performed using a 1.80 m×3 mm I.D. glass column packed with 3% SP-2250 on Supelcoport 100–120 mesh (Supelco Inc., Bellefonte, PA, USA). The column was maintained at 220°C and the injection port and detector were operated at 250°C and 280°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 30 ml min^{-1}, which developed a head column pressure of about 1.5 bars. The hydrogen and air flow-rates were 30 and 300 ml min^{-1}, respectively. The chart speed of the integrator was 5 mm min^{-1}. The injection volume of samples and standards was 10 μl.

2.3. Preparation of standards

Three stock standard solutions were obtained by dissolving individually racemic Mtd hydrochloride, EDDP perchlorate (metabolite) and lidocaine hydrochloride (I.S.) in methanol at 1.0 mg ml^{-1} (free base). Stock solutions were stable for several months at 4°C. Standard calibration solutions were prepared by spiking drug-free human serum, saliva or urine with stock standard solutions further diluted to achieve final concentrations between 0.05 and 2.0 μg ml^{-1} of Mtd and of EDDP and stored at −20°C. An I.S. working solution at 15 μg ml^{-1} was obtained by dilution of an aliquot of lidocaine stock solution with methanol.

2.4. Extraction procedure

One ml of thawed biological samples or standard calibration solutions were added into a 15 ml centrifuge glass tube, then followed successively by 100 μl of I.S., 500 μl of 10% anhydrous sodium carbonate in water and 4 ml of hexane. 0.5 ml of 2-propranol was added in the cases of serum and saliva samples for avoiding emulsion. The tubes were tightly capped and vortex-mixed for 2 min. After centrifugation for 10 min at 1500×*g*, the upper organic layer was transferred into a 5 ml glass tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue extract was regenerated with 50 μl of methanol and vortex-mixed for 30 s. A 10 μl aliquot of the extract was then injected onto the column.

2.5. Validation of the method

It was performed by assaying two human serum, saliva and urine standards and samples determined in a day (n=6) and from replicate analysis on six separate days in order to achieve the intra-day and inter-day relative standard deviations (R.S.D.), respectively. The linearity of standard calibration curves of biological fluids was performed by simple

measures of spiked standard samples over 6 days in the range of 0.05–2.0 μg ml^{-1}. The validation of the method was also tested by comparing the results of six serum and twelve urine samples from two treated patients obtained by this GC method with those achieved by enantioselective HPLC as previously described [16]. To calculate the recovery, spiked serum, saliva or urine samples (0.1 and 0.5 μg ml^{-1}) were extracted as described above. Peak areas from extracted samples (n=6) were compared to peak areas from injection of appropriate standard solutions diluted in methanol. The interference with this method was determined by injection of different drugs of abuse or common drugs onto the column either directly or after alkaline extraction.

2.6. Application of the method

Four patients under maintenance treatment for opiate dependence received orally a single dose of

