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## Development of a LC-MS/MS Method for the Determination of Narcotic Drugs and Psychotropic Substances in Human Hair

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Narcotic drug use is an important problem in our country and all over the world. Hair is a valuable specimen for monitoring these long-term drug uses. For this purpose, a sensitive method was created and validated for the identification and quantification of benzoylcegonine (morphine, codeine, 6-monoacetylmorphine, heroin, tetrahydrocannabinol, amphetamine, 3,4-methylenedioxyamphetamine, buprenorphine, methamphetamine, 3,4-methylenedioxy-N-methylamphetamine, and cocaine in human hair by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The sample preparation step includes washing, standard addition, liquid-phase extraction with methanol, and solid-phase extraction steps. Analysis for substances identified from a pretreated hair took a maximum of 3.64 min. In the method, the LOD values of each substance ranged from 0.11-0.87 ng mg<sup>-1</sup>, and the linearity was quite good ( $r^2 > 0.99$ ). The concentration ranges for quantification were 0.50 and 8.00 ng mg<sup>-1</sup> for all substances. In addition, the intraday and interday accuracy and precision values of this method were acceptable (<12.81%) and the recovery was found to be between 93.72%-104.78% at different concentrations. The results of this study showed that the developed very fast and reliable LC-MS/MS method is suitable for use as a validated method in the analysis of illicit drugs simultaneously in hair.

**Keywords:** Narcotic drug, Psychotropic substance, Hair, LC-MS/MS, Method validation

### INTRODUCTION

The world's largest drug smuggling route is the Balkan route, where narcotic stimulants are taken from Afghanistan via Iran, Turkey, and the Balkans to Western and Central European markets [1]. Turkey, which is both a destination and a transit country, is affected by the traffic of opium and derivatives originating from Afghanistan, psychotropic substances originating from Europe, and the chemicals used in their production.

The doubling of the quantity of drugs seized within the borders of the European Union and the increase in the

quantity of drugs seized in Turkey in recent years are alarming [2]. While cannabis still ranks first (51.9%) among the drugs used in Turkey, the rate of use of heavier quality drugs has increased compared to the previous year [3]. In addition, the results of fatal overdose cases reported by Turkey indicate a younger profile of drug-related deaths in Turkey than the average age of those in the European Union [2].

Examination of human biological materials in order to detect the presence of these Narcotic drugs and psychotropic substances that especially affect young people in Turkey is an important task of forensic and clinical toxicology laboratories. In these laboratories, narcotic drugs and psychotropic substances analyzes are mostly performed on urine and blood samples. However, the results obtained can

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only provide information about drug use for a few days. Information on longer-term use can only be obtained by analyzing hair samples.

Toxicological analysis of hair in different countries; is performed for various purposes such as postmortem toxicology, drug screening in the workplace, license renewal, and doping control [4,5]. To combat drug abuse, hair analysis, in addition to traditional urinalysis, has become an indispensable tool in drug administration procedures in some countries. Narcotic drug analysis in hair is used in court as evidence of chronic use. The advantage of hair as a test matrix is that it can provide historical details of drug exposure, samples are easy to collect under close surveillance, and are relatively noninvasive [6].

Hair analysis for narcotic-stimulant substances is increasingly applied, especially in the field of forensic medicine. In such an analysis, gas and liquid chromatography-mass or tandem mass spectrometry (GC-MS LC-MS or GC-MS/MS and LC-MS/MS) are considered the "gold standard" for the determination of drug-stimulant substances in hair due to their good sensitivity and specificity [6,7]. However, in our country, drug analysis in hair can only be done in the laboratories of the Forensic Medicine Institute, which is responsible for responding to the demands of the prosecutor's office and the courts, and the test results are reported very late due to the heavy workload. For this reason, undertaking this service by alternative laboratories will both accelerate the justice mechanism and respond to other private and legal requests apart from judicial authorities.

