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ANALYSIS OF METHADONE AND METABOLITES IN BIOLOGICAL FLUIDS WITH GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

The analysis of methadone and its metabolites in biological fluids by gas chromatography—mass spectrometry is described with deuterated methadone and metabolites as internal standards. The method allowed the determination of 20 ng methadone in 0.5 ml of plasma or saliva. Mean saliva to plasma ratio of methadone for two patients was determined to be 0.51 ± 0.13. Methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in urine were measured by selected ion monitoring. Gas chromatography—mass spectrometry was found to have advantages over conventional gas chromatographic methods in terms of ratio analysis. 1,5-Dimethyl-3,3-diphenyl-2-pyrrolidone previously reported as a metabolite was shown to result primarily from the decomposition of EDDP free base.

INTRODUCTION

In order to study methadone metabolism, many analytical methods have been developed to quantify methadone and its metabolites. Gas chromatography (GC), which is the method of choice for the analysis of human samples, has achieved the analysis of 5—15 ng/ml of methadone and the major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) [1—4]. 2-Ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP), a minor metabolite, could not be quantified in human urine because of a lack of sensitivity and selectivity of GC [5]. A selected ion monitoring (SIM) assay by gas chromatography—mass spectrometry (GC—MS) under electron impact (EI) conditions was described for the quantitation of methadone by monitoring *m/e* 294 (methadone), *m/e* 297 ([$^2H_3$]methadone), and *m/e* 308 for the internal standard, 2-dimethylamino-4,4-diphenyl-5-octanone [6]. By this method, methadone in plasma was assayed with a sensitivity limit of 5 ng/ml. [$^2H_5$]Methadone has been synthesized and utilized for the SIM assay of methadone in human

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plasma using chemical ionization (CI) [7]. The sensitivity limit of this method was similar to that reported using the EI—SIM method. The CI—GC—MS method has also been applied to the enantiomeric assay of [$^2H_3$]methadone and [$^2H_5$]methadone using the $^2H_8$-analogue as the internal standard [8]. Although SIM analysis is adequate to accurately quantitate 5 ng/ml levels of methadone in human plasma, the low abundance of the ions monitored under EI conditions has hindered the development of a more sensitive method. The relatively low sensitivity achieved by using [$^2H_5$]methadone even under CI conditions may be a factor of instrument conditions.

We have previously described the synthesis of deuterated methadone and metabolites [9]. These compounds have been used for the detection of metabolites and for studies of methadone interaction with other drugs. In this report we describe the applicability of the synthesized compounds to the SIM analysis of methadone and its metabolites in biological samples. Our emphasis was placed on the use of a high abundance ion for the analysis of methadone and to the use of ratio analysis.

EXPERIMENTAL

Materials

2-Dimethylamino-4,4-diphenyl-5-nonanone was prepared by the method of Lynn et al. [3]. The product was not distilled but was crystallized from diethyl ether solution as the perchlorate salt. Recrystallization from diethyl ether—ethanol gave crystals, m.p. 137—139°C. Methadone·HCl, [$^2H_3$]methadone·HCl, [$^2H_{10}$]methadone·HCl, EDDP perchlorate, [$^2H_3$]EDDP perchlorate, [$^2H_{10}$]EDDP perchlorate, EMDP·HCl, [$^2H_{10}$]EMDP·HCl, 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (DDP) and [$^2H_{10}$]DDP were obtained as described [9]. The purity and melting point of EDDP perchlorate were determined by differential scanning calorimetry; m.p. 177°C, recrystallized from diethyl ether—ethanol (literature value [10] m.p. 167—168°C).

Samples

Plasma, saliva, and urine samples were obtained from a pharmacokinetic study of four female methadone maintenance patients which was conducted by the Alcohol and Drug Commission, Vancouver, Canada. Maintenance dosage levels were: patient A, 30 mg/day; and patient B, 90 mg/day. Plasma and saliva samples were taken at 0, 2, 4, 6, 8, 11, 12 and 24 h after the usual daily dose. Urine samples were obtained at 1, 3, 5, 7, 9, 13 and 24 h. A special protocol was used to obtain saliva samples. After the oral dose of methadone was taken, the patients were instructed to rinse the mouth with 250 ml of water. Patients were not allowed to eat or drink just prior to providing a saliva sample. The mouth was rinsed thoroughly with water before the sample was taken. All samples were stored frozen until analysed.