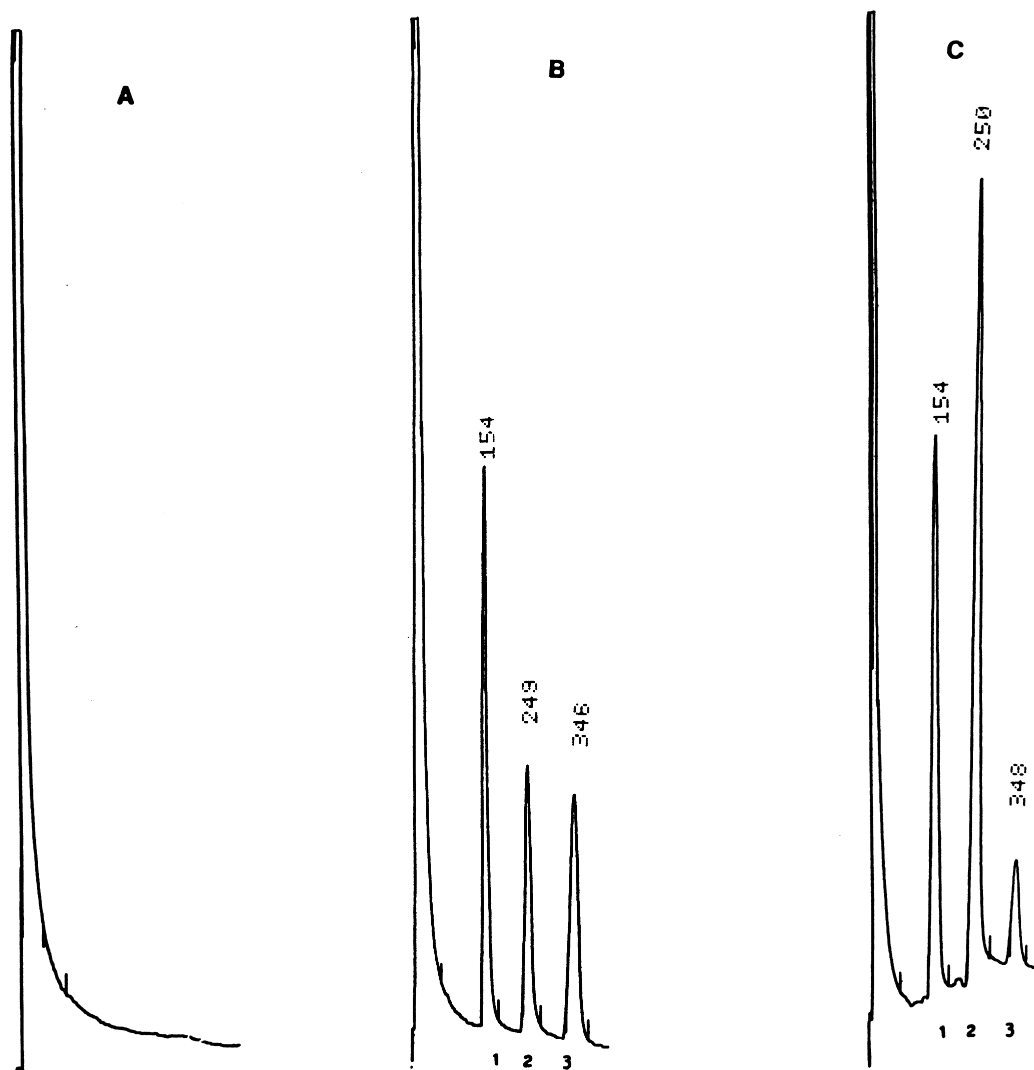

Fig. 1. Chromatograms of methadone (Mtd) and EDDP metabolite in human urine after extraction. A=Blank urine; B=Human urine standard spiked with 200 ng ml^{-1} of Mtd and EDDP; C=Patient urine 3h after oral administration of 90 mg Mtd. Peaks 1, 2, 3=I.S, EDDP, Mtd, respectively.

Table 1
Precision, linearity, recovery of methadone (Mtd) and EDDP standards ($n=6$)

Compound	Precision (R.S.D.%)				Linearity of calibration standards: 0.05–2.0 μg ml^{-1}	Recovery (%)	
	0.1 μg ml^{-1}		0.5 μg ml^{-1}			0.1 μg ml^{-1}	0.5 μg ml^{-1}
	Intra-day	Inter-day	Intra-day	Inter-day			
Serum							
Mtd	2.75	5.26	1.45	3.75	r=0.9990 y=1.15x+0.050	89.4±4.3	91.5±2.4
EDDP	2.12	3.40	1.15	2.96	r=0.9992 y=1.31x+0.036	91.2±3.4	92.4±4.8
Saliva							
Mtd	3.46	5.52	2.84	4.60	r=0.9992 y=1.67x+0.021	92.7±4.7	93.2±3.1
EDDP	2.52	5.14	2.20	3.95	r=0.9993 y=1.70x+0.036	91.8±3.9	94.7±5.4
Urine							
Mtd	2.35	3.92	1.82	4.24	r=0.9999 y=1.58x−0.010	91.0±4.1	92.5±2.7
EDDP	2.24	3.25	1.11	3.98	r=0.9997 y=1.64x+0.016	92.8±3.1	94.2±2.6

racemic Mtd solution (from 60–100 mg per dose) in the morning. A number of urine, serum and saliva samples were collected at different times in the day and used to test the applicability of this method. All biological samples were frozen at −20°C before use.

