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Determination of morphine in urine samples using gas chromatography–mass spectrometry (*GC/MS*)

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ABSTRACT

The detection and quantitation of drugs of abuse in urine is of growing interest in forensic and clinical toxicology. With the development of highly sensitive chromatographic methods, such as gas chromatography–mass spectrometry (GC–MS), more and more substances can be determined in urine. This review includes method for the determination of the morphine in biological samples, where morphine obtained by acidic hydrolysis from urine samples was extracted using mixture of solvents followed by trimethylsilylation. The derivatized extract was submitted to GC/MS analysis of EI-SIM mode. Different factors were studied to five the optimum condition for determination as pH, temperature, solvent of extraction and time hydrolysis. The calibration curves of morphine derivatized (MOR-TMS) in urine samples were linear in the concentration range from 5 to 100 ng/ml. The limit of quantitation was 5 ng/ml. With measuring the optimum makes this method is high sensitive to morphine and become good confirmatory method for determination of morphine in urine.

KEYWORDS

Confirmatory method;
GC/MS;
Morphine;
Trimethylsilylation;
Biological samples.

INTRODUCTION

Morphine is an alkaloid molecule, a term given to natural occurring nitrogen containing bases found mainly in plants. It is one of twenty-four such alkaloids found within the resin of the opium poppy plant – Papaver somniferum and it usually comprises 10% of all opium extract. Designated with the chemical formula $C_{17}H_{19}NO_3$,HCl,3H_2O, morphine exists mainly as a bitter, white crystalline compound, one that is water insoluble. It has appeared and continues to appear in a variety of other forms, however, including, but not limited to: pharmaceutical concoctions (*i.e.* Patent medicines), morphine acetate (salt), morphine hydrochloride (salt), and morphine sulfate (salt)[1].

Morphine is the principle alkaloid of opium, ranging in concentration from 4-21%, the usual range being

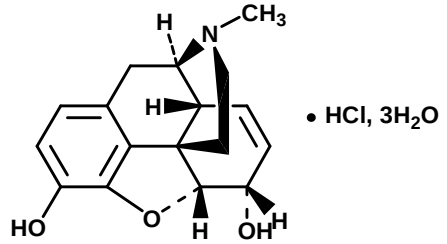

Figure 1 : Chemical structure of morphine

8-14%. Licitly produced raw opium, know as Indian Opium, contain not less than 9.5% morphine, calculated as anhydrous morphine. (See Figure 1 for the chemical structures of related to morphine).

Testing for drugs of abuse is becoming more prevalent in various environments including forensics and social justice environments as well as in sporting events.

Morphine is a mainstay in the treatment of acute and chronic pain. Glucuronidation is the main route of morphine metabolism, producing morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Simultaneous determination of morphine concentrations has practical application in both pharmacokinetic studies and forensic assessment.

A variety of techniques have been used to quantify Morphine. Immunoassays offer adequate sensitivity for morphine but lack the specificity to distinguish opiates from their corresponding glucuronides[2-11]. Gas chromatography mass spectrometry (GC–MS) provides the needed sensitivity and selectivity[12-18], high-performance liquid chromatography (HPLC)[19-28] and HPLC/MS[29-32].

This paper describes a method to confirm and determine urinary morphine; this method includes a hydrolysis of conjugates by strong acid (HCL), extraction and derivatization of morphine.

The hydrolysis is necessary to free the morphine conjugated and this can be performed either by acidic or enzymatic hydrolysis, acidic hydrolysis is considered more efficient and reproducible than acid or enzymatic hydrolysis[33].

The extraction procedure should be efficient and selective. A good recovery is important, since the total amount of morphine present is very low value. Due to the low sensitivity of the mass detector to morphine, derivatization is used to stabilize ions formed in the mass spectrometer to favor structurally informative fragmentation mode. So the Derivatization of morphine with N,O-bistrimethylsilytriflutoacetamide (BSTFA (was needed in this method.

In this method determined the optimum conditions to determination of morphine by GC-MS, the pH of extraction, solvent of extraction, time of derivatization and temperature of derivatization.

