# ANALYSIS OF VARIOUS OPIATES INCLUDING HYDROCODONE OR HYDROMORPHONE IN BIOSAMPLES BY GC/MS AFTER SILYLATION

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**ABSTRACT:** A GC/MS method suitable to conduct practical toxicological analyses of unspecified opiates including hydrocodone or hydromorphone is presented. Silylation can be a convenient reaction to derivatize unknown analytes in toxicological samples. However, silylation of drugs with tautomeric keto/enol groups in molecular structure can cause analytical problems due to poor reproducibility. After using N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) acceptable results were achieved.

**KEY WORDS:** Hydrocodone; Hydromorphone; GC/MS.

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## INTRODUCTION

The method for the simultaneous analyses of various opiates in blood or urine samples by GC/MS is described. The universal electron impact scan mode is useful for the detection of unknown compounds. Nevertheless the targeted confirmation of specified opiates is more sensitive in electron impact SIM mode and it is preferred for quantitation. Various ways of derivatization of opiates for GC/MS analyses have been recommended [2, 3, 5, 7, 8, 9]. In toxicological samples with unknown analytes, silylation can be convenient to derivatize polar groups of various nature. However, analysis of drugs with tautomeric groups in molecular structure as in hydrocodone or hydromorphone can be rather difficult and under certain conditions can cause poor reproducible results due to the occurence of both keto/enol forms. In our experiments we have found, that after using N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) no underivatized free ketoforms of hydrocodone or hydromorphone were detected. Moreover the efficient GC separation of silylated codeine, hydrocodone, morphine and hydromorphone was achieved. The separation was sufficient also in case of hydroxy-epimers which can originate during biotransformation of some ketoopioids by their reduction [1, 4, 5, 6]. No practical problems with moisture sensitivity of derivatives apeared in our conditions. Results of validations of the assays of various 4.5-epoxymorphinans (dihydrocodeine, codeine, hydrocodone, hydromorphone, mor-

phine, norcodeine) in spiked serum or urine samples using deuterated morphine as internal standard, solid phase extraction and silylation procedure are compared in our contribution.

## EXPERIMENTAL

All solvents and reagents were of analytical grade quality. Bond Elut Certify columns for solid phase extraction (SPE) were obtained from Varian Inc. Silylating reagent N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Fluka Chemie AG. The following reference standard substances were used for GC/MS:

- dihydrocodeine tartrate, m. w. 451.5, Napp Research Centre Ltd., UK;
- codeine phosphate sesquihydrate, m. w. 406.4, UNDCP Vienna, Austria;
- hydrocodone tartrate, m. w. 449.5, Sigma Chemical Co., USA;
- hydromorphone hydrochloride, m. w. 321.8, Sigma Chemical Co., USA;
- morphine hydrochloride trihydrate, m. w. 375.8, UNDCP Vienna, Austria;
- norcodeine hydrochloride trihydrate, m. w. 375.8, Makor Chem. Ltd., Israel;
- morphine (N-methyl-D3) monohydrate, m. w. 306.4, Lipomed A. G., Switzerland.

Precision and recovery experiments were conducted with blank bovine serum or human urine samples spiked with opiate standard mixture in methanol solution to resulting concentration levels 10 or 100 ng/ml. The concentrations values are related to salts as given above.The aliquot of methanol solution of opiates added to 1 ml serum or urine sample was 5 μl. Deuterated morphine as internal standard 200 ng in 10 μl methanol was added to each sample before extraction. 100% extraction recovery samples were simulated by derivatization of evaporated standard opiate mixture at the relevant concentration with internal standard. Three operators performed the sample extraction and derivatization always in triplets. The GC/MS measurements of these nine extracts of each sample kind and concentration level were performed during three days and repeatibility of these data were evaluated. Standard calibration curves were obtained by spiking 1ml blank serum samples with the opiate standard mixture to concentration 0, 5, 50, 500, 1000, 2000 ng. The serum calibration samples were prepared in doublets.

The following abbreviations were used in the text:

- DHC: dihydrocodeine;
- NDHC: nordihydrocodeine;
- CO: codeine;
- HC: hydrocodone;
- HM: hydromorphone;
- MO: morphine;
- NCO: norcodeine;
- NHC: norhydrocodone;

– NHM: norhydromorphone;
– MO-D3: morphine (N-methyl-D3);
– ISTD: internal standard;
– TMS: trimethylsilylderivative;
– MSTFA: N-methyl-N-trimethylsilyltrifluoracetamide;
– BSTFA: N,O-bis-trimethylsilyltrifluoracetamide;
– TMCS: trimethylsilylchlorosilane.

**Sample preparation, derivatization**

1 ml serum or urine samples were extracted on Bond Elut Certify Varian columns (130 mg). 1 ml sample with 200 ng internal standard mixed with 1 ml TRIS buffer pH 9 was applied slowly on a SPE column conditioned with 2 ml methanol and 2 ml water. Then the column was washed with 2 ml destilled water, 1 ml 0.1 M acetate buffer pH 4 and 2 ml methanol. The analytes were eluted from the dry column into a vial with 2 x 1 ml mixture of dichloromethane/isopropanol/ammonium hydroxide (25%), 40/10/1, v/v/v, prepared daily. After evaporating solvents, the dry sample extract was silylated with 100 μl MSTFA at 80°C 20 min. 1 μl was analyzed by GC/MS.