2.7. Calculation

The Mtd and EDDP metabolite concentrations in the biological samples was calculated on the peak area ratio of each analyzed compound to the I.S.. The ratio values found were reported on a standard calibration curve performed under the same conditions as described above.

3. Results and discussion

3.1. Chromatography

Typical chromatograms of drug-free human urine, standard calibration urine and treated patient urine are shown in Fig. 1. Their peak form was symmetric.

Table 2
Precision of methadone (Mtd) and EDDP metabolite in biological fluids of two treated patients ($n=6$)

Compound	Patient no. 1			Patient no. 2		
	Concentration (μg ml^{-1})	R.S.D.% (n=6)		Concentration (μg m^{-1})	R.S.D.% ($n=6$)	
		Intra-day	Inter-day		Intra-day	Inter-day
Serum						
Mtd	0.105	3.12	5.20	0.215	2.14	4.50
EDDP	0.045	3.85	6.64	0.062	2.86	5.85
Saliva						
Mtd	0.175	2.77	4.80	0.320	1.72	3.50
EDDP	N.F.	N.D.	N.D.	0.020	4.50	7.80
Urine						
Mtd	2.160	1.92	3.94	4.310	1.25	3.80
EDDP	6.524	1.71	2.98	9.725	1.62	3.54

Legends: N.F.=not found; N.D.=not determined.

3.2. Statistical data

The standard calibration curves of serum, saliva and urine exhibited good linearity for Mtd and EDDP over the range of concentrations tested with correlation coefficients greater than 0.999 for both compounds (Table 1). The intra-day and inter-day R.S.D.s (n=6) of two serum, saliva and urine standards (0.1 and 0.5 μg ml^{-1} Mtd and EDDP) and of serum, saliva and urine samples from two treated patients are shown in Tables 1 and 2. The detection limit (signal-to-noise ratio >3) of the assay after extraction was about 1.0 ng ml^{-1} for EDDP and 1.5 ng ml^{-1} for Mtd. The recovery of Mtd and EDDP metabolite from two previous serum, saliva and urine standards is good and shown in Table 1 and that of I.S. is about 90%.

A comparison between the Mtd and EDDP concentrations in six serum samples and 12 urine ones from two treated patients determined by this GC method and the levels of the sum of Mtd enantiomers and of metabolite in the same samples obtained from an enantioselective HPLC previously described [16] gave congruent results with correlation coefficients r=0.989 and 0.994 for Mtd and EDDP in serum, respectively and 0.992 and 0.998 for these two compounds in urine, respectively (Table 3).

3.3. Interferences

Most drugs of abuse such as morphine, codeine, dionine, narcotine, nalorphine, cocaine, benzoylecgonine, cocaethylene, dextropropoxyphene were shown not to interfere with I.S., EDDP and Mtd by direct injection or after alkaline extraction of standard solutions of these compounds. All barbiturates, some antiepileptic drugs (phenytoin, valproic acid), salicylic acid and acetylsalicylic acid did not appear on the chromatogram after alkaline extraction. Some benzodiazepines such as diazepam, nitrazepam, oxazepam did not interfere with this method. No endogenous interfering peaks were observed with drug-free human serum, saliva and urine at the retention times of I.S., EDDP and Mtd (Fig. 1A).