LC-MS/MS is highly preferred since there is no derivatization step in the analysis of narcotic drugs in the hair. However, some past studies have included an incubation time of 18 h or more, and analysis times longer than 10 min have been reported [8,9,10]. In many studies, method validation studies were carried out for only one or a maximum five-item analysis at the same time [11,12]. In the methods where more substances were analyzed, the recovery values decreased to 25% [13]. For this reason, it is very important to create reliable methods for drug analysis in hair, where the sample preparation and analysis time is lower, and many drugs can be analyzed at the same time.

In our study, an LC-MS/MS method was developed to detect almost all narcotic drugs and psychotropic substances ((benzoylecgonine (BEG), morphine (MOR), codeine,

6-monoacetylmorphine (6-MAM), heroin, tetrahydrocannabinol (THC), amphetamine (AMPH), 3,4-methylenedioxyamphetamine (MDA), buprenorphine (BUP), methamphetamine (mAMP), 3,4-methylenedioxy-N-methylamphetamine (MDMA), and cocaine (COC)) in hair extracts of users of these drugs simultaneously and even at very low concentrations. This method will contribute to the literature as the first alternative scientific method established to perform multiple drug analyses in hair simultaneously in clinical laboratories in Turkey.

## EXPERIMENTAL

### Materials and Chemicals

Standards of BEG, MOR, codeine, 6-MAM, heroin, THC, AMPH, MDA, BUP, mAMP, MDMA, and cocaine were purchased from Lipomed (Lipomed AG, Arlesheim, Switzerland). Nalorphine, THC-d3, AMPH-d5, MDA-d5, mAMP-d5, MDMA-d3 used as internal standards were also obtained from lipomed (Lipomed AG, Arlesheim, Switzerland). Methanol, acetonitrile, acetone, and ammonium format were obtained from Merck (Merck KGaA, Darmstadt, Germany). Solid phase extraction (SPE) columns with a sorbent of a permeable reactive barrier (60 mg/3 ml) were obtained from Welch/WL00522-20009 (WelchMaterials Inc., USA).

### Sample Collection and Application

Blank hair samples, which will be used in the validation of the narcotic drugs and psychotropic substances analysis method in hair, were collected from volunteers (from 5 people over 18 years old). Each volunteer signed the informed consent form. Hair samples were quickly washed with water, methanol, and acetone respectively in triplicate. Hair samples were washed to remove hair care products, sweat, and surface materials that could interfere with the analysis or reduce extraction efficiency, and to remove the risk of potential external contamination of drugs [14,15]. The samples were dried at room temperature and stored dry until further analysis.

### Preparation of Standards and Hair Quality Control Samples

From the reference stock solutions at a concentration of 1 mg ml<sup>-1</sup>, two separate stock solutions at a concentration of

10 mg l<sup>-1</sup> were prepared. One of these stock solutions was used for calibration curves (linearity and sensitivity) and the other for validation studies (recovery, precision and accuracy).

The MOR internal standards used were nalorphine for BEG, codeine, 6-MAM, heroin, BUP, COC; THC-d3 for THC; AMPH-d5 for AMPH; MDA-d5 for MDA; mAMP-d5 for mAMP; MDMA-d3 for MDMA. For internal standard substances, a mixed stock solution was prepared at a concentration of 10 mg l<sup>-1</sup> and the solvent was added to the extraction solution with methanol at a final concentration of 40 mg ml<sup>-1</sup>.