**GC/MS conditions**

Instrument used was HP GC/MSD 6890-5973 equipped with automatic liquid sampler.

Splitless injector was held at 250°C, auxiliary 270°C, oven temperature programmed from 85°C which was held 2 min, then 30°C/min till 220°C, then 3°C/min till 260°C, then 15°C/min till 280°C, then held 3.5 min. Capillary used was HP5-MS 30 m x 250 μm x 0.25 μm, carrier gas He at constant flow 1 ml/min.

Electron impact SIM mode has been used for precision and recovery experiments and to evaluate calibration parameters. Analytes tested were monitored at these conditions:

| Analyte | Starting sampling time [min] | M/z monitored | Dwell time [ms] |
|---|---|---|---|
| DHC.TMS | 13.00 | 373 315 282 236 | 50 |
| CO.TMS | 14.50 | 371 356 343 234 | 50 |
| HC.TMS | 14.85 | 371 356 313 234 | 50 |
| HM.2TMS | 15.30 | 429 414 357 234 | 50 |
| MO.2TMS | 15.53 | 29 414 401 236 | 40 |
| MO-D3.2TMS | 15.53 | 32 | 40 |
| NCO.2TMS | 15.75 | 429 414 292 250 | 50 |

Electron impact scan mode in the m/z range 45–550 with the rate 1 scan/sec has been used to to check the completness of silylation of keto-compounds and to compare scan mode and SIM limits of detection in case of need of trace analysis in certain cicumstances.

## RESULTS

The stability of opioid silylderivatives prepared from mixed reference standard substances tested has been checked by reanalysing them after storage in tightly capped vials at 4°C for 8 days. The mean area ratio values of these silylated standard mixtures measured after 8 days fitted into the range of deviations from mean value (n = 9) determined in fresh derivatives originally, except for norcodeine. The stability of silylderivates was considered as sufficient for our short term experiments. The data obtained for norcodeine could not be evaluated from reasons of poor reproducibility of silylation as explained below.

The precision experiments in serum, three days repeatibility of area ratio values on both levels of concentration tested (10 and 100 ng/ml), resulted in variation coefficients (CV) values in the range from 3 to 16% for individual silylated opioids, except of norcodeine. No significant shifts in CV values with concentration were observed. The yield of silylation of active hydrogen in nitrogen group of norcodeine molecule was not reproducible and resulted in coefficient of variation exceeding 20% even in silylated reference standard substance only, and more than 30% including SPE step in spiked serum samples (Table I).

The precision experiments in urine, three days repeatibility of area ratio values on both levels of concentration tested, resulted in CV values from 4 to 23% for individual opioids with no significant shifts between concentration values tested. The repeatibility was poor for norcodeine also in urine as can be expected (Table II).

TABLE I. PRECISION EXPERIMENTS IN SERUM. REPEATIBILITY OF GC/MS DETERMINATION IN SIM MODE, n = 9

| Analyte | CV [%] | |
|---|---|---|
| | c = 10 ng/ml | c = 100 ng/ml |
| DHC | 7 | 7 |
| CO | 7 | 3 |
| HC | 9 | 16 |
| HM | 13 | 14 |
| MO | 6 | 3 |
| NCOD | 44 | 33 |

TABLE II. PRECISION EXPERIMENTS IN URINE. REPEATIBILITY OF GC/MS DETERMINATION IN SIM MODE, n = 9

| Analyte | CV [%] | |
|---|---|---|
| | c = 10 ng/ml | c = 100 ng/ml |
| DHC | 6 | 10 |
| CO | 6 | 4 |
| HC | 23 | 21 |
| HM | 20 | 23 |
| MO | 7 | 4 |
| NCOD | 4 | – |

The recovery values in serum or urine on two concentration levels (100 and 10 ng/ml) were calculated from mean area ratio values from each series of nine experimental extracts related to those of silylated mixed reference standard substances (n = 9). The recovery value of internal standard was estimated from mean area value corresponding to appropriate series of nine extracts related to mean area value of silylated reference standard substance (n = 9). The calculated recovery of internal standard in serum was 85% and in urine 101%. (The repeatibility of internal standard area measurements in silylated reference substance expressed as CV was 7.8% and in extracts 13%). The recovery values for individual opioids from serum or urine are summarized in Tables III and IV. The recovery values from serum ranged 68–89%, without significant shifts with concentration. The recovery values from urine ranged from 72–101%, except of norcodeine. Due to poor reproducibility of norcodeine determination both in serum and urine, its recovery could not be evaluated, and it seems only to be low.