3.4. Application of the method

The results show that in urine samples from four treated patients, EDDP levels were always higher (about 1.5–5 fold) than those of Mtd. In contrast, the metabolite concentrations were undetectable or very little in saliva samples and always lower (about 2–4 fold) in serum samples than those of Mtd. Mtd levels in saliva in most cases were shown greater than those in serum. It is noted that there is a polymorphism of

Table 3
Comparison between Methadone (Mtd) and EDDP concentrations in 6 serum samples and 12 urine ones determined by this CGL and a HPLC method

Samples	Serum (ng ml^{-1})				Urine (μg ml^{-1})			
	Methadone		EDDP		Methadone		EDDP	
	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC
No. 1:	105	112	40	45	1.04	0.95	1.89	1.78
No. 2:	215	232	68	64	1.05	1.17	2.85	2.92
No. 3:	695	740	98	85	1.59	1.49	3.25	2.87
No. 4:	898	932	150	158	2.15	2.04	3.29	3.34
No. 5:	1260	1370	209	218	2.17	2.35	3.38	3.12
No. 6:	1927	1830	415	375	2.31	2.58	3.71	3.64
No. 7:	N.D.	N.D.	N.D.	N.D.	2.53	2.37	3.80	3.95
No. 8:	N.D.	N.D.	N.D.	N.D.	3.08	2.95	4.60	4.75
No. 9:	N.D.	N.D.	N.D.	N.D.	3.87	3.67	5.04	4.90
No. 10:	N.D.	N.D.	N.D.	N.D.	4.00	4.20	6.52	6.43
No. 11:	N.D.	N.D.	N.D.	N.D.	4.31	4.52	9.72	9.10
No. 12:	N.D.	N.D.	N.D.	N.D.	6.70	6.32	12.30	11.90

Results: r =0.989 and 0.994 for Mtd and EDDP, respectively in serum (n=3), and r=0.992 and 0.998 for Mtd and EDDP, respectively in urine (n=3).
Table legend as per Table 1.

CYP 2D6. In people with CYP 2D6 deficiency (5–10% of the Europeans), EDDP metabolite will probably not appear, whereas, in ultra rapid metabolizers (2%), Mtd will possibly disappear. Three representative Mtd and EDDP kinetic curves in serum, saliva and urine samples from a patient (no.

