HADMÉRNÖK

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DETERMINATION OF MORPHINE AND CODEINE IN SERUM AFTER POPPY SEED CONSUMPTION USING GAS CHROMATOGRAPHY – MASS SPECTROMETRY

Absztrakt

An analytical method is presented here for the assessment of morphine and codeine concentrations in human serum by employing mass spectrometry in the selective ion monitoring mode following its separation with gas chromatography. Deuterated (marked with heavy water) analogies of the analytes were used as surrogate internal standards. Samples were cleaned up using solid phase extraction. The target compounds were analyzed as pentafluoropropionic esters. The monitored ions were m/z=414, 577 and 361 [morphine], m/z=417, 580 and360 [D_3-morphine], m/z=282, 445 [codeine], m/z=274, 448, [D_3-codeine]. The recovery of the analytes from solid phase extraction was 70 to 80% (morphine) and 75 to 85% (codeine) at a concentration of 10 ng/mL. The limit of quantitation (confidence limit) was 0.87 ng/mL for morphine and 0.9 ng/mL for codeine. Calibration was accomplished employing blank human serum spiked with the analytes at various concentrations.

Egy analitikai eljárás került kidolgozásra a vérszérum morfin és kodein tartalmának meghatározására gázkromatográfiás-tömegspektrometriás technikával szelektív ionmonitorozás technikával. Az eljárással kísérő sztenderdként a vizsgált vegyületek deutériummal jelölt molekuláit alkalmaztuk. A minták tisztítása és kivonása szilárdfázisú extrakcióval történt. A vizsgált hatóanyagokat penta-fluoropropionsavanhidriddel származékoltuk. A mért inonok (n/z) morfin: 414, 577 és 361; morfin-D3: 417, 580 és 364; kodein: 282, 445; kodein-D3: 285 és 485 voltak. Az analitikai eljárás visszanyerési hatásfoka 10 ng/mL koncentrációknál morfinnál 70-80 % illetve kodeinnél 75-85 %. Az eljárás mennyiségi kimutatási határértékei morfinnál 0,87 ng/mL illetve kodeinnél 0,9 ng/mL. A kalibrációhoz humán szérumot használtunk úgy, hogy a vizsgált anyagnak a kalibrációhoz szükséges koncentrációjú elegyével jelöltük meg.

Keywords: *tiltott kábítószerek, anyagcsere, mák, GS-MS, morfin, ingyenes morfin, kodein ~ illicit drugs, metabolites, poppy seed, GS-MS, serum morphine, free morphine, codeine*

INTRODUCTION

Serum and urine samples are frequently tested to identify illicit drug consumption. The presence of illicit drugs or their metabolites in urine is proof of drug use, while their concentrations in blood are proportional to their effects upon the user. *[1-2]* The consumption of these drugs by the personnel of the Hungarian Defence Forces is strictly prohibited. In order to enforce this rule, urine samples are analyzed for the presence of compounds which would indicate heroin, morphine or codeine abuse. *[3-4]*

EXPERIMENT

Testing for illicit substances in biological samples is very difficult due to the fact that there are very small quantities involved, and the fact that these biological samples themselves contain many different substances, some of which could cause interference during the testing. It is therefore very important to perform some in vitro tests to determine how reliable the testing we do is. We need to test for these substances in normal human volunteers. Unfortunately, we run into a quandary here, because if we give illegal substances to human volunteers, it would be in fact unethical. In this situation, however, Hungary finds itself in a unique situation. Poppy seed (Papaver Somniferum), which contains morphine and related compounds in substantial amounts, is a staple item in the Hungarian cuisine, and it is routinely and regularly ingested by a large part of the Hungarian nation. No law restricts the amount of poppy seed contained in food and for example, up to 120 g is usually contained in a piece of poppy seed cake. Investigations performed in our laboratory show that adults excrete morphine and codeine in urine above the approved cut-off level for up to 3 days after eating one piece of this cake. *[5-6]* However, in Hungary there is a law *[7]* regulating the level of narcotics a Poppy-seed can contain that can be sold to the public *(Table: 1)*.

