

Method Development for the Detection of Basic/Weak Basic Drugs in Hair by LCMSMS: Comparison between Methanolic and Alkaline Extraction on Real Samples

Jaber Al Jaber^{1,2}, David Holt³, Atholl Johnston^{1,3}

¹The William Harvey Research Institute, Barts & The London School of Medicine & Dentistry, Queen Mary University of London, London, UK; ²Department of Pharmacology and Toxicology, Faculty of Medicine, Kuwait University, Jabriya, Kuwait; ³Analytical Services International Ltd., St. George's University of London, London, UK.

Email: j.aljaber@qmul.ac.uk

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ABSTRACT

Detection of drugs in hair has become popular in recent years. The significantly long drug detection window (months) in hair has allowed the retrospective investigation and measurement of past consumption of drug. As the majority of drugs are basic, an extraction method was developed based on a methanolic solution for detection of basic/weak basic drugs in hair. It was compared with alkaline digestion (NaOH) followed by LLE. A filtration step with filtration vials was added and their materials were compared. After filtration, extracts were injected directly onto a C18 column coupled to Sciex ABI 2000 MSMS. The mobile phase was 50% methanol, 0.1% formic acid and 2 mM ammonium acetate (isocratic). Both methods were compared by applying them to real samples. Results showed that calibration was linear with r^2 of 0.991 - 0.999 for 20 tested analytes. The matrix effect was assessed to be between 91.4% - 110.2% for 18 analytes. PTFE filter material showed better recoveries over the GMF and PVDF based filters. Stability of analytes during extraction in general was better with methanolic incubation than alkaline digestion. With regard to real sample recovery, 6 out of 10 analytes recovered better with alkaline digestion. In conclusion, the methanolic method is capable of extracting most basic drugs in hair samples but only part of the total incorporated drug. Therefore, these results suggest that a combination of both methods (methanolic and alkaline extractions) in hair sample processing for general detection of basic and weak basic drugs may produce better results. However, not all basic drugs are stable under alkaline digestion.

Keywords: Hair Analysis; Basic Drugs; LCMSMS; Benzodiazepines; Method Development; LLE

1. Introduction

Drug analysis in hair has grabbed the attention of toxicology analysts and researchers in recent years. This is mainly because it has provided some ability of proving drug ingestion when conventional samples could not. Hair differs from other traditional biological samples used for human toxicological analysis such as urine, blood, liver or saliva with its significantly longer detection window (months) allowing retrospective investigation and measurement of drug consumption. Hair analysis is becoming accepted in many developed countries for substance consumption related issues in a wide range of sectors; the medico-legal sector, workplace testing, treatment monitoring, schools, forensics, research, insurance companies, environmental biomonitoring and driving licensing [1-4].

Extraction of drugs from hair is considered one of the

most important steps in hair analysis. Apart from external drug deposition on hair, drugs are mainly enclosed tightly in the hair shaft and to a certain extent maybe bound to proteins, melanin or lipids of the cell membrane complex. Therefore, hair matrix type, structure of the drug, method and duration of extraction, and solvent used are all important factors affecting the final extraction yield [5].

There are numerous reports of screening strategies published for analysing different forensic basic drugs groups. Hypnotic drugs such as benzodiazepines are at the top of the list of drug facilitated crimes. These are weak basic drugs and have been reported to be extracted from hair by different methods, with phosphate buffers, methanol or digestive enzymes [6-8]. Other basic drug groups like antipsychotics, antidepressants and amfetamines have frequently been reported to be extracted with alkaline digestion (NaOH) [9-12]. However, recently

there has been some focus on using methanolic solutions as an extraction medium consisting of varying ratios of methanol, acetonitrile and formate buffer for extraction of several groups of basic drugs simultaneously [13-15]. However, the extent of their efficiency was not examined most of the time. Therefore, in the present study the aim was to develop a method to enable the detection and quantification of basic and weak basic drugs in hair simultaneously based on the methanolic solution extraction technique and to compare its efficiency with the alkaline digestion technique which was followed by liquid-liquid extraction (LLE).