The extraction method was revised from previous studies [16-18]. Hair samples were cut to a length of approximately 1-2 mm before weighing. 50 mg of the ready-made hair samples were weighed and transferred to each glass tube which corresponds to three concentrations of quality control samples. 2 ml of methanol, standard substances, and internal standard substances were added to the samples and mixed in a shaker for 30 min. Methanol was preferred in our study, as studies have shown that methanol is the most effective organic solvent in hair [19]. 2 ml of 0.1 M HCl was added and incubated for 3 h at 60 °C. After the centrifuge (1780 x g for 10 min) the upper phase was transferred to a clean second tube. 2 ml of 1 M NaOH was added to the first tube and incubated at 90 °C for 45 min, after the centrifuge the upper phase was transferred to the same second tube. The pH of the consolidated solution in each tube was neutralized and then subjected to SPE extraction. The cartridge was conditioned with 1 ml of methanol and 1 ml of ultrapure water. The sample was then loaded into the cartridge and 1 ml of 5% methanol solution was passed through. After the cartridge was left at room temperature for 20 min, 1.5 ml of washing solution (0.5 ml 100% methanol; methanol with 2% ammonia; methanol with 2% acetic acid) was passed and the solution coming out of the cartridge was collected. The eluted samples were concentrated under a nitrogen evaporator, dissolved in 100 µl of methanol, and transferred to the analysis vials. Measurements were made by injecting 10 µl into the LC-MS/MS device.

### LC-MS/MS Conditions

Sample analysis was carried out on a Shimadzu LCMS-

8030-plus (UFLC XR model LC-MS/MS, ESI technology). Analytes were separated on a Shim-Pack Column (FC-ODS 150mmX2.0mmID, 3µm, Shimadzu) *via* gradient elution using 10 mM ammonium format (mobile phase A) and 100% methanol (mobile phase B).

The separation of narcotic drugs and psychotropic substances was achieved following oven temperature of 40 °C, nebulized gas flow of 1.5 l min<sup>-1</sup>, desiccant gas flow of 10 l min<sup>-1</sup>, and total flow of 0.4 ml min<sup>-1</sup> in dual gradient mode. Oven gradient flow was as follows; achieving 95% concentration for pump B in 3 min and holding for 5 min, finally decreasing to 5% and stopping in one min. A and B pumps maximum pressure was 660 bar.

A heated electrospray ionization (ESI) source was used in the mass spectrometer system at the mode of positive ionization. Argon (collision) and nitrogen (carrier) gases were used in the analysis. By performing multiple reaction monitoring (MRM) optimization (dwell time 50) of each analyte, collision energies, main ions, and mass analysis of product and precursor ions were determined.

### Validation Parameters

Method validation in our study was performed according to the guidelines of the Society of Hair Testing (SoHT), the European Workplace Drug Testing Society (EWDTS), the European Medicines Agency's Guideline on bioanalytical method validation, and following the accepted criteria for bioanalytical method validation[20-25].

