TECHNICAL NOTE

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Simultaneous Gas Chromatography/Mass Spectrometry Assay of Methadone and 2-Ethyl-1, 5-Dimethyl-3,3-Diphenylpyrrolidine (EDDP) in Urine

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ABSTRACT: An efficient extraction and gas chromatography/mass spectrometry (GC/MS) procedure has been developed for the simultaneous determination of methadone and 2-ethyl-1, 5-dimethyl-3,3-diphenylpyrrolidine in urine samples. The merits of this procedure include (1) effective high-volume sample processing; (2) excellent gas chromatography characteristics; (3) high precision for quantitative methadone determination—1.0% coefficient of variation (CV) for GC/MS injection replicates and 1.2% for extraction replicates; (4) excellent linearity within the range (0 to 1200 ng/mL) studied; and (5) adequate detection limits (50 ng/mL) for most practical purposes. The detection limit for methadone may be improved 40-fold by using a different internal standard.

KEYWORDS: toxicology, abuse drugs, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), chemical analyses, urine, GC/MS, methadone, selected ion monitoring, urinalysis

Urinalysis of drugs [*1*] normally consists of an immunoassay screening step and a confirmatory, quantitative analysis step for those specimens which screen positive in the first step. Among the various techniques presently available, gas chromatography/mass spectrometry (GC/MS) procedures are considered the most reliable and are the preferred confirmatory method [*2*]. An effective confirmatory procedure should be specific and sensitive, yet still be able to process a large number of specimens in a relatively short time. In most instances, an extraction or an extraction-derivatization procedure is necessary to prepare the specimen for the GC/MS analysis. The purpose of this study was to develop an effective method that can process a large number of urine samples for the simultaneous determination of methadone and one of its major metabolites, 2-ethyl-1, 5-dimethyl-3,3-diphenylpyrrolidine (EDDP).

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Materials and Methods

Methadone, 1,1,1-$^2H_3$-methadone hydrochloric acid (HCl) (d_3-methadone), EDDP perchlorate, and 2-[ethyl-2,2,2-$^2H_3$]-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate (d_3-EDDP) were obtained through the National Institute on Drug Abuse (NIDA) Drug Supply System.

Samples and Controls

Urine samples from five patients on a methadone maintenance program at levels of 3, 20, 50, 58, and 82 mg/day were obtained from the Treatment Alternatives to Street Crime Program (Department of Psychiatry, Substance Abuse Programs, University of Alabama at Birmingham, Alabama). Standards and controls were prepared in-house from stock solutions (0.1 mg/mL) of both methadone and EDDP at concentrations of 150, 300, 600, and 1200 ng/mL. Drug-free urine controls were also included in the analysis.

Extraction Procedure

The sample preparation scheme shown in Fig. 1 consists of a liquid-liquid extraction procedure. Initially, 400 μL of working internal standard (1 mg/mL d_3-methadone) was added to each 5-mL sample and then shaken to mix. The pH of the resulting solution was adjusted to between 9 and 10 with 1 mL of 1.5*M* carbonate buffer (pH 9.5), which