Extraction procedures and standard curve preparation

Plasma and saliva. Saliva was centrifuged in order to remove solids. To plasma or saliva samples (0.5 ml) was added 0.2 ml of internal standard (I.S.), 2-dimethylamino-4,4-diphenyl-5-nonanone perchlorate (a stock solution of I.S.

was prepared to contain 10 mg/ml in methanol which was diluted with water to make a solution equivalent to 200 ng in 0.2 ml water). The solution was diluted to 3 ml with water and 0.1 ml of 1 *N* sodium hydroxide was added. After adding methylene chloride (15 ml) the solution was vortex mixed for 3 min. The aqueous layer was aspirated off and the methylene chloride layer was dried over anhydrous sodium sulfate. The dried methylene chloride was decanted (approximately 13 ml) and evaporated under nitrogen. The residue was dissolved in 50—100 μl of methanol and a 2—5 μl aliquot was injected onto the GC—MS system. Standard curves were prepared by spiking control samples of plasma and saliva (0.5 ml) with methadone in the amounts of 0, 20, 40, 100, 200 and 500 ng. The peak area ratios of methadone/internal standard obtained by monitoring *m/e* 72 were plotted vs. the concentration of methadone.

Urines. After thawing the sample, to 1 ml of the urine was added 0.2 ml of solution containing the internal standards at concentrations of 20 μg of [$^2H_{10}$]-methadone, 10 μg of [$^2H_{10}$]EDDP, and 10 μg of [$^2H_{10}$]DDP per ml. The mixture was diluted to 5 ml with distilled water and the pH was adjusted to 7—8 with 0.1 *N* sodium hydroxide. The mixture was extracted by vortex mixing for 2 min with methylene chloride (15 ml). The methylene chloride extract (13 ml) was dried over anhydrous sodium sulfate and taken to dryness under nitrogen. The residue was taken up in methanol (0.1—0.4 ml) and a 2—5 μl aliquot was injected onto the GC—MS system. For standard calibrations varying amounts of methadone (0.5—1 μg) were added to control urines (1 ml). Blank samples containing only the deuterated internal standards were also prepared in order to subtract background interferences resulting from isotopic impurity and column bleeding. Calibration curves were prepared by plotting the peak area ratios of unlabelled/labelled compound at each of the two ions monitored vs. the known concentration ratio of unlabelled compound to its corresponding labelled internal standard. Monitoring ions were *m/e* 223/*m/e* 233 (methadone), *m/e* 277/*m/e* 280 (EDDP), *m/e* 208/*m/e* 218 (EMDP), and *m/e* 265/*m/e* 275 (DDP).

For the GC analysis of methadone and EDDP in urines, 2-dimethylamino-4,4-diphenyl-5-nonanone (10 μg in 0.2 ml water) was used as the internal standard. The extraction procedures were essentially the same as those described for the SIM analysis of urine samples.

Stability of EDDP

EDDP perchlorate stock solution (1.5 ml, 1 mg/ml in methanol) was evaporated under nitrogen. After adjusting to pH 12 with 1 *N* sodium hydroxide, diethyl ether (25 ml) was added to extract the free base. An aliquot of the ether layer (20 ml) was transferred to a 25-ml volumetric flask and made up to 25 ml with the same solvent. Samples (0.2 ml) were taken for analysis at 0, 1, 2, and 3 days after preparation of the sample which was kept on the bench at room temperature. An internal standard stock solution was prepared with methanol to contain 10 μg of [$^2H_{10}$]DDP and 20 μg of [$^2H_3$]EDDP perchlorate per ml. Sample solution (0.2 ml) was mixed with internal standard solution (0.2 ml) and the mixture was analyzed for DDP and EDDP by monitoring *m/e* 265 and *m/e* 275 for DDP and *m/e* 277 and *m/e* 280 for EDDP. The

standard curves were prepared using various concentrations of EDDP perchlorate and DDP dissolved in methanol. The concentrations of EDDP were expressed as the free base.

Gas chromatography—mass spectrometry and gas chromatography

GC—MS. GC—MS was performed using a Varian MAT 111 gas chromatograph—mass spectrometer. A modified accelerating voltage supply was controlled by a Varian 620L computer to allow ion monitoring. The mass spectrometer was operated with an electron ionization voltage of 70 eV and a source temperature of 285°C. Trap current was 30 μA. A glass column (1.6 m × 2 mm I.D.) packed with 3% OV-17 on 80—100 mesh Chromosorb W HP was used with carrier gas (helium) at 20 ml/min. Column temperature was 200°C, programmed to 270°C at 10°C/min; separator and inlet line temperatures were 290°C, and injector temperature 220°C.