Drugs	Food group, type of food	Maximum Allowable Consumption
1. Morphine	poppy seed	30 mg/kg
2. Narcotin	poppy seed	20 mg/kg
3. Morphine & narcotin	poppy seed	40 mg/kg
4. Thebaine	poppy seed	20 mg/kg
5. Codeine	poppy seed	20 mg/kg

Table 1. The maximum allowable consumption of controlled material from natural origin allowed in food groups

Extracts of Poppy-seed were tested for the amount of Morphine is contained, at the level varied between 0-70 mg/kg. We decided to run tests on human volunteers after the ingestion of food articles containing poppy-seed in order to determine the abilities of our lab to detect small quantities of morphine and related substances. In this way, we can get adequate substation that gives real values. It also is ethical, but even then, to double check the ethics, we submitted our proposal to the Committee of Ethical Studies of the Institute of Health Protection, Hungarian Defense Forces, and it was dually approved prior to the beginning of the investigations.

EQUIPMENT

Analysis was performed on an Agilent 6890 gas chromatograph (GC) coupled with an Agilent 5973 single quadrupole mass spectrometer (MS; Agilent Technologies, Wilmington, DE). Samples were injected using an Agilent autosampler unit. The extraction of analytes was performed on a Bond Elute Certify cartridge (CP-Analitika Kft., Budapest, Hungary). Disposable equipment was used for sample preparation whenever possible, including pipette tips, vials, caps and solid phase extraction cartridges.

Gas chromatography conditions

1.0 µL splitless injections were made at 280 °C injector temperature. Carrier gas was Helium 6.0 grade (Messer Hungarogáz Kft., Budapest, Hungary) introduced at a flow rate of 1.0 mL/min. Separation was performed on a Varian FactorFour™ VF-5MS capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 µm; CP-Analitika Kft., Budapest, Hungary). Oven temperature was kept at 100 °C for 1 min, followed by an increase of temperature of 20 °C/min to 220 °C, then 3 °C/min to 240 °C, then at 20 °C/min to 300 °C and was finally held constant for 5 min.

Mass selective detection

The transfer line temperature was 280 °C. Electron impact ionization was performed at 70 eV energy, 230 °C ion source temperature. Quadrupole temperature was 150 °C. The MS was operated in single ion monitoring (SIM) mode. Monitored ions are displayed in *Table 2*.

Name of compound	Purpose	Target ion (m/z)	Qualifier ion #1 (m/z)	Qualifier ion #2 (m/z)
Morphine pentafluoropropionic ester	analyte	414	577	361
Codeine pentafluoropropionic ester	analyte	282	445	
D_3-Morphine pentafluoropropionic ester	internal standard	417	580	364
D_3-Codeine pentafluoropropionic ester	internal standard	285	448	

Table 2. Monitored ions of analytes and internal standard compounds

Data acquisition and processing.

Data was acquired with the Agilent Chemstation Software (Kromát Kft., Budapest, Hungary). Peak areas were used for quantification.

Analytical results were produced using B.E.N. Software (a program designed to run on Windows Excel™ determining the analytical parameters of the GC-MS measurements) *[8]*.

MATERIALS AND METHODS

Chemicals

Morphine [100µg/mL in methanol], D_3-morphine [100µg/mL in methanol], codeine [100µg/mL in methanol] and D_3-codeine [100µg/mL in methanol] solutions were obtained from Cerilliant Company (Austin, TX).

Pentafluoropropionic anhydride (PFPA) and pentafluoropropanol were purchased from Sigma Aldrich Hungary Kft. (Budapest, Hungary).

Standard human serum was acquired from Promochem (Budapest, Hungary).

All other chemicals and solvents were obtained from Merck Kft. (Budapest, Hungary) and were analytical grade.

Solutions

Calibrations were done using the standard serum and were taken through the same preparation steps as the blood samples. Serum standards and blood samples were spiked with standard solutions A and B as well as the surrogate internal standard solution *(Table 3)*.