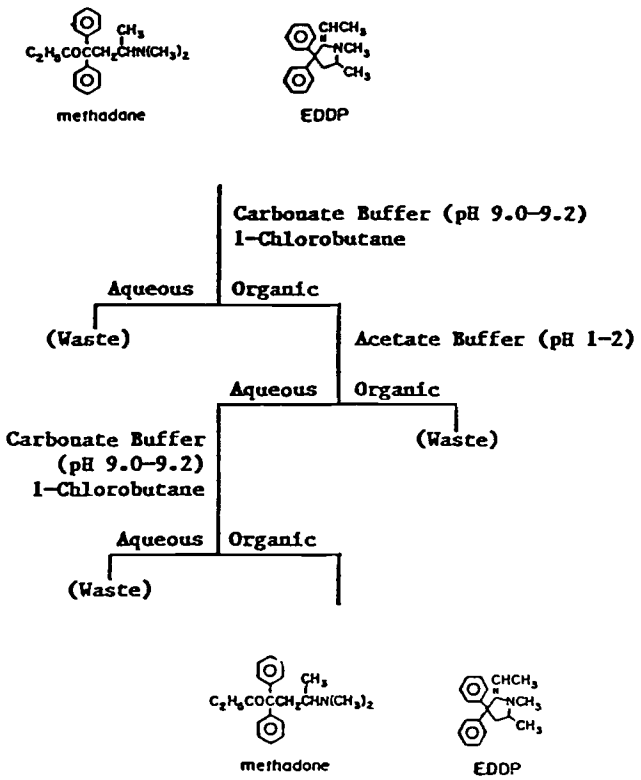


FIG. 1—*Extraction flowchart.*

was prepared by combining 16 g of sodium carbonate (Na_2CO_3) and 18 g of sodium bicarbonate ($NaHCO_3$) and diluting the solution to a final volume of 250 mL.

Then, 15 mL of 1-chlorobutane was added, and the drugs were extracted into the organic phase by shaking on a platform shaker for 30 min. The samples were centrifuged to ensure complete separation of the layers. The upper organic layer was transferred to a 50-mL plastic tube by freezing the lower aqueous layer in a dry ice/isopropanol bath and decanting the organic layer.

The drugs were extracted back into the aqueous phase by the addition of 3 mL of sodium acetate (0.2*N*) and shaken for 30 min on a platform shaker. After centrifugation of the sample, the upper organic phase was aspirated to waste and the aqueous phase was transferred to a 15-mL round-bottom glass tube. The pH of the aqueous phase was adjusted to between 9 and 10 with 1.5*M* carbonate buffer and 1*N* sodium hydroxide (NaOH).

The drugs were re-extracted into the organic phase by adding 2 mL 1-chlorobutane and vortex-mixing the solution for 2 min. The upper organic phase was transferred to a 5-mL conical tube by freezing the lower aqueous phase in a dry ice/isopropanol bath and decanting the organic layer. Each sample was evaporated to dryness under a stream of nitrogen gas (N_2) at 50 to 60°C and stored in the freezer at −20°C until the GC/MS analysis was performed.

Gas Chromatography/Mass Spectrometry Procedure

A Hewlett-Packard 5890 gas chromatograph coupled with a 5970B mass selective detector (MSD) mass spectrometer was used for the analysis. A 12-m (0.251-mm inside diameter) J & W DB-5 (0.25-μm film thickness) capillary column (J & W Scientific, Folsom, California) was connected to the MSD through a direct capillary interface. The injection port was a capillary split injector with a silanized glass insert. The carrier gas, helium, was at a flow rate of approximately 1.0 mL/min with a split ratio of 10:1. The oven temperature was 190°C. The MSD was used in the selective ion monitoring mode. The following ions (*m/z*) were monitored: Methadone 294, 223, and 295; deuterated methadone 297 and 226; and EDDP 277, 262, and 276. The first ion listed for each compound was used for quantitation.

Results and Discussion

Methadone is metabolized by mono- and di-*N*-demethylation with subsequent cyclization metabolites to form EDDP and 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP). Since about 33% of methadone ingested is excreted in the urine unchanged and about 43% as EDDP [*3*], the simultaneous detection of both compounds provides evidence of methadone ingestion and allows pharmacokinetic studies.

Several confirmation procedures for the detection of methadone have been published in the scientific literature [*4,5*]. The procedure developed in this study is improved over these reported with (1) the implementation of a back extraction step, which greatly improves the cleanliness of the extract and the quality of the gas chromatogram, (2) the use of a short capillary column to facilitate efficient separation in the short retention time needed for high-volume work, and (3) selected ion monitoring of three ions for the analytes and two ions for the deuterated internal standard, to optimize the sensitivity and reliability of quantitation.

Selection of Ions for Monitoring

Initially, the mass spectra of methadone, EDDP, and their deuterated analogs (Figs. 2 and 3) were obtained to determine which ions would be most suitable for the detection