Gas chromatography. The GC analysis was carried out using a Hewlett-Packard Model 5830A chromatograph equipped with a hydrogen flame detector. The glass column, 1.8 m × 2 mm I.D. was packed with 3% OV-17 on 80—100 mesh Chromosorb W HP. Injection temperature was 250°C, oven temperature 210°C, and detector temperature 300°C. The carrier gas (helium) flow-rate was 50 ml/min.

RESULTS AND DISCUSSION

Analysis of methadone in plasma and saliva

Methadone levels in plasma and saliva were frequently found to be too low to use the selective ion at *m/e* 223 for monitoring since with EI the relative abundance of this ion is only 1.6%. In order to enhance the sensitivity of the method the less selective but strong base peak at *m/e* 72 was chosen for monitoring. As shown in Fig. 1, SIM at *m/e* 72 showed high selectivity for methadone with 2-dimethylamino-4,4-diphenyl-5-nonanone as the internal standard, the base peak of which is also *m/e* 72. The lower limit of reproducible quantitation of methadone in 0.5 ml of plasma or saliva taken for extraction was 20 ng.

The sensitivity achieved by this method is comparable to the methods which are routinely used to analyze methadone in human plasma (Table I).

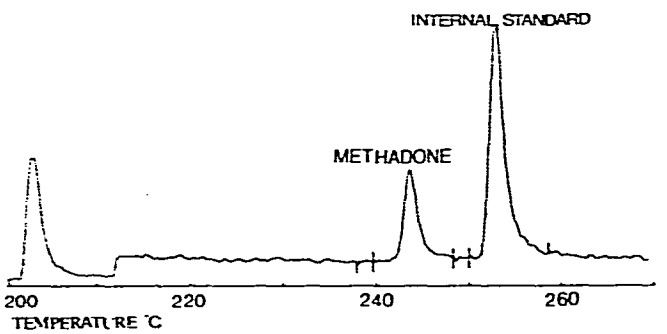

Fig. 1. SIM chromatogram (*m/e* 72) of methadone from saliva; internal standard, 2-dimethylamino-4,4-diphenyl-5-nonanone.

TABLE I

COMPARISON OF QUANTITATIVE METHODS OF METHADONE FOR HUMAN BLOOD SAMPLES

Method*	Sensitivity (sample, final dilution)	I.S.	Reference
GC—FID	15 ng/ml (4 ml plasma, 20 ml chloroform)	SKF-525A	1
GC—FID	15 ng/ml (3 ml plasma, 0.2 ml *n*-butyl chloride)	*n*-Docosane	2
GC—FID	5 ng/ml (15 ml whole blood, 5 μl chloroform)	Methadone analogue	3
GC—ECD	4 ng/ml (1 ml plasma, oxidation to benzophenone) 4-(4-chlorophenyl)-4-phenyl-2-dimethyl-aminobutane (I.S.) forms 4-chlorobenzo-phenone by oxidation		4
GC—MS (EI)	5 ng/ml (4 ml plasma, 0.1 ml toluene)	Methadone analogue	5
GC—MS (CI)	5 ng/ml (1 ml plasma, 20 μl ethyl acetate)	Methadone-d_5	6

*Abbreviations: FID = flame ionization detector; ECD = electron-capture detector.

The low sensitivity observed is contrary to expectations of using the base peak *m/e* 72 for monitoring and is due to instrumental conditions, especially ion source conditions, variation of which was found to seriously limit sensitivity. Special care was therefore taken to reduce background. The temperatures of separator, inlet line, and ion source were elevated overnight to remove retained impurities before samples were analyzed. This method is more than adequate to analyze the patients' saliva or plasma samples, the concentrations of which were found to vary over a wide range between 0.05—1.0 μg/ml during the 24-h period of the study.