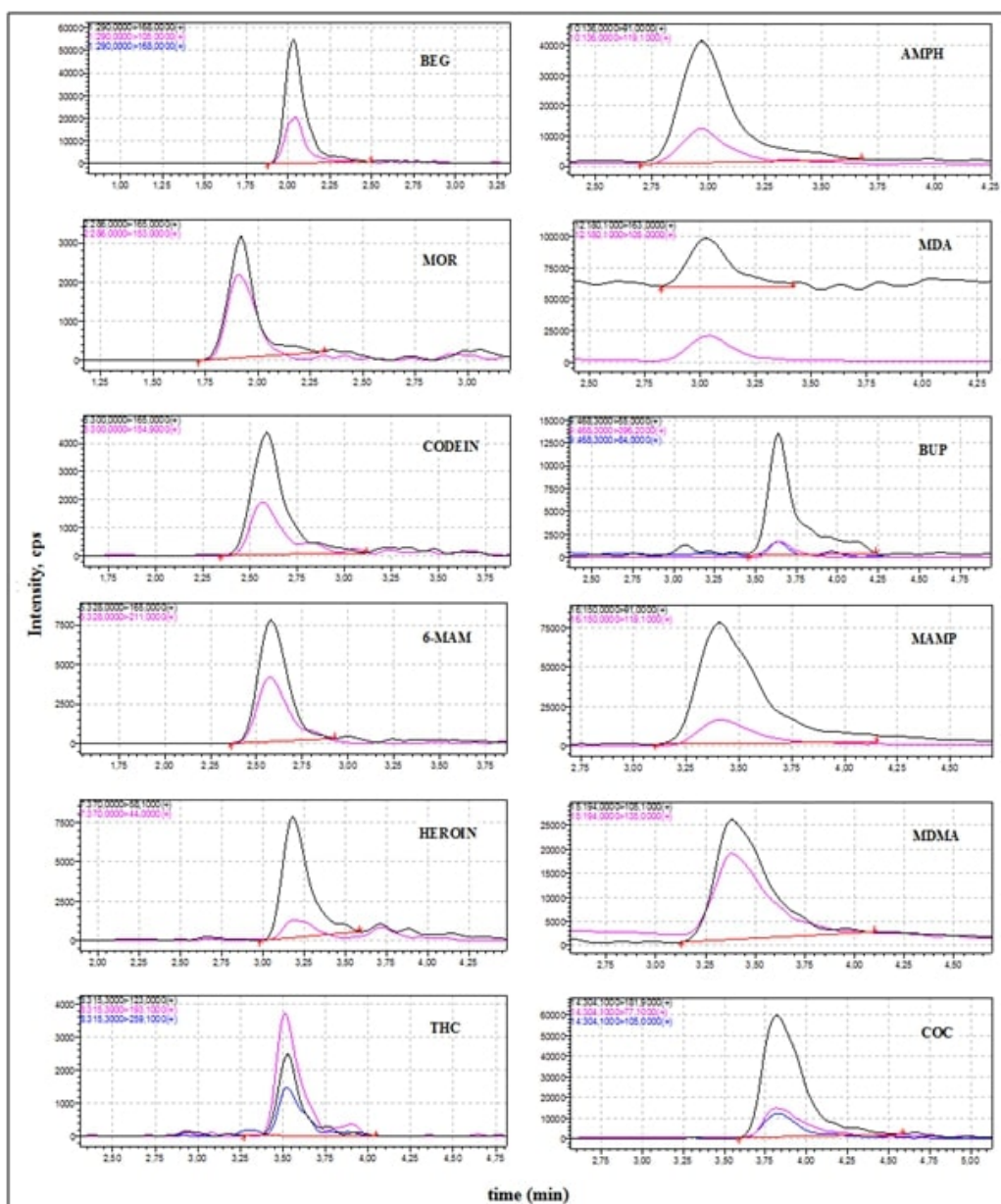
Necessary validation tests were carried out to make the method as selective as possible. Before starting the development of a quantitative method, the analytical range was determined, which should cover all the concentration levels expected to be present in the sample analyzed to ensure consistent results. The analytical range of this method was defined as the range with appropriate precision, accuracy, and linearity. The sensitivity of the method was also measured, as a small difference in analyte concentration is very significant in this method. Linearity, recovery, selectivity, intra-, and inter-assay precision, and accuracy were evaluated.

All analyzes were performed at least in triplicate. The linearity range was established between 0.5-8 ng ml<sup>-1</sup>. For the sensitivity, The LOD and LOQ were measured relative to the signal-to-noise ratio at approximately 3 and 10. For

recovery, low, medium, and high concentrations (1 ng ml<sup>-1</sup>, 4 ng ml<sup>-1</sup>, 8 ng ml<sup>-1</sup>) were used. The bias was obtained by dividing the difference of the result from the target value by the target value. Relative standard deviation was calculated by dividing the standard deviation of values by the average of the values.

## RESULTS AND DISCUSSION

The retention times of 12 analytes in the hair samples are given in Fig. 1. The retention times, Q1 and Q3 ions, CE (V) values of each analyte and internal standard are given in Table 1.



**Fig. 1.** Chromatograms were obtained in the analysis of narcotic drugs and psychotropic substances (4 ng ml<sup>-1</sup>). (Abbreviations: BEG: Benzoylcegonine; MOR: Morphine; 6-MAM: 6-Monoacetylmorphine; THC: Tetrahydrocannabinol; AMPH: Amphetamine; MDA: 3,4-methylenedioxyamphetamine; BUP: Buprenorphine; mAMP: Methamphetamine; MDMA: 3,4-Methylenedioxy-N-methylamphetamine; COC: Cocaine).