2. Materials

2.1. Chemicals

All chemicals were of HPLC or analytical grade. Methanol, acetonitrile, acetone, propanol, methyl-tert-butyl-ether (MTBE), citalopram, clobazam, clonazepam, cocaine, codeine, desmethyldiazepam, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), 3,4-Methylenedioxy-methamphetamine (MDMA), midazolam, sulpiride, zaleplon and zolpidem were purchased from Sigma-Aldrich Co, Dorset UK. 7-aminoclonazepam, amphetamine, amphetamine-D11, benzoylecgonine-D8, benzoylecgonine, codeine-D3, diazepam, diazepam-D5, ecognine methyl ester, medazepam, oxazepam and temazepam were purchased from LGC standards, Middlesex UK. Other prescription drug standards were supplied from their manufacturer. Deionised water was prepared on site (ELGA Limited). Dichloromethane, 7.5 M ammonium acetate solution and all consumables (tubes, etc) are from VWR International Ltd, Lutterworth UK. The rest of the chemicals used in the solutions below were from Sigma-Aldrich Co, Dorset UK.

2.2. Solutions

2.2.1. Standard Solutions

1000 mg/L or 100 mg/L working standards made with MS grade methanol of each analyte and internal standard. All stored at -20°C and diluted to the required concentration prior to the experiment.

2.2.2. 0.1 M Phosphate Solution

One litre of solution was prepared by dissolving 1.7 g of anhydrous disodium orthophosphate and 12.14 g of sodium dihydrogen orthophosphate monohydrate in 800 mL of de-ionized water. The pH was adjusted to 6 with 1 M potassium hydroxide and the solution made up to volume with de-ionized water.

2.2.3. 0.1% Formic Acid (v/v)

100 μL of concentrated formic acid (98/100) was mixed

with 99.9 mL of de-ionized water, mixed well and stored at room temperature.

2.2.4. Methanolic Extraction Solution

60 mL of methanol, 10 mL of acetonitrile and 30 mL of 0.1% formic acid were measured into measuring cylinder, mixed well and stored at room temperature.

2.2.5. 1 M NaOH Solution

40 g of NaOH (MW 40.0) pellets were measured into a 1000 mL volumetric flask and made up to the 1000 mL mark with de-ionized water. This was mixed thoroughly and stored at room temperature.

2.2.6. 2 M Ammonium Acetate

266.6 mL of 7.5 M ammonium acetate solution were measured into a 1000 mL volumetric flask and made up to the 1000 mL mark with de-ionized water.

3. Methods and Results

3.1. Hair Preparation

The decontamination process was by using three washing steps with two solvents and one aqueous solution: 0.01 M phosphate solution (pH 6), dichloromethane and propanol/acetone (1:1). The uncut hair was put in a 6 mL glass tube, then 2 mL of the first solution added and vortex mixed for 1 minute. After that the solvent was decanted and then the next solvent was added. After the last washing step (2 mL propanol/acetone) the hair was left to dry at room temperature or the process was speeded up with the speed-vac. This decontamination method was found to be quick and very efficient in cleaning post-mortem hair samples.

After the hair had dried segments of ~ 0.6 cm each were cut into small pieces (1 - 3 mm) with scissors and ideally 25 mg were weighed directly. If this was not possible any amount between 10 and 80 mg was considered and placed into a 2 mL glass tube with a screw cap.

3.2. Methanolic Extraction

Five hundred micro-litres of the methanolic extraction solution (described earlier) was added to the hair in each tube and left in a sonicator for 16 hours at 40°C . In the case of calibrators, the required concentrations were made based on adding 25 μL of drugs standard (e.g. 1 mg/L in methanol) to the calibrators' tubes containing 25 mg blank hair each and those 25 μL were deducted from the extraction solution (475 μL). Also 25 μL of internal standards (bromperidol and the deuterated standards amphetamine-D11, benzoylecgonine-D8, codeine-D3 and diazepam-D5) were added as well at a concentration similar to the middle calibrator. After centrifugation for 10 min-

utes the extracted solutions were transferred to auto-sampler vials and evaporated to dryness followed by re-constitution with 125 μL of 25% methanol and filtered with the syringeless mini-uniprep filters (Whatman GE). Finally 20 μL of this filtrate was injected into the LCMSMS system.