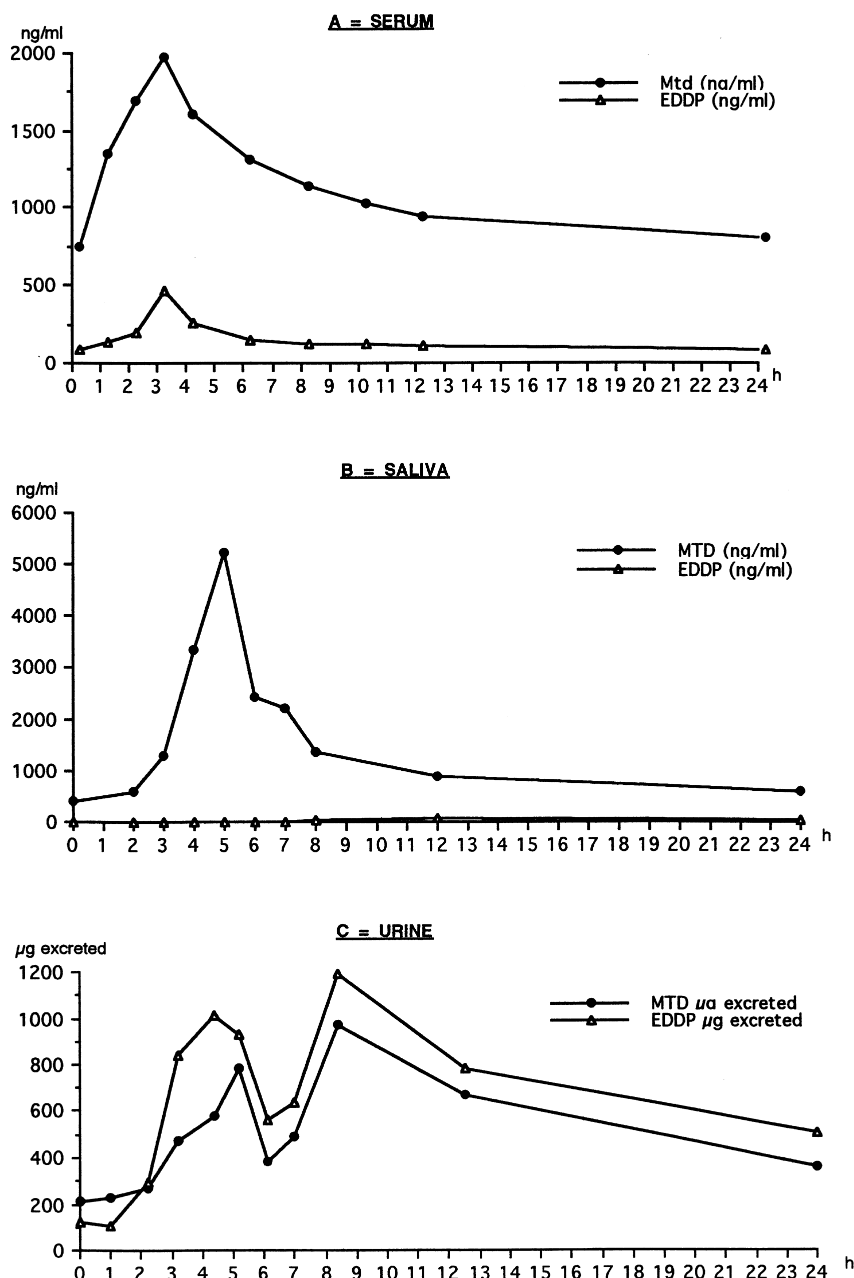

Fig. 2. Concentration-time curves for methadone (Mtd) and EDDP in patient no. 4 after the oral ingestion of 70 mg Mtd-HCl, at time 0 and after one month under Mtd maintenance treatment A=Serum (n=3 with R.S.D.<6.5%); B=Saliva (n=3 with R.S.D.<3.8%); C=Urine (n=3 with R.S.D.<4.5%).

4) after oral administration of 70 mg Mtd HCl and after one month under Mtd maintenance treatment are shown in Fig. 2A, B and C, respectively. In four patients, the times of peak levels (Tmax) of Mtd and EDDP were approximately the same and found between 3 and 4 h for serum and 4 and 10 h for urine; those of Mtd only for saliva varied between 2 and 5 h.

The method described in this paper shows improvement over existing GC assays in several aspects. Most of procedures [3–8] did not study the interferences of main drugs of abuse such as narcotic drugs which are often present in biological matrices of addicts and could interfere with Mtd, EDDP or I.S. and some of them did not analyze EDDP metabolite [5,6,8]. The simultaneous determination of Mtd and EDDP metabolite is important in the therapeutic control of treated patients because the presence of the mother compound is sometimes undetectable in urine. Moreover, the knowledge of EDDP levels allows to explain the inter-individual differences in Mtd pharmacokinetics and the effects of enzymatic inducer compounds associated with this drug. Besides, the proposed method presented a simple extraction procedure and a short chromatographic analysis time which are two important parameters for the routine determination in series of these two compounds. The choice of lidocaine as I.S. is justified by its retention time shorter than that of Mtd and EDDP, its availability and its absence in drugs used for the addiction therapy. Some other techniques used either dextropropoxyphene as I.S. [8], which is a drug often administered in maintenance treatment for opiate dependence, or synthesized I.S. such as 2-dimethylamino-4,4-diphenyl-5-nonanone [4,7] and SKF 525-A [3] which are not available. The thermionic detection used in this technique is more sensitive than the flame ionization detection used by some previous GC methods (3–6) for the Mtd and EDDP determination and can also reduce the solvent peak of the sample extracts.

In conclusion, because of its rapidity, its simplicity and its specificity, the present procedure is suitable for routine determination of Mtd and EDDP metabolite in therapeutic monitoring and for overdosage treatment in emergency toxicology.

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