ID	matrix	volume	Volume of standard solution spikes (µL)			Morphine and codeine concentration (ng/mL)
			Standard solution B	Standard solution A	Surrogate internal standard solution	
Chemical standard	methanol	1 mL	0	5	1	50
Serum blank	blank serum	1 mL	0	0	0	0
Calibrator #1	blank serum	1 mL	1	0	1	1
Calibrator #2	blank serum	1 mL	2.5	0	1	2.5
Calibrator #3	blank serum	1 mL	5	0	1	5.0
Calibrator #4	blank serum	1 mL	10	0	1	10
Calibrator #5	blank serum	1 mL	0	2.5	1	25.0
Calibrator #6	blank serum	1mL	0	5.0	1	50
Calibrator #7	blank serum	1mL	0	10	1	100
Blood sample	serum	1 mL	0	0	1	

Table 3. Composition of calibrators, blanks and serum samples

pH=9 carbonate buffer solution. To 20 mL 0.2 mol/L sodium carbonate aqueous solution 230 mL 0.2 mol/L Sodium Hydrogencarbonate aqueos solution was added. The mixture was diluted with distilled water (LichroSolv® grade) to 1000 mL.

Morphine and codeine concentrations that were obtained during the analysis of the chemical standard were considered 100% in recovery experiments.

Standard solution A. 100 µL Cerilliant Morphine Solution and 100 µL Cerilliant Codeine Solution was diluted to 800 µL with methanol in a 2 mL silanized vial (CP-Analitika Kft., Budapest, Hungary). The solution was used for 1 week and stored at -20 °C.

Standard solution B. 100 µL Standard solution A was diluted 900 µL to with methanol in a 2 mL silanized vial (CP-Analitika Kft., Budapest, Hungary). The solution was used within 24 hours.

Surrogate internal standard solution. 100 µL Cerilliant D3-Morphine Solution and 100 µL Cerilliant D3-Codeine Solution was diluted to 800 µL with methanol in a 2 mL silanized vial (CP-Analitika Kft., Budapest, Hungary).

The solution was used for 1 week and stored at -20 °C.

Blood collection

Blood was collected in tubes with walls previously coated with sodium fluoride from healthy volunteers who had given their written informed consent in advance. The tubes were gently shaken for 30 seconds and then kept at 5 °C for 10 minutes. Centrifugation was done at 3000 rounds per minute for 10 minutes using an Eppendorf 584 Centrifuge (Eppendorf GmBH, Hamburg, Germany). 1 mL supernatant was then transferred to a 2 mL silanized vial for clean-up and derivation.

SAMPLE PREPARATION AND DERIVATION

Sample clean-up was performed by applying the supernatants to solid phase extraction cartridges containing a mixed special C_8 and strong cation exchanger sorbent *[9]*. Recovery of the analytes from solid phase extraction was assessed by processing three standard serum samples spiked with both compounds at a concentration of 10 ng/mL each. The column was pre-conditioned with 3 mL methanol and 3 mL pH=9 carbonate buffer solution. Serum samples previously spiked with the standard solutions were allowed to drip through at a rate of 1 mL/min.

After the sample passed through the sorbent the latter was washed with 3 mL distilled water and allowed to dry for 35 minutes *in vacuo* (-50 mm Hg). The analytes were eluted at a rate of 1 mL/min into a 2 mL silanized vial using 1 mL dichloromethane:propanol:ammonia 80:20:2 mixture. The elution process was repeated once. The eluted fractions were combined and desiccated at 36 °C under a gentle stream of nitrogen, using a Pierce Reacti-Therm™ Heating Module (Dr. Wéber Consulting Kft., Budapest, Hungary). The dry residue was reconstituted with 100 µL PFPA and 70 µL pentafluoropropanol, the vial was closed and kept at 60 °C for 30 minutes. After this period the solution was allowed to cool to room temperature and was then forwarded for analysis.

RESULTS AND DISCUSSION

The illicit consumption of drugs and psychotropics is a serious problem in modern societies. The specific and sensitive identification and quantification of these chemicals and their metabolites, as well as utilization of analytical data for the verification of the influence of illicit acute and chronic drug consumption on individuals involved in felonies therefore remains an important challenge.