3.3. Alkaline Digestion

Twenty five milligrams of washed blank/patient hair was incubated with 0.5 mL of 1 M NaOH for 3 hours in a water bath (50°C) to allow for digestion of the hair. Samples were then extracted after digestion with 2 mL MTBE by 30 minutes rotary mixing and 10 minutes centrifugation. Solvent layers were transferred into clean tubes containing 150 μL 1% formic acid, then mixed on a rotary mixer for 20 minutes and removed after 10 minutes of centrifugation by aspiration. From the remaining aqueous layer 20 μL was injected into the LCMSMS system.

3.4. Method Development with Some Validation for Selected Analytes

3.4.1. Instrumentation and Ion Identification

The LCMSMS analysis was carried out with an HPLC system consisting of a Perkin Elmer PE200 series auto-sampler, pump and column oven. The MSMS part was a SCIEX API 2000 Triple Quadrupole MS-MS instrument (Applied Biosystems) equipped with an electrospray interface (Turbo Ion Spray) set on the positive mode. Ion spray voltage was set to 5500 V. Nitrogen was used as the nebulizer gas-GS1 (30 psi), auxiliary gas-GS2 (20 psi heated to 300°C), curtain gas (20 psi) and as the CAD gas (set on 3).

Analytes were infused at 0.25 mL/minute to the LCMSMS system at a concentration of 1 mg/L in 50% methanol/2mM ammonium acetate solution. The precursor and product ion identification and parameter tuning were completed by automatic and manual tuning. Optimum tuning parameters, precursor and product quantitation ions are shown in **Table 1**. Mobile phase was 50% methanol, 0.1% formic acid and 2 mM ammonium acetate (isocratic). Separation with Altech Alltima C18 column (15 cm \times 2.1 mm, 5 μm) maintained at 50°C.

3.4.2. Comparison of Mini-Uniprep Filter Media

Three filter materials were compared by using spiked blank hair samples at concentrations of 0.5 ng/mg hair. 25 mg of blank hair were weighted out in glass tubes. Then the whole extraction/incubation method was applied to all the samples. The three materials were 0.45 μm pore size Glass Microfiber (GMF), 0.2 μm pore size Polytetrafluoroethylene (PTFE) and 0.2 μm pore size Polyvinylidene fluoride (PVDF) used in manufacturing

Whatman Mini-UniPrep Syringeless Filters. The filters efficiency was calculated by comparing the percentage of extraction recovery through their peak area ratio of product ion for each drug after filtration with unfiltered calibrators.

Table 2 shows clearly that the PTFE filter medium has the greatest recovery (mean = 100%) of all the analytes while the GMF and PVDF filters (mean = 89% and 72%, respectively) reduced significantly the response of amisulpride and citalopram. Also the responses of medazepam, midazolam and zolpidem were considerably reduced by the PVDF filter.

3.4.3. Linearity

The linearity study reflects the relationship between change of spiked analyte concentration and detector response to that change [16]. It was attained by spiking hair samples with 50, 75, 100, 500, 1000 and 2000 pg/mg of each analyte. The linear correlation coefficients (r^2) values were better than 0.99 for all analytes.

3.4.4. LOD and LOQ

Limit of detection and limit of quantification were assessed by spiking blank hair with the analytes in decreasing concentrations. The LOD and LOQ were calculated at a signal to noise ratio of 3 and 10, respectively. LOD values for all analytes were 0.5 - 23 pg/mg and LOQ values were 1.7 - 76.7 pg/mg (**Table 3**).

3.4.5. Selectivity

Selectivity in drug analysis is the ability of an analytical method to differentiate and quantify the analyte of interest in the presence of other components in the sample [17]. In order to achieve that a low standard (0.1 ng/mg) of each drug from the group was injected individually into the LCMSMS system and all the other drug detection windows and retention times were observed to see if there were any interfering peaks or false positive results that might appear in the same retention time and same window as the injected analyte. No interference was found on the same detection window and retention time for any of the analytes. Also drug-free hair samples showed no false positive results.