Calibration measurements in serum performed in doublets resulted in determination of lineariry in the range 0–500 ng/ml with correlation coefficient > 0.995 for individual opioids obtained on dailly basis (Figure 1). LOQ, limit of quantitation (S/N >10) was about 10 ng/ml. LOD, limit of detection (S/N > 3) could be lower for some opioids than 5 ng/ml as it is apparent from SIM chromatograms of spiked serum samples (Figure 2). The least favourite results were obtained for norcodeine. In urine, limit of detection is estimated to 10 ng/ml for hydrocodone, to 5 ng/ml of other opioids.

We consider the precision results obtained even for hydrocodone and hydromorphone as acceptable for practical toxicological analyses. We checked the course of silylation reaction especially of these compounds in various conditions (70, 80, 90°C and 20, 30, 60 min) and no difference in reaction yields was found. In GC/MS electron impact scan mode no free underivatized ketoforms were detected. (This was not the case trying to use BSTFA with 1% TMCS for silylation in our preliminary experiments, the results are not shown here).

The concentration levels both in serum or urine chosen for our experimental testing were rather low, but the choice was intentional with respect to verify and evaluate the possibilities of practical analyses not only in overdose cases but also in cases checked

Fig. 1. Determination of opiates in silylated forms in serum with internal standard deuterated morphine. Calibration curves based on GC/MS in SIM mode.

for professional fittness, e.g. drivers blood. In these cases the expected serum levels are generally low. As an example we demonstrate the results od serum and urine analyses obtained from a volunteer (60 kg body weight) after peroral application of 10 mg of hydrocodone bitartrate. In Figures 3 and 4 the results of GC/MS analyses of these human samples are demonstrated. The sensitive EI-SIM mode (Figure 3) has been used for targeted analysis of the serum sample obtained 2 hours after hydrocodone application. Only parent compound was detected, no metabolites.The concentration of hydrocodone in this serum sample determined by our SIM method was 19 ng/ml. Nevertheless the

Fig. 2. Limit of detection of hydrocodone SIM chromatograms, ion 371, spiked sera to the concentrations 0, 5, 50 ng/ml.

analysis of the same sample was performed also in scan mode for comparison. The urine sample of the volunteer was obtained after 10 hours and analysed only in EI-scan mode (Figure 4) to detect not only the parent compound but various metabolites as well. In this

case the urine sample hydrolyzed by hydrochlorid acid was used for SPE. The discussion of metabolism of hydrocodone and description of metabolites have been published elsewhere [1, 4, 5]. As it can be expected, the achieved concentration of hydrocodone in this urine sample is much higher than in serum and therefore its detection and detection of appropriate metabolites in scan mode need not cause problems. The spectra obtained

Fig. 3. SIM and SCAN chromatograms, ion 371, peaks of hydrocodone. Serum sample obtained from a volunteer of 60 kg body weight 2 hours after peroral dose of 10 mg hydrocodone tartrate. In SIM mode concentration determined 19 ng/ml (as tartrate).

at these higher concentration levels are of good quality, convenient for reliable identification of unknown analytes. The separation of hydrocodone and its metabolites is efficient. Nevertheless the confirmation and quantification of low levels of specified opiates in serum is much more convenient in SIM mode.

TABLE III. SPE RECOVERY IN SERUM, n = 9

| Analyte | Recovery [%] | |
|---|---|---|
| | c = 10 ng/ml | c = 100 ng/ml |
| DHC | 68 | 79 |
| CO | 80 | 79 |

Fig. 4. Total ion chromatogram and spectrum of hydrocodone. SCAN mode GC/MS of hydrolyzed urine sample obtained from a volunteer of 60 kg body weight 10 hours after peroral dose of 10 mg hydrocodone tartrate. Peaks (min): α-DHC (13.74), β-DHC (14.03), NDHC (14.75), HC (14.99), HM (15.48), ISTD (15.67), maybe NHC (16.13), maybe NHM (16.52).

| | | |
|---|---|---|
| HC | 77 | 72 |
| HM | 70 | 89 |
| MO | 70 | 74 |

TABLE IV. SPE RECOVERY IN URINE, n = 9

| Analyte | Recovery [%], c = 100 ng/ml |
|---|---|
| DHC | 94 |
| CO | 92 |
| HC | 63 |
| HM | 61 |
| MO | 88 |

## CONCLUSIONS

The GC/MS method described using SPE and silylation in our conditions is suitable for sensitive toxicological detection and confirmation of various opiates including those with problematic keto-group in molecular structure. The limit of detection in SIM mode in serum is of about 5 ng/ml, in urine 10 ng/ml. The method is not quite satisfactory for reliable detection of traces of N-demethylated opiates, as norcodeine, due to poor reproducibility of silylation yield in these compounds.

The method in SIM modification is convenient for determination of traces of specified opiates, e. g. in blood serum with limit of quantitation of about 10 ng/ml and CV of individual analytes in the range 3–16% at this low level. The linearity of calibration in serum was in the concentration range 0–500 ng/ml with correlation coeficient > 0.995 obtained on daily basis.

However the reliability of identification of unknowns in toxicological samples with uncertain history, which must be analyzed in scan mode, requires concentration levels of opiates of at least 50 ng/ml to receive mass spectra with quality factor > 90% using library comparisons.

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