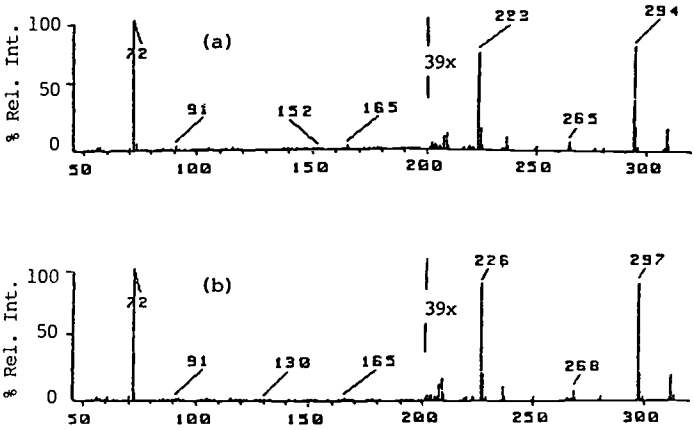


FIG. 2—*Mass spectra of* (a) *methadone and* (b) d_3-*methadone. The ion intensities of all ions greater than* m/z *200 were enhanced 39 times.*

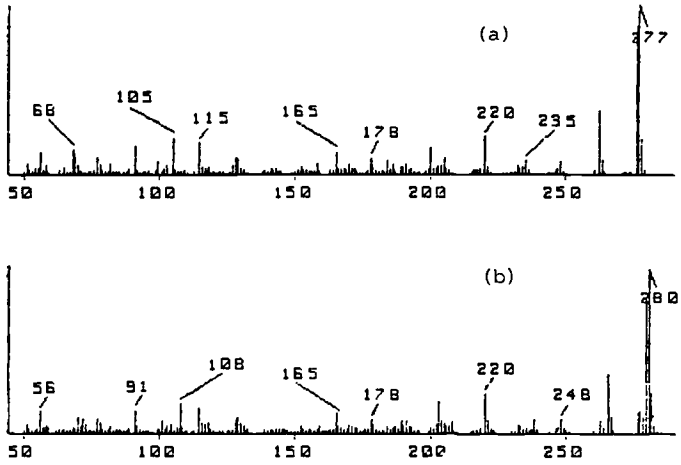


FIG. 3—*Mass spectra of* (a) *EDDP and* (b) d_3-*EDDP.*

of methadone and EDDP in urine extracts. In Fig. 2, the intensities of significant ions with *m/z* higher than 200 are low, their relative intensities are expanded 39-fold for display in Fig. 2 to facilitate comparison. Since the *m/z* 72 ion is present in both methadone and d_3-methadone (the intended internal standard) and these two compounds are poorly resolved by the GC, the *m/z* 72 ion could not be used in the GC/MS analysis. A closer examination of the spectra indicated that the following ions (*m/z*) might be suitable: methadone 294, 223, and 295; and d_3-methadone 297 and 226. (Since EDDP was sufficiently separated and deuterated EDDP was not used as the internal standard, the selection of ions to be monitored for EDDP was less critical; the following ions were selected: *m/z* 277, 262, and 276.) The intensity ratios of these ions obtained from controls with various concentration levels and sample replicates (as shown in Table 1) were found to be consistent and suitable for use.

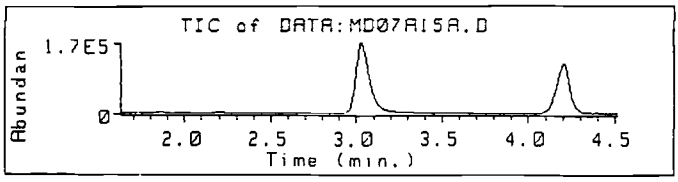
TABLE 1—*Reproducibility of intensity ratios of ions selected for monitoring.*

Sample	Ion Intensity Ratio of EDDP		Ion Intensity Ratio of Methadone	
	262/277	276/277	223/294	295/294
300 ng/mL control	0.40	0.90	0.90	0.23
extraction replicates	0.42	0.93	0.92	0.23
	0.42	0.92	0.91	0.22
600 ng/mL control	0.39	0.92	0.90	0.23
extraction replicates	0.40	0.93	0.93	0.23
1200 ng/mL control	0.39	0.93	0.92	0.23
extraction replicates	0.38	0.92	0.92	0.23
Patient sample	0.39	0.91	0.89	0.23
extraction replicates	0.40	0.92	0.88	0.22
	0.38	0.91	0.89	0.23
	0.40	0.93	0.90	0.23
600 ng/mL GC/MS	0.40	0.92	0.90	0.23
replicates	0.39	0.92	0.90	0.23
	0.39	0.92	0.89	0.22
	0.38	0.91	0.91	0.23

Qualitative and Quantitative Determination

A total ion (of all the ions monitored) chromatogram obtained from a test sample is shown in the top part of Fig. 4. Qualitative identification of an analyte is based on the fulfillment of the following conditions: (1) the appropriate ions for the analyte must be present at the correct retention time and (2) the intensity ratios of these ions must be within 20% of these ratios established by an authentic control analyzed under identical conditions. Under the GC conditions used in this study, the retention times are 3.0 min for EDDP and 4.10 min for methadone and d_3-methadone. A ± 0.1-min variation is allowed for chromotograms obtained at different injections.