**Table 1.** Multiple Reaction Monitoring (MRM) Conditions for each Analyte and Internal Standard

Analyte	t <sub>R</sub> (min)	Q1 <sup>a</sup> (m/z)	Q3 <sup>b</sup> (m/z)	CE (V)
BEG	2.03	290.0	168.0, 105.0, 168.0	-20, -31, -20
MOR	1.91	286.0	165.0, 153.0	-40, -43
CODEIN	2.58	300.0	165.0, 154.9	-43, -38
6-MAM	2.57	328.0	165.0, 211.0	-38, -28
HEROIN	3.19	370.0	44.0, 58.1	-40, -32
THC	3.52	315.3	123.0, 193.1, 259.1	-35, -22, -21
AMPH	2.96	136.0	91.0, 119.1	-19, -14
MDA	3.01	180.1	163.0, 105.0	-12, -23
BUP	3.64	468.3	84.0, 55.0, 396.2	-49, -52, -45
mAMP	3.40	150.0	119.1, 91.0	-15, -20
MDMA	3.40	194.0	105.1, 135.0	-25, -20
COC	3.84	304.1	182.0, 181.9, 77.1, 105.0	-20, -20, -55, -33
<i>Nalorphine</i> *	2.76	312.0	201.0, 184.9, 164.9	-30, -32, -42
<i>THC-d3</i> *	3.36	348.1	302.0, 196.0, 330.1	-21, -28, -17
<i>AMPH-d5</i> *	3.77	141.0	93.0, 124.1	-19, -14
<i>MDA-d5</i> *	3.95	185.0	168.0, 110.1	-12, -23
<i>mAMP-d5</i> *	4.51	155.0	92.0, 93.0	-22, -27
<i>MDMA-d5</i> *	4.58	197.0	105.0, 77.0	-25, -44

(Abbreviations: t<sub>R</sub>: retention time; CE: Collision Energy; BEG: Benzoylcegonine; MOR: Morphine; 6-MAM: 6-Monoacetylmorphine; THC: Tetrahydrocannabinol; AMPH: Amphetamine; MDA: 3,4-methylenedioxy-amphetamine; BUP: Buprenorphine; mAMP: Methamphetamine; MDMA: 3,4-Methylenedioxy-N-methylamphetamine; COC: Cocaine). <sup>a</sup>Precursor ion. <sup>b</sup>Product ion. \*Internal Standard.