EXPERIMENTAL

Chemicals

All chemicals used through the work were of analytical reagents grade. The reagent included morphine HCL (1gm/mL) was obtained from Sigma-aldrich gmbh steinheim, germany. Stock solutions of morphine were prepared in methanol (50 ug/ml) and stored at 5°C. The silylation reagent BSTFA obtained from Sigma-aldrich gmbh steinheim, germany, Chloroform, Carbon tetrachloride, Ethyl acetate, Isopropanol, Toluene, Benzene, n-Hexane, Cyclohexane, Petroleum ether, Diethyl ether and Methylene chloride (BDH), Sodium hydroxide (10N) and (1N), (BDH), HCl concentrated and (2N(, (BDH).

Urine samples

Some precautions have been considered in urine samples, the sample was collected in duplicate in two 30 mL plastic bottles. Each plastic bottle was filled at least 2/3 full. Immediately after collection, the temperature (32°C- 38°C) within 4 min., pH, specific gravity and creatinine value of the fresh samples were recorded. Urine samples were stored at ?5 ^{0}C until the analysis.

Instrumentation

The analysis of extracted morphine, the system performed with a Agilent Technologies, 6890N Gas Chromatograph (Agilent Technologies, USA) and mass detector GCMS-5973N (Agilent Technologies, USA), connected to a desktop computer with MSD Chemstation D.01.00 software. The column employed Hp-5MS (30-m x 0.25-mm i.d., 0.25-µm film thickness of 95% dimethyl–5% diphenyl polysiloxane copolymer column) Restek, Bellefonte, PA, USA). The carrier gas consisted of ultra-pure grade helium (Air Products, Parkersburg, WV, USA) at a flow rate of 0.7 ml/min. The injector temperature was held at 250 ^{O}C. For MOR-TMS detection the oven started with 50 ^{O}C to 0.5 min. and was ramped from 50 to 150 ^{O}C at 25 ^{O}C min and from 150 to 300 ^{O}C at 10^{O}C/min and hold for 5 min. to give a run time 24.50 min., the total ion chromatograph of MOR-TMS is reported in Figure 2, analysis was accomplished by selected ion monitoring of ions (high resolution mode) between 3 and 24.50 min. at m/z = 59,

73, 124, 162, 204, 287, 342, 399, 400, 401, 402 and EMV at 1400.

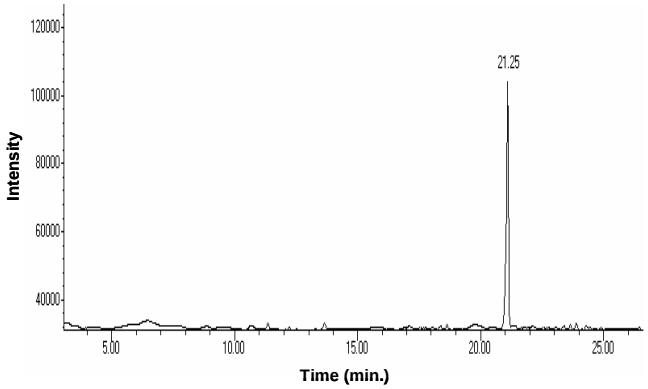

Figure 2 : The total ion chromatogram of MOR-TMS

Sample preparation

Hydrolysis

Urine (10) ml was mixed with 2 ml of Concentrated HCL, the sample was hydrolysed at 100°C in a heating unit for 30 min. with occasional stirring. After cooling to room temperature, 2 ml of 10N NaOH were added and added few drops of HCL until adjust pH (8-9).

Effect of pH

The effect of pH is studied to reach to optimum pH for the extraction metabolite of Morphine from urine sample, so the effect of pH was study using spiked urine with 30 ng/ml as shown in Figure 3.