3.4.6. Matrix Effect

The matrix effect (ME) is basically the ion suppression or enhancement of the analyte peak caused by co-eluting compounds from the biological matrix (urine, blood, hair, etc). The aim of this experiment was to acquire more accurate data on the extent of matrix influence on the results when matrices come from different types of hair. As proposed by Matuszewski *et al.* the ME was assessed by comparing the mean peak area of product ion of ex-

Table 1. Optimized MRM and MS/MS parameters for analytes and internal standards.

Analyte	Q1 (m/z)	Q3 (m/z)	Internal Standard	Retention Time (min)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
7-Aminoclonazepam	285.92	120.94	Diazepam_D5	2.9	51	370	10.5	41	2
Alprazolam	308.98	280.84	Diazepam_D5	11.2	71	240	10.5	35	4
Amfetamine	135.99	91.15	Amfetamine_D11	2.7	11	340	5.5	21	8.9
Amfetamine_D11	147.29	98.20	-	2.6	36	360	4	25	12
Amisulpride	370.05	242.07	Diazepam_D5	2.2	51	370	10.5	37	10
Amlodipine	408.90	237.90	Bromperidol	8.5	35	330	3.6	35	10
Aripiprazole	448.23	176.20	Bromperidol	13.6	76	370	6.5	43	2
Benzoylcegonine	289.92	168.04	Benzoylcegonine_D8	3.4	26	350	10	27	6.5
Benzoylcegonine_D8	298.15	171.10	-	3.3	20	200	10	30	6.6
Bromperidol	420.17	123.20	-	6.3	66	370	8.5	59	0
Carbamazepine	237.02	194.06	Diazepam_D5	7.3	56	290	10.5	29	4
Chlordiazepoxide	299.98	227.03	Diazepam_D5	6.8	56	170	10.5	35	4
Citalopram	325.05	109.14	Diazepam_D5	4.3	61	170	10.5	37	2
Clobazam	300.97	259.18	Diazepam_D5	9.1	56	370	10.5	31	4
Clonazepam	315.91	269.98	Diazepam_D5	7.5	66	250	10.5	37	4
Cocaine	303.92	182.04	Benzoylcegonine_D8	3.2	26	350	10	27	6.8
Codeine	300.09	165.00	Codeine_D3	2.2	26	350	10.5	63	12
Codeine_D3	303.00	165.00	-	2.2	31	370	10.5	63	16
Dehydroaripiprazole	446.02	284.90	Bromperidol	11.9	76	350	10	33	8
Desmethyldiazepam	270.97	140.02	Diazepam_D5	14.1	66	350	8.5	41	6
Diazepam	284.98	193.05	Diazepam_D5	18.1	46	350	8.5	45	8
Diazepam_D5	290.02	198.04	-	18.1	46	330	8.5	43	4
Ecgonine M.Ester	200.07	182.10	Benzoylcegonine_D8	2.0	51	340	5.5	25	28
Lorazepam	320.86	274.89	Diazepam_D5	13.3	46	360	10.5	33	6
Lormetazepam	334.89	288.87	Diazepam_D5	13.6	46	280	10.5	31	6
MDA	180.10	105.10	Amfetamine_D11	2.6	26	340	4.5	31	14
MDEA	208.11	163.10	Amfetamine_D11	2.7	36	350	6	19	24
MDMA	194.22	163.10	Amfetamine_D11	2.5	36	360	6	17	8
Medazepam	270.99	91.00	Diazepam_D5	5.5	56	350	10.5	49	2
Midazolam	325.95	290.89	Diazepam_D5	5.6	81	270	10.5	39	6
Mirtazapine	265.92	195.11	Bromperidol	3.4	36	350	8.5	35	8
Nitrazepam	282.01	235.89	Diazepam_D5	7.4	66	370	10.5	33	4
OH-Risperidone	427.11	207.09	Bromperidol	3.6	46	360	7.5	39	8
Oxazepam	286.96	240.89	Diazepam_D5	10.2	96	350	8.5	35	8
Phenazepam	350.79	206.04	Diazepam_D5	15.3	61	350	8	49	24
Quetiapine	384.22	253.24	Bromperidol	7.2	76	350	10.5	31	12.8
Risperidone	411.18	191.05	Bromperidol	4.5	51	120	10.5	39	11.3
Sulpiride	342.02	213.90	Bromperidol	2.1	41	370	10	51	24
Temazepam	300.99	255.03	Diazepam_D5	12.4	41	270	8.5	33	10
Zaleplon	305.92	235.96	Diazepam_D5	5.5	56	350	8.5	39	8.1
Zolpidem	308.06	234.93	Diazepam_D5	3.2	66	270	10.5	49	4