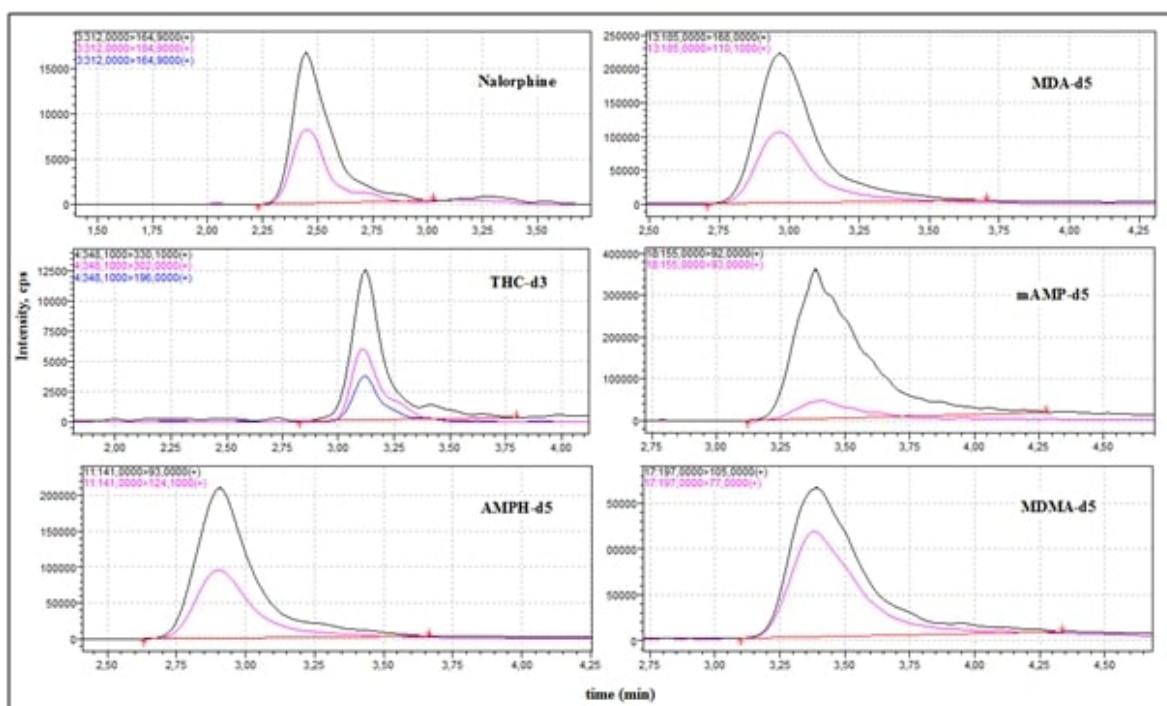
The specificity of the method was evaluated by comparing the blank hair and standard solution added hair samples with the mass spectra obtained by LC-MS/MS. Figure 1 shows the chromatograms of each narcotic drug and psychotropic substance, which indicate that there was no significant interference at their respective retention times. These data indicate that the method is capable of separating all of the 12 analytes under the given gradient conditions. Chromatograms obtained in the analysis of internal standards are also given in Fig. 2. There is no interference in the chromatogram of the internal standards.

### Linearity and Sensitivity

For linearity, standard solutions at five different concentrations (0.50-1.00-2.00-5.00-8.00 ng mg<sup>-1</sup>) were used and calibration curves were drawn for each narcotic drug and psychotropic substance. The determination of the

conformity of the points obtained here to the line equation was shown with the coefficient of determination ( $r^2$ ). The linearity ranges were shown in Table 2 and calibration curves showed good linearity ( $r^2 > 0.9993$ ) within these ranges.

Limit of detection (LOD) and limit of quantity (LOQ) were defined for sensitivity. The LOD and LOQ for all narcotic drugs and psychotropic substances were measured relative to the signal-to-noise ratio at approximately 3 and 10. The LOQ was determined as the lowest concentration level that can be verified with acceptable values for recovery and precision. The values should not exceed 15%. The recommended cut-offs for substances in the hair to determine their use are in the range of 0.01-0.5.00 ng mg<sup>-1</sup>. LOD values were found between 0.11 and 0.87 ng mg<sup>-1</sup> for all narcotic drugs and psychotropic substances. LOQ values were found, close to the cut-off levels of the Society of Hair



**Fig. 2.** Chromatograms of internal standards. (Abbreviations: THC: Tetrahydrocannabinol; AMPH: Amphetamine; MDA: 3,4-methylenedioxyamphetamine; mAMP: Methamphetamine; MDMA: 3,4-Methylenedioxy-N-methylamphetamine).