Monitoring drug concentrations in saliva may better reflect the time course of a drug at the receptor site. This was found to be the case for the drug procainamide for which a parallel relationship between time course of the drug in saliva and cardiac action of the drug was observed [11]. This should especially be true for drugs which act on the central nervous system. For drugs such as methadone which are extensively bound to plasma protein, monitoring drug concentration in saliva could better define the activity because the concentrations of a drug in saliva frequently reflect the unbound fraction which crosses the blood brain barrier. It appeared therefore useful to initiate monitoring saliva levels of the drug in steady-state maintenance patients especially in view of the fact that a lack in correlation between methadone concentrations in plasma and symptom complaints of patients have been reported [12].

The mean ratio of saliva to plasma of patient A was 0.55 ± 0.15 (S.D.) with a range of 0.40—0.79. Patient B showed a mean ratio of saliva to plasma of 0.48 ± 0.10 (S.D.) with a range of 0.30—0.58 (Fig. 2). Salivary pH could

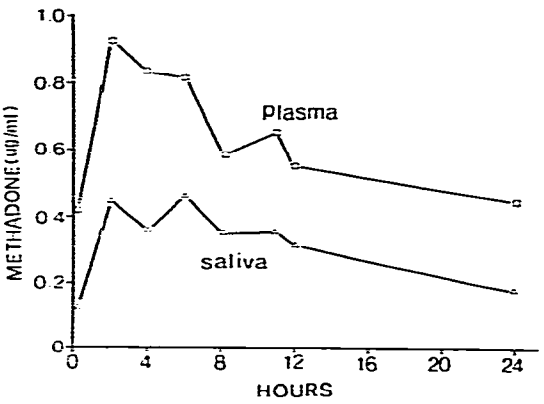

Fig. 2. Methadone concentration in plasma and saliva of a maintenance patient, dose 90 mg/day.

possibly account for the intra-individual variation in the results but pH values were not available. Patient A had difficulty in providing saliva samples and chewing gum (Dentyne, Adams Brands Inc.) was used to stimulate saliva production. Interference from the chewing gum in the analysis was not observed. Adsorption of the methadone to the gum was not proved but was considered to be minimal. If salivary concentrations of methadone in these two patients are a reflection of unbound methadone in plasma then our results agree well with those reported by Horns et al. [12], in which 50% binding of methadone to plasma was reported. This contrasts with the results reported by Lynn et al. [3] where salivary concentrations were found to be much higher than those obtained in whole blood. Our result however was not adequate to draw pharmacokinetic conclusions because of the limited sample size and number of samples.

Analysis of methadone and metabolites in urine

Retention times by SIM of methadone and metabolites were 3.24 (EMDP), 4.0 (EDDP), 4.78 (methadone), and 6.23 min (DDP). When we analyzed methadone and metabolites in urine, GC—MS was a time consuming method compared with GC methods because a separate injection of the sample was required to analyze each metabolite. This was due to the limited range of the ion monitoring mechanism used. GC—MS quantitation with deuterium-labeled internal standards, however, provided ease in work-up procedures. Extractability of labelled and unlabelled compounds from urine using methylene chloride was found to be the same. At pH 7.5 (n=4, 2.5 μg each in 1.0 ml), percent recoveries of methadone, [$^2H_{10}$]methadone, EDDP and [$^2H_3$]EDDP were 84.9 ± 2.6, 83.6 ± 0.2, 94.2 ± 3.8 and 93.9 ± 1.1, respectively. EDDP and [$^2H_3$]EDDP were analyzed in the recovery studies using [$^2H_{10}$]EDDP as the internal standard; methadone and [$^2H_{10}$]methadone using [$^2H_3$]methadone as internal standard.

The calibration equations prepared for methadone and EDDP are shown in Table II. The results indicate that calibration equations can be expressed by using only slope values because intercept values were found to be not significant. Also, after determining the ratio of unlabelled to labelled compound,

TABLE II

CALIBRATION EQUATIONS FOR METHADONE AND EDDP FOR URINE ANALYSIS

Slope and intercept values are calculated by means of computer program (Triangular Regression Package, Computing Centre, The University of British Columbia). Methadone (n=7), EDDP (n=10). Ratio (drug/internal standard) = slope × drug concentration + intercept. Internal standards are [$^2H_{10}$]methadone (4 μg) and [$^2H_{10}$]EDDP (2 μg).