The quantitation of an analyte in a test sample is determined by the use of the internal standard, as described below. The abundance of the analyte's quantitation ion is divided by the abundance of the internal standard's quantitation ion in any given test sample.



COMPOUND	RT	MASS	AREA	AMT(NG)	TARGET RANGE	RATIO
EDDP	3.03	277	4235524	1603.22		1.00
	3.03	276	3730743	1572.58	0.72 - 1.08	0.88
	3.03	262	1620908	1450.64	0.34 - 0.50	0.38
MEHTADONE	4.21	294	1888856	1177.53		1.00
	4.21	223	1704924	1128.70	0.75 - 1.13	0.90
	4.22	295	435152	1003.06	0.22 - 0.32	0.23
ISTD	4.19	297	1269162	800.00		1.00
	4.19	226	1138026	792.75	0.72 - 1.08	0.90

FIG. 4—*Total ion chromatogram with all the data and calculations obtained using the TARGET software package.*

This is the intensity ratio for that analyte. This intensity ratio is then divided by the same intensity ratio obtained from the calibration control. The concentration of the analyte in that given test sample is obtained by multiplying this ratio by the concentration of the analyte in the calibration control (300 ng/mL). The TARGET™ software package (Thru-Put Systems, Orlando, Florida) performs the integration of the peak areas and the calculations and generates the output, as shown in the lower part of Fig. 4.

Precision

The precision of the procedure was tested at two levels: (1) the precision within the GC/MS analysis of a single extract and (2) the precision among different extracts of the same sample. The precision within the GC/MS analysis was tested by injecting the same extract four separate times. The results, shown in the upper portion of Table 2, indicate better precision for methadone than for EDDP, as a result of the use of deuterated methadone as the internal standard.

The precision of the variation between extractions was determined using a sample collected from a patient known to use a 58-mg methadone daily dose. Since the analyte concentrations in this specimen are relatively high, the sample was diluted ten-fold and four replicates were extracted separately. The results, shown in the lower portion of Table 2, indicated better precision for methadone than for EDDP, again as a result of the deuterated analog of methadone as the internal standard.

Linearity

The linearity of the extraction-GC/MS procedure was tested by analyzing control samples of methadone and EDDP at the following concentrations: 0, 150, 300, 600, and 1200 ng/mL. Since the same amount of the internal standard is used in all samples, the linearity can be examined by plotting the quantitation ion intensity ratios (*m/z* 277/297 for EDDP and *m/z* 294/297 for methadone) obtained from these samples. Results shown in Fig. 5 indicate an excellent linear responses for both compounds within the range tested.

Sensitivity

With the experimental procedure and conditions used, the detection limits for EDDP and methadone were estimated to be approximately 50 ng/mL, one third of the 150 ng/mL sample. This detection limit is estimated based on the abundance of the quantitation ions (Fig. 6*b*) generated by the 150 ng/mL samples for methadone (*m/z* 294) and EDDP (*m/z* 277), as shown in Fig. 6. It is reasonable to assume that a 50-ng/mL sample will

TABLE 2—*Precision of GC/MS analysis and extraction procedure.*

	EDDP, ng/mL				Methadone, ng/mL			
Sample	Result	Mean	S.D.	CV, %	Result	Mean	S.D.	CV, %
600 ng/mL GC/MS injection replicates	582				592			
	566				603			
	648				604			
	573	592	38	6.4	596	599	6.0	1.0
Patient sample extraction replicates	621				589			
	808				579			
	664				592			
	570	666	102	15	595	589	7.0	1.2