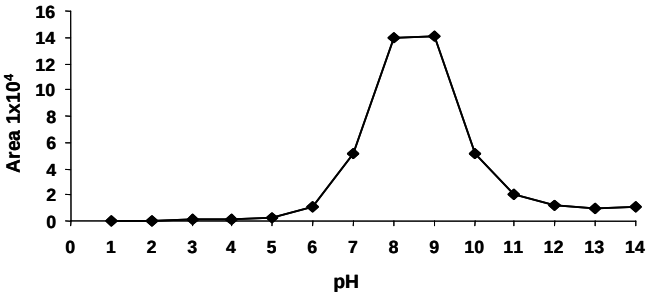

Figure 3 : Effect of pH on extraction of morphine.

Extraction

Liquid –liquid extraction with 80 ml mixture of solvent and was shaking for two minutes and allowed to separate into two phases. The organic layer was collected and evaporated to dryness with stream of air. Added small amount of the same solvent of extraction and transfer into small vial, evaporate to dryness with stream of air at room temperature.

Effect of solvent on extraction of morphine

The polarity of solvent affects on extraction efficiency. Several water immiscible organic solvents chloroform, benzene, Carbon tetrachloride, Chloroform, Chloroform : Isopropanol (9:1), Cyclohexane, Diethyl ether, Ethyl acetate, Methylene chloride, n.Hexane, Petroleum ether, and Toluene. The effect of extracting solvents on extraction efficiency are represented graphically in Figure 4.

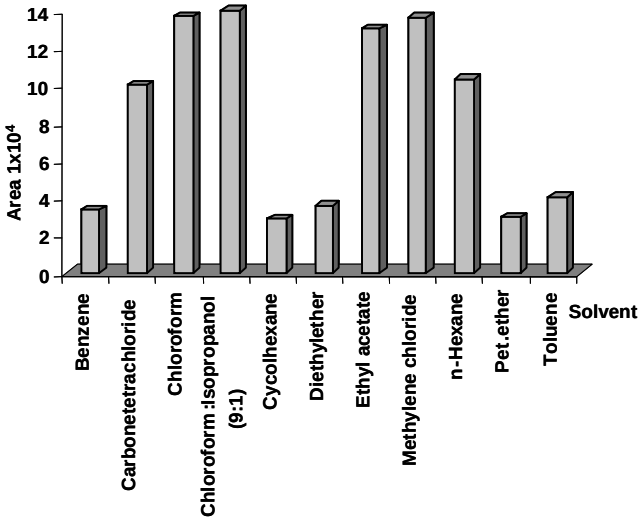

Figure 4 : Effect of solvent on extraction of morphine.

Derivatization

To the vial containing urine extract after complete evaporation added 50 ul of ethyl acetate and 50 ul BSTFA are added, the vial which is vortexed and heated at 80°C for 30 min.

Effect of temperature derivatization

The proper choice of the Derivatization agent and of its optimal amount appeared to be decisived for the sensitivity of the method. To establish the thermal stability of the MOR-TMS, the effect of temperature of Derivatization on the Formation of the MOR-TMS was

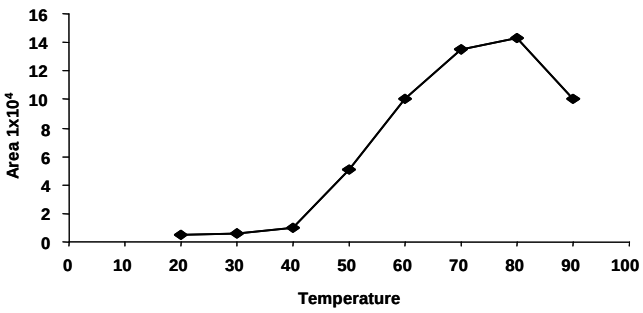

Figure 5 : Effect of temperature on formation of MOR-TMS.

studied by measuring Conc. of the MOR-TMS formed at increased temperature intervals between 20 and 90 ^{O}C at constant time as shown in Figure 5.

Effect of time of derivatization

To establish the thermal stability of the MOR-TMS, The effect of time of derivatization on the formation of the MOR-TMS was studied by measuring concentration of the MOR-TMS formed at increased time intervals between 5 and 40 min. at constant temp.80 ^{O}C as shown in Figure 6.