A number of approaches have been established for the verification of illicit drug consumption. Urine is one of the most widely used sample for such investigations *[10-13]*. However, the collection of urine is not always feasible without invading the subject's privacy. In addition to this, urine analysis does not always provide evidence of a single intake since it takes time, usually a few hours, for urinary metabolite concentrations to reach the limit of detection *[10]*. Assessed concentrations are affected by fluid intake, nutritional regimen, age, consumption of pharmaceuticals and health status especially noting whether the person has circulatory or renal disorders, which raises the risk of obtaining false negative results.

The presented method was developed for the determination of morphine and codeine concentrations in serum. The linear dynamic range was found to range from 1 to 100 ng/mL for both compounds. For each curve, seven different concentrations were used, not including the standard matrix. The regression line *(Table 4)* was calculated by the German Industrial Standards(DIN) 32645 rules*[14]*.

Compound	Intra-assay Calibration curve	Correlation coefficient(r2)	Intra-assay Calibration curve	Correlation coefficient(r2)
Morphine	y=0,1085x-0,0399	0,99999	y=0,1059x-0,0019	0,99998
Codeine	y=0,1029x+0,0634	0,99999	y=0,102x+0,395	0,99999

Table 4. Results of the regression lines(n=5)

Statistical tests were performed with confidence level of 95%. The limit of quantification was 0.87 ng/mL for morphine and 0.9 ng/mL for codeine. Determination coefficients (r^2) of the calibration curves were higher than 0.9999 in all cases. Recovery of the analytes from solid phase extraction was estimated by processing three standard serum samples spiked with both compounds at a concentration of 10 ng/mL each. The recovery was found to be 70-80%

for morphine and 75-85% for codeine. The recovery of the internal standards did not differ significantly (p=0.05) from that of the analytes. Running a solvent standard *(see Table 2)* allows for the calculation of recoveries after processing each set of samples.

Several measures were taken to assure specificity during the analysis. Disposable equipment was used for sample preparation whenever possible, including pipette tips, vials, caps and solid phase extraction cartridges. The absence of interfering peaks was verified for each sequence of samples that were analyzed by running serum standards *(see Table 2)* concurrently.

The identity and purity of ion chromatogram peaks were checked by matching the retention time, peak shape and the ratio of target and qualifier ions *(Table 5)* with those of the standards *[14-16]*.

Name of compound	Retention time (min)	Qualifier #1 / Target ion	Qualifier #2 / Target ion
Morphine pentafluoropropionic ester	8.93	20±4 %	10±2 %
Codeine pentafluoropropionic ester	9.53	55±6 %	
D_6-Morphine pentafluoropropionic ester	8.89	28±4 %	12±2 %
D_3-Codeine pentafluoropropionic ester	9.50	60±6 %	

Table 5. The accepted ranges of the ratios of target and qualifier ions, provided as percentage

Peak symmetry was also a requirement. As a result of these efforts, the detection of the analytes and the internal standards was specific after chromatographic separation. The evaluation of the analytical data obtained from serum blank runs indicated that no interfering ion peaks were present in the matrix. In order to further reduce the chance of misidentification caused by interference occurring at the retention time of the analyte peaks, narrow time reference peak windows (±0.5%) were set *[18]*.

Spiking the deuterated analogs of the analytes to the samples as surrogate internal standards prior to sample preparation eliminated all difficulties arising from the diversity of matrix components and the loss of analyte during the extraction process. This approach renders the analytical method equivalent to reference methods as it can be categorized as a special kind of isotope dilution techniques with the labelled internal standards being analogous to the analytes. The minimal difference between the molar masses of the analyte molecules and the internal standard chemicals, along with the overwhelming similarities in physical and chemical properties, results in virtually identical retention of analyte and its deuterated analog on the chromatographic column. The probability of the appearance of interfering peaks is therefore reduced, just as any inaccuracies that would otherwise be inevitably introduced during sample preparation. Standardization was performed using standard serum specimens and were taken through the same sample preparation steps as the samples. This eliminated analytical errors arising from adverse chemical reactions between analytes and molecules normally present in human serum. Using surrogate internal standards also promoted a one-step calculation of analyte concentrations by placing the ratio of the target ion peaks of the analyte and the internal standard into the equation obtained during calibration.