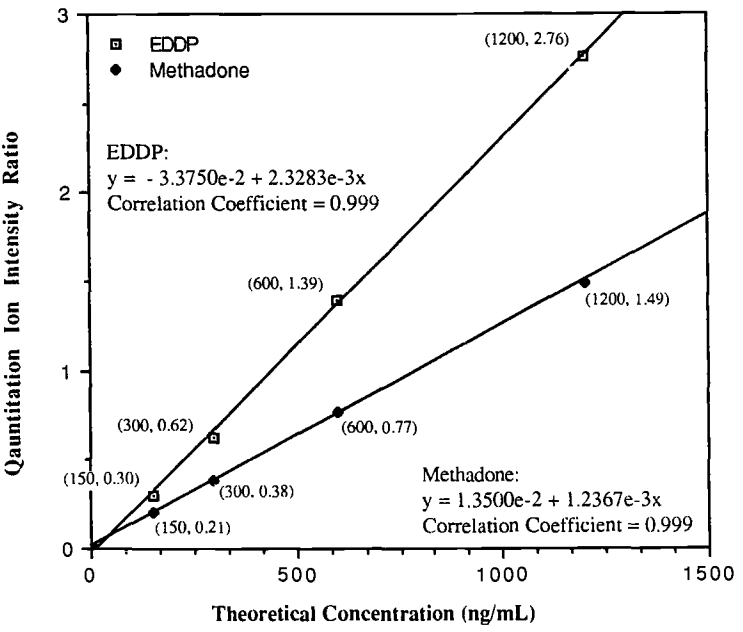


FIG. 5—*Correlation of quantitation the ion intensity ratios for EDDP* (m/z *277/297) and methadone* (m/z *294/297) with the theoretical concentrations.*

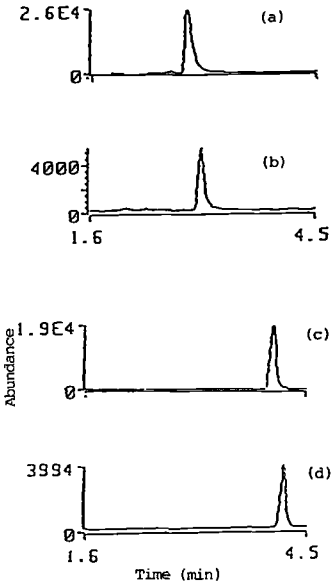


FIG. 6—*Abundance of the quantitation ions of EDDP* (m/z *277) at* (a) *300 ng/mL and* (b) *150 ng/mL, and those for methadone* (m/z *294) at* (c) *300 ng/mL and* (d) *150 ng/mL.*

generate ion abundance at approximately one third of these shown in Fig. 6*b*, and can be quantitated without difficulty. It should be noted that, in this figure, the retention times of these ions for the 300 ng/mL samples do not exactly match those of 150 ng/mL sample because of slight operator variations between injections of the two samples.

The sensitivity can, of course, be improved by increasing the initial sample volume or by decreasing the reconstitution solvent volume of the extract prior to GC/MS analysis. For monitoring the drug levels of patients in a maintenance program, a detection limit of 50 ng/mL is more than sufficient, since the concentrations of both EDDP and methadone in these samples normally are present at much higher concentrations. However, the capability of detecting these drugs at a lower concentration level may occasionally be needed for the detection of methadone abuse.

Considering that the ion intensities of the three ions monitored for methadone were present at levels of about 3% of the base ion (*m/z* 72), it is conceivable that the detection limit could be improved by as much as 40-fold if the ion *m/z* 72 were used for quantitation. However, in order to use this ion for quantitation, a compound other than deuterated methadone must be used as the internal standard. A deuterated analog of EDDP is a logical choice.

Conclusions

In summary, data presented in this study demonstrate an effective extraction-GC/MS procedure for the simultaneous determination of methadone and EDDP in urine specimens. The procedure achieves (1) good precision for GC/MS analysis [6.4% and 1.0% coefficient of variation (CV) for EDDP and methadone] and extraction (15% and 1.2% CV for EDDP and methadone), (2) an excellent linear response (0.999 correlation coefficient for both EDDP and methadone) within the range tested, and (3) detection limits of 50 ng/mL for both methadone and EDDP which are sufficient for most practical purposes. If necessary, these detection limits could be moderately improved by using a larger sample volume and a smaller reconstitution volume. Furthermore, the detection limit for methadone may be greatly improved by using deuterated EDDP as the internal standard.

Acknowledgment

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