Q1 = precursor ion, Q3 = product ion, DP = declustering potential, FP = focusing potential, EP = entrance potential, CE = collision energy, CXP = collision cell exit potential.

Table 2. Mini-UniPrep filter media: comparison of results for % recovery of 0.5 ng/mg spiked analytes.

Analyte	GMF	PTFE	PVDF
7-Aminoclonazepam	114	94	93
Alprazolam	100	98	101
Amisulpride	7	92	21
Carbamazepine	108	98	94
Chlordiazepoxide	116	99	84
Citalopram	7	113	<1
Clobazam	106	100	94
Clonazepam	103	104	93
Desmethyldiazepam	99	100	101
Diazepam	103	99	98
Lorazepam	104	97	99
Lormetazepam	109	96	94
Medazepam	86	100	2
Midazolam	59	104	25
Nitrazepam	109	97	93
Oxazepam	105	102	92
Phenazepam	105	103	92
Temazepam	100	101	99
Zaleplon	106	96	98
Zolpidem	100	96	18
Mean	92.3	99.5	78.5

GMF = Glass Microfiber, PTFE = Polytetrafluoroethylene, PVDF = Polyvinylidene fluoride.

Table 3. Linearity, LOD, LOQ and Absolute Recovery (RE) results for 0.5, 1 and 2 ng/mg of analytes (n = 3).

Analyte	Linearity (r ²)	LOD pg/mg	LOQ pg/mg	Absolute Recovery (RE)						
				0.5 ng/mg	%CV	1 ng/mg	%CV	2 ng/mg	%CV	%CV for 3 Conc.
7-Aminoclonazepam	0.991	2.5	8.3	67.4	7.3	62.6	5.6	64.2	1.9	3.8%
Alprazolam	0.997	1.5	5	98.3	0.8	93.7	1.3	93.1	0.7	3.0%
Amisulpride	0.997	1	3.3	28.4	3.4	29.0	6.0	29.8	2.6	2.4%
Carbamazepine	0.996	1.3	4.3	98.0	2.7	93.0	1.6	94.0	1.8	2.8%
Chlordiazepoxide	0.999	2.6	8.7	78.9	4.1	75.6	6.8	72.5	4.0	4.2%
Citalopram	0.991	4.8	16	12.9	11.3	16.5	34.7	13.9	2.5	12.9%
Clobazam	0.999	1.2	4	103.8	1.6	98.7	2.5	96.8	2.1	3.6%
Clonazepam	0.999	2.2	7.3	94.6	5.0	89.3	5.5	90.7	0.9	3.0%
Desmethyldiazepam	0.999	12	40	98.4	0.2	92.8	4.8	91.3	3.9	4.0%
Diazepam	0.999	2.6	8.7	87.6	3.8	87.6	9.9	82.4	3.6	3.5%
Lorazepam	0.996	2.7	9	79.9	4.2	72.4	6.3	75.3	6.0	5.0%
Lormetazepam	0.995	3.4	11.3	101.0	7.4	94.8	4.8	94.3	4.7	3.9%
Medazepam	0.996	7	23.3	104.4	3.9	103.1	6.3	94.8	2.0	5.2%
Midazolam	0.998	1	3.3	60.4	13.4	57.8	6.6	60.1	6.4	2.4%
Nitrazepam	0.998	6.5	21.7	102.0	2.0	98.1	3.2	100.3	4.3	2.0%
Oxazepam	0.996	23	76.7	118.8	5.0	100.1	2.3	100.1	4.2	10.2%
Phenazepam	0.998	6.5	21.7	90.2	5.9	79.0	6.2	81.1	6.6	7.1%
Temazepam	0.999	2.6	8.7	95.8	0.4	93.1	2.1	91.8	2.8	2.2%
Zaleplon	0.998	1.3	4.3	80.5	1.4	77.7	2.8	78.7	1.8	1.8%
Zolpidem	0.999	0.5	1.7	73.5	4.0	71.2	2.2	68.3	3.1	3.7%