**Table 2.** Sensitivities, Recoveries, and Linearities of the 12 Narcotic Drugs and Psychotropic Substances

Analyte	LOD (ng mg <sup>-1</sup> )	LOQ (ng mg <sup>-1</sup> )	Linearity 0.50-8,00 (ng mg <sup>-1</sup> ), r <sup>2</sup>	Recovery		
				1.00 (ng mg <sup>-1</sup> )	4.00 (ng mg <sup>-1</sup> )	8.00 (ng mg <sup>-1</sup> )
BEG	0.24	0.81	0.9999	98.00	98.38	96.66
MOR	0.15	0.50	0.9997	99.18	101.87	100.59
CODEIN	0.14	0.47	0.9999	99.32	95.85	96.13
6-MAM	0.11	0.36	0.9999	96.50	95.38	98.72
HEROIN	0.15	0.50	0.9929	101.52	94.78	97.96
THC	0.19	0.63	0.9997	101.34	101.14	99.67
AMPH	0.15	0.50	0.9999	99.22	100.52	99.07
MDA	0.16	0.53	0.9998	101.90	98.00	98.39
BUP	0.14	0.46	0.9995	101.72	96.02	95.83
mAMP	0.41	1.35	0.9997	104.78	101.26	102.18
MDMA	0.35	1.18	0.9993	104.92	102.24	103.33
COC	0.87	2.91	0.9997	95.48	93.72	97.14

(Abbreviations: LOD: Limit of Detection; LOQ: Limit of Quantification; r<sup>2</sup>: Regression Coefficient; BEG: Benzoylcegonine; MOR: Morphine; 6-MAM: 6-Monoacetylmorphine; THC: Tetrahydrocannabinol; AMPH: Amphetamine; MDA: 3,4-methylenedioxyamphetamine; BUP: Buprenorphine; mAMP: Methamphetamine; MDMA: 3,4-Methylenedioxy-N-methylamphetamine; COC: Cocaine).

Testing Guidelines, in the range of 0.36-2.91 ng mg<sup>-1</sup> [22, 23]. LOD and LOQ values are shown in Table 2.

### Recovery

For recovery, low, medium, and high concentrations (1.00 ng mg<sup>-1</sup>, 4.00 ng mg<sup>-1</sup>, 8.00 ng mg<sup>-1</sup>) of standard substances and internal standard were added to clean hair samples. The overall mean recovery values of the 12 analytes from human hair were found at 93.72-104.92% (Table 2).

### Precision and Accuracy

For precision and accuracy, intra-day and inter-day reproducibility measurements were examined triple on the same day and were repeated on three days. Low, medium and high concentrations (1 ng mg<sup>-1</sup>, 4 ng mg<sup>-1</sup>, 8 ng mg<sup>-1</sup>) of standard substances were studied for precision and

accuracy. The accuracy of the method was determined and calculated as intra-day and inter-day bias. The low % bias indicated that the method was correct within the ≤15% acceptance limit. At medium concentration, intra-day and inter-day bias values were found to be the lowest and highest at 1.99/7.08%, and 1.75/-11.25%. Intra-day and inter-day precision values are given as relative standard deviation (RSD) values which were also found below 15%. At medium concentration, intra-day and inter-day RSD values were found to be the lowest and highest at 2.91/3.70% and 5.18/-10.70%. This method showed acceptable accuracy and precision. The results are shown in Table 3.

As in the rest of the world, the use of narcotic drugs and stimulants continues to be an individual and social problem in our country. Analysis of these substances from hair samples has gained importance in recent years as it gives

**Table 3.** Intra-day and Inter-day Bias (%) and RSD (%)

Analyte	1.00 (ng mg <sup>-1</sup> )				4.00 (ng mg <sup>-1</sup> )				8.00 (ng mg <sup>-1</sup> )			
	Bias		RSD		Bias		RSD		Bias		RSD	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
BEG	6.11	6.11	2.76	7.54	2.67	4.00	3.51	9.13	3.18	1.88	6.86	8.93
MOR	-3.78	-3.78	6.98	12.47	3.39	-6.75	3.05	10.46	2.75	-0.35	6.63	5.18
COD	3.18	3.18	7.95	11.63	4.46	7.25	3.65	8.16	4.77	7.14	7.29	6.53
6-MAM	2.77	2.77	5.25	11.68	6.57	-11.25	2.91	10.70	4.65	-4.88	6.29	8.99
HEROIN	1.45	1.45	6.98	12.81	2.93	-2.50	3.27	10.26	4.99	2.59	6.90	12.42
THC	3.93	-0.03	12.93	9.00	7.08	2.75	3.44	9.25	4.88	4.71	7.05	5.68
AMPH	-2.65	-2.65	5.21	3.08	2.70	-3.50	3.19	5.18	1.87	-0.43	6.64	0.84
MDA	-8.82	-8.82	12.20	6.58	3.50	-2.75	3.24	3.60	2.70	-0.64	6.64	1.29
BUP	-2.77	2.77	6.47	6.17	3.70	8.50	3.70	7.83	3.17	2.09	6.89	5.49
mAMP	-4.28	-4.28	3.67	5.22	2.78	-1.75	3.26	3.82	2.38	0.99	6.72	1.88
MDMA	-2.37	-2.37	5.12	4.10	1.99	-2.75	3.21	5.40	1.68	1.76	6.76	1.71
COC	-12.81	-12.81	3.23	5.73	2.61	-8.00	3.05	9.51	3.12	1.27	6.81	8.26