The calculation of the limits of detection, identification and quantitation were based on average concentrations obtained during intraassay and interassay comparison studies *(Table 6)*.

	Intra-assay		Inter-assay	
	Morphine	Codeine	Morphine	Codeine
Limit of detection (ng/mL)	0.23	0.24	0.25	0.25
Limit of identification (ng/mL)	0.47	0.48	0.51	0.52
Limit of quantitation (ng/mL)	0.87	0.90	0.93	0.98

Table 6. Limits of detection, identification and quantitation of morphine and codeine (n=5)

These studies were conducted as defined by the German Industrial Standard DIN 32645 *[14]*. The aim of these studies was to estimate the accuracy of the analytical procedure. The analysis was performed on the lowest concentration level (1 ng/mL morphine and codeine; n=5) *(Table7.).*

Compound	Calculated concentration (ng/mL)	Relative standard deviation (%)	Relative error (%)
Morphine	1.004	7.6	0.4
Codeine	0.914	7.0	8.6

Table 7. Results of the intra-assay accuracy analysis (n=5)

Nominal concentration of both morphine and codeine was 1.0 ng/mL. Calculated concentrations are the means of concentrations obtained in 5 sequential runs. Relative error is the deviation of the calculated concentration from the nominal concentration *(Table8.).*

Compound	Calculated concentration (ng/mL)	Relative standard deviation (%)	Relative error (%)
Morphine	1.01	10.5	1
Codeine	0.92	11.5	8

Table 8. Results of the inter-assay accuracy analysis (n=5)

Nominal concentration of both morphine and codeine was 5 ng/mL. Calculated concentrations are the means of concentrations obtained in runs performed on 5 consecutive days. Relative error is the deviation of the calculated concentration form the nominal concentration. The bias was less than 10% in the case of the lowest concentration calibrator. .In studies of accuracy the differences between measured and expected concentrations were not higher than 10.5% in the case of morphine and not higher than 11.5% in the case of codeine *[15-16]*.

System applicability analysis indicated that the task presented herein could be accomplished using GC-MS, therefore this technique was selected *(1. Figure).*

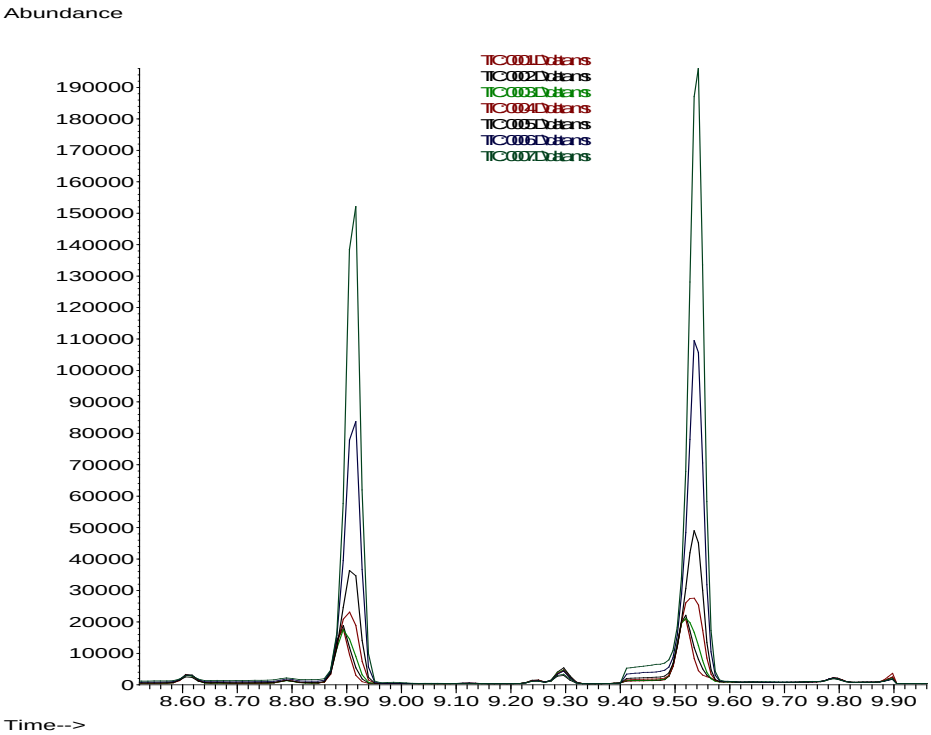

Figure 1. Overlay chromatograms of morphine and codeine belonging to the same series of calibration