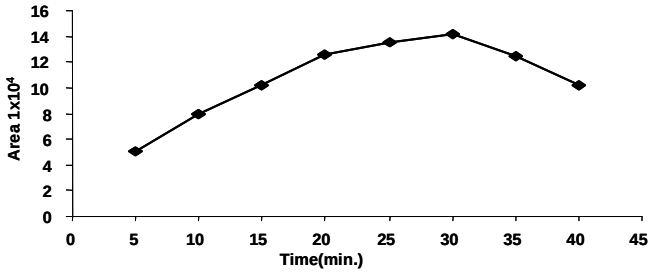

Figure 6 : Effect of time on formation of MOR-TMS.

METHOD VALIDATION

The linearity of the method was verified using of human urine samples spiked at nine levels (5, 10, 20, 30, 40, 50, 60, 80, and 100 ng/ml) spiked. The urine samples were spiked with Morphine from a stock solution 50 µg/ml), linear regression line was obtained by plotting the peak area versus the MOR-TMS concentration as show in Figure 7. Coefficients of variation (CV) for intra-day and inter-day precisions were calculated at three concentrations. The limit of detection was determined by estimating the minimum concentration equivalent to, or greater than, three times of the background noise. The Limit of quantification was defined as fives times the background noise.

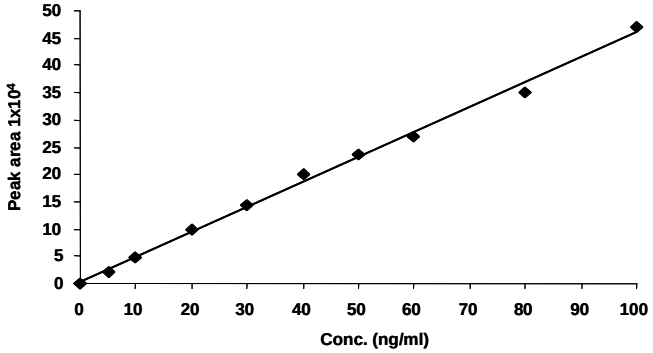

Figure 7 : Standard curve of MOR-TMS.

Precision and accuracy

The intra-day precision was evaluated by replicate analysis (n = 5) containing the following concentration of 5, 20 and 40 ng/ml. For inter-day precision, the samples were analyzed in triplicate on 5 different days over a 3-weekperiod (n = 15) and Coefficients of variation (CV) was calculated as shown in TABLE 1. Accuracy was established by comparing the peak area ratios for amounts of MOR-TMS and the peak area ratios for the same analyte in the Standard preparation. It is expressed as a recovery percentage (Recovery %). Values were processed according SFSTP recommendations[34].

TABLE 1 : Recovery and precision for MOR-TMS at three target concentrations.

Intra day (n =5)			Inter-day (n =15)	
MOR-TMS (ng/ml)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
5.00	96	0.22	95	0.25
20.00	97	0.12	92	0.54
40.00	95	0.29	97	0.62

n = number of replicates

RESULT AND DISCUSSION

Several series of experiments were carried out for optimization of the different parameters of the procedure described in Section 2.4. The optimum pH of extraction of Morphine from urine samples is pH = 8-9 as shown in Figure 3. (Chloroform : Isopropanol) (9:1) v/v is the best solvent to give good yield as shown in Figure 4 and we can use another solvent like chloroform, Methylene chloride and Ethyl acetate if (Chloroform : Isopropanol) (9:1) v/v not available. From the Figure 5, the optimum temperature 80^{O}C give high yield at constant time 30 min. and also we found 30 min. is the good time for derivatization at constant temp. 80^{O}C. as shown in Figure 6.

Figure 7 shows the Standard curve obtained which was found to be liner from 5-100 ng/ml, the good recovery indicates that the good conditions for extraction and derivatization as shown in TABLE 1 and the good recovery.

TABLE 1 shows that the Good recovery from 92 to 97 % for three concentration. The method proved to

be precise in terms of both intra-day and inter-day analyses, with coefficients of variation less than 1%.

The (LOD) was estimated with decreasing the concentration as shown in Figure 7 was 5 ng/ml.

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