(Abbreviations: RSD: Relative Standard Deviation; BEG: Benzoylcegonine; MOR: Morphine; 6-MAM: 6-Monoacetylmorphine; THC: Tetrahydrocannabinol; AMPH: Amphetamine; MDA: 3,4-methylenedioxy-amphetamine; BUP: Buprenorphine; mAMP: Methamphetamine; MDMA: 3,4-Methylenedioxy-N-methyl-amphetamine; COC: Cocaine).

information about the range of use of these substances, but studies on hair samples are limited. For this reason, it is aimed to establish and validate a narcotic drug and psychotropic substance analysis method in hair with LC-MS/MS device in our laboratory under international standards.

Difficulties in studies of hair samples have often resulted from inefficient extraction of substances from the hair, unacceptable detection limits, or limited method validation [23]. However, it has been found that narcotic drugs and psychotropic substances are distributed over the cortex and medulla in the hair, and therefore, the physical destruction of the hair structure will facilitate the removal of these substances from the hair [26]. In the light of this information, hair samples were subjected to a short preliminary preparation process, including grinding. In our method, most laboratory materials on which hair samples are prepared were disposable, which reduces the occurrence of external contamination.

The established method is very fast for drug-stimulant analysis and analysis of these 12 substances from the LC-MS/MS device takes less than 3.9 min, except for the preliminary preparation of the hair. Moreover, the established method is very useful for quantitative analysis, as the method requires a small amount of hair (50 mg) and up to a dozen samples can be prepared at the same time. The validation studies of the new method established for hair stimulant drugs were also carried out and the validation results were found in accordance with international standards. The LOD values found for each narcotic drug and psychotropic substance in the hair are less than 0.87 ng ml<sup>-1</sup> and this value is below the concentration value accepted for the analysis of these substances [24,27]. The linearity for each analyte is very high (>0.9929) and high recovery was achieved by finding the recycling values in the range of 96.66 and 104.92 at low, medium, and high concentrations. Accuracy is much lower than the acceptable limit of 15%, both intraday and interday, and even less than 5% for many analytes.

The establishment of this validated hair analysis method, which uses advanced techniques per international standards that will be useful in the diagnosis, treatment, and follow-up of both clinical and forensic cases, will contribute to the literature. This method, which we developed and validated,

not only enabled the accurate determination of these analytes with improved sensitivity without lengthy sample prep processes but also enabled us to accurately distinguish narcotic drugs and psychotropic substances from each other.

## CONCLUSIONS

In this study, a reliable, accurate, simple, and sensitive method was developed for the quantitation of 12 narcotic drugs and psychotropic substances in hair using LC-MS/MS. To our knowledge, this is the first scientific validation study in our country in which 12 substances in hair were analyzed simultaneously using LC-MS/MS. It is very important to find the concentration in the hair to determine human exposure to narcotic drugs and psychotropic substances long ago. In addition, the number of studies that can analyze these substances with reliable results at such a low concentration is limited. Furthermore, our results are important for the advancement of LC-MS/MS methods and the use of the validated method is the basis for monitoring exposure to illegal drugs in hair.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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