Employing PFPA as a derivation agent provided several benefits. It is a very reactive chemical, allowing a simple, rapid and complete derivation of the analytes and the internal standards. The adducts are volatile and have a molecular mass considerably higher than that of the analytes. Electron ionization mass spectra of adducts were pure with an intensive molecular ion in the case of all monitored compounds. The adducts are highly fluorinated, so they can be detected with a high degree of sensitivity using negative chemical ionization.

Exploratory measurements were performed for the following reasons:

- The concentrations of the analytes in the samples were expected to be less than the low end of the therapeutic range (2 ng/mL),
- Blood is a complex matrix containing endogenous chemicals and macromolecules, which causes difficulties during separation,
- Retention parameters of both the target compound and the internal standard and the reproducibility of these parameters (<2% relative standard deviation) was evaluated,
- Tailing factors of the chromatographic peaks of the target analyte and the internal standard were determined and it was verified the value of these factors was below 5%.

System applicability analysis indicated that the task presented herein could be accomplished using GC-MS, therefore this technique was selected.

CONCLUSIONS

The presented method can be applied successfully for the fast and accurate determination of morphine and codeine concentrations in human serum. The method meets quality control requirements. With the use of calibrators prepared by spiking the analytes and the internal standards that were added to standard serum, and by employing deuterated analogs of the analytes as internal standards, the important analytical parameters (limits of detection, identification and quantification) can be inferred after running each sequence of samples.

Running serum extracts allow the most favourable signal-to-noise ratio among all the biological matrices. Therefore, serum is the matrix of choice in pharmacokinetic studies. The small volume required for analysis makes the method compliant with ethical requirements.

Deuterated analogs of the analytes were used as surrogate internal standards (isotope dilution technique). This allows for the elimination of many errors normally introduced during sample preparation and analysis. The other important factor that contributes to the elimination of errors is that calibration was performed using standard serum spiked with the analytes. The way that this was evaluated was the ratio of signal intensities.

Solid phase extraction was employed for cleaning up the samples. This is the most widely used sample preparation method as adsorbents are available with various polarities and selectivities. Solid phase extraction is a simple, easy-to-learn technique with high sample throughput. It also allows remarkable selectivity and reproducibility for analysis.

The analysis was performed using a fully computer-controlled GC-MS equipped with an auto-sampler unit. Samples could be analyzed with using both electron impact ionization and chemical ionization. The latter increased sensitivity three times.

This method developed by our team is suitable not only for therapeutic drug-level processing but also for investigating and confirmation of drug abuse.

This method was also applied to distinguish the consumption of foods containing poppy seed from abuse of different kinds of drugs produced from the poppy plant (opium, poppy straw, poppy tea etc.). The results of blood analysis of the participants taking part in the experiment are shown in the *Table 9.*

Partici-piants' ID No.	Morphine (free) (ng/mL)			Codeine (ng/mL)		
	in 1h	in 2h	in 4h	in 1h	in 2h	in 4h
I.	1.10	1.80	<1.00	<1.00	<1.00	-
II.	1.18	2.00	<1.00	<1.00	-	-
III.	1.20	2.10	<1.00	<1.00	<1.00	-
IV.	1.15	1.78	<1.00	<1.00	-	-
V.	1.00	1.80	<1.00	<1.00	-	-
VI.	1.15	1.50	<1.00	<1.00	<1.00	-
VII.	1.40	1.90	<1.00	<1.00	-	-
VIII.	1.10	1.40	<1.00	<1.00	-	-
IX.	<1.00	1.15	<1.00	<1.00	-	-
Blank	0.00	-	-	-	-	-

Table 9. The participants' blood analysis results for the morphine and its metabolites

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