	Methadone	EDDP
Slope	0.326	0.483
Slope standard error	0.0574	0.0033
Intercepts	0.0513	0.0054
Intercept standard error	0.0503	0.0117
Coefficient of determination (r^2)	0.9957	0.9990
Standard ratio (S.D.)* (n=6)	1.305 ± 0.1272	0.968 ± 0.0484
Slope calculated from standard ratio**	0.326	0.484

*Ratios of equal amount of drug to internal standard (standard ratio).
**Standard ratio × 1/internal standard.

quantitation of either methadone or EDDP can be obtained by multiplying the standard ratio, the reciprocal value of the amount of added internal standard, and the observed ratio. Slope values of calibration curves and the slopes calculated from the standard ratio were found to be the same (Table II). The calibration method was used for the analysis of methadone because of instability of the measured ratio which occurred when ions of 10 mass units difference were monitored. The ratio was found changeable depending upon the SIM conditions, particularly due to the instability of the magnetic field. The coefficient of variation of the methadone standard ratio was twice (9.7%) that of EDDP (5.2%).

GC—MS of methadone and EDDP was compared with GC (Table III). The two methods were found to be well correlated for the analysis of urine samples.

Previous experiments with the GC analysis of EMDP in the urine of maintenance patients indicated that the EMDP peak is frequently overlapped either with caffeine or with hydroxycotinine, a metabolite of nicotine [5]. When we attempted to analyze maintenance patient urines for EMDP by monitoring m/e 208 for EMDP and m/e 218 for the internal standard, [$^2H_{10}$]EMDP, we found that the amount of EMDP appeared to be less than 100 ng/ml. We concluded that determination of this metabolite was not necessarily important to study demethylation mechanisms of methadone.

TABLE III

COMPARISON OF GC—FID AND GC—MS (SIM) IN THE ANALYSIS OF METHADONE AND EDDP IN HUMAN URINES

Methadone (0.4—6.8 μg/ml) and EDDP (8—32 μg/ml) were analyzed; n = 14 in both cases.

Methadone	SIM = 0.998 GC + 0.347	r^2 = 0.963
EDDP	SIM = 1.26 GC — 2.13	r^2 = 0.962

STABILITY OF EDDP

In an attempt to obtain information on the minor metabolic pathways of methadone, the level of DDP in urine was determined by monitoring *m/e* 265 for DDP and *m/e* 275 for [$^2H_{10}$]DDP as the internal standard. When the pH of the urine was adjusted to 10.4 for extraction, inconsistency in the reproducibility of the concentration of DDP in a urine sample was found. To investigate this problem, EDDP perchlorate was made alkaline and left to stand at room temperature. A total ion current profile of the methylene chloride extract showed DDP as a significant decomposed product. DDP was identified by thin-layer chromatography (TLC) at R_F 0.8 on Silica Gel F 254 (Brinkman) with ethanol—acetic acid—water (6:3:1). Further study revealed that EDDP free base is easily decomposed to DDP in diethyl ether. Stoichiometric conversion of EDDP to DDP was observed when EDDP and DDP in the solution were measured using labelled compounds as internal standards (Fig. 3). A similar result for the conversion of EDDP to DDP by apparent oxidation reactions was separately reported by Bowen et al. [13]. Minor metabolic transformation of methadone to 2,2-diphenyl-4-dimethylaminopentanoic acid and further metabolism to DDP was suggested [14]. 2,2-Diphenyl-4-dimethylaminopentanoic acid was not detected in urines of maintenance patients by our studies using mass chromatograms in which *m/e* 167 $(C_6H_5)_2$ C^+H for the acid and *m/e* 226 $(C_6H_5)_2$ C^+COOCH_3 for the diazomethane-treated acid were monitored. Our results strongly suggest that DDP is a metabolic artefact and that pH adjustment is important when biological samples are extracted to detect new metabolites if artefact formation is to be avoided.

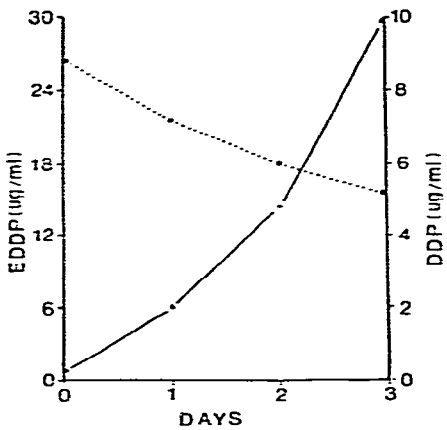

Fig. 3. Air oxidation of EDDP base to DDP (— — — EDDP, —— DDP).

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