tracted drug-free hair samples from 5 different sources (black, baby-brown, black dyed, white and blonde) spiked with 0.2 ng/mg low standard solution following filtration (*i.e.* after extraction/incubation) with the mean peak area of product ion of neat unextracted standards prepared in the mobile phase [18]. The percentage of the ME was calculated by dividing the mean of the extracted samples by the mean of unextracted ones according to Equation (1).

$$\text{ME}(\%) = \frac{\text{spiked after incubation}}{\text{unextracted standard}} \times 100 \quad (1)$$

This percentage represents the absolute ME (both ion suppression and ion enhancement).

The ME results shown in **Table 4** indicate that there was no significant ion suppression or enhancement affecting the analytes, except with amisulpride and the metabolite of clonazepam the 7-amino. Some of the analytes showed slight ion enhancement such as diazepam and clobazepam, but the rest of the drugs were within 91.4 - 110.2% ME. Also the %CV was in the range of 3.2% - 11.1% showing a good agreement level between the different hair samples.

3.4.7. Absolute Recovery with Methanolic Incubation

Recoveries were estimated by determining peak areas of analytes obtained when the drugs spiked with blank hair (in extraction solution) at three concentrations 0.5, 1, and 2 ng/mg ($n = 3$) where compared with unextracted/unfiltered standards. Therefore, this recovery takes into account all the extraction steps including filtration, incubation and matrix effect (absolute). The recovery was calculated as a percentage by dividing the average peak area ratio of the extracted standards over the average peak area ratio of the unextracted standards and multiplied by 100.

The results in **Table 3** show that all the analytes had good recoveries (>58%) at all three concentrations with %CV below 13.4%, except for 2 analytes which gave low recoveries (12.9% - 29.8%).

3.4.8. Stability during Methanolic Extraction

All analytes were prepared at 0.5 mg/L (\approx ng/mg) of the extraction solution (mentioned in solutions section) and incubated/sonicated with slightly harsher conditions at 50°C for 18 h without the blank hair. Then the mean peak area ratio of product ion/internal standard was compared

Table 4. Matrix effect (ME) results for 0.2 ng/mg hair samples spiked with the analytes ($n = 5$).

Analyte	Black	Brown	Dyed	White	Blond	%ME	%CV
7-Aminoclonazepam	91	92	65	62	75	77	18
Alprazolam	103	104	93	92	91	97	7
Amisulpride	46	52	58	28	62	49	27
Carbamazepine	120	106	96	98	98	103	10
Chlordiazepoxide	109	104	93	95	94	99	7
Citalopram	97	120	99	99	109	105	9
Clobazam	121	121	108	112	110	114	5
Clonazepam	112	99	95	93	95	99	8
Desmethyldiazepam	118	111	107	100	106	109	6
Diazepam	117	120	113	113	116	116	3
Lorazepam	118	112	100	102	102	107	7
Lormetazepam	113	114	106	110	108	110	3
Medazepam	98	116	96	102	94	101	8
Midazolam	100	109	95	93	96	99	6
Nitrazepam	112	105	95	96	100	101	7
Oxazepam	113	111	97	97	100	104	7
Phenazepam	117	114	92	97	98	104	11
Temazepam	119	115	109	109	109	112	4
Zaleplon	99	99	87	86	87	91	8
Zolpidem	95	98	91	92	94	94	3
Mean	106	106	95	94	97	99	6

with the mean peak area ratio of product ion/internal standard of unextracted/unincubated standards prepared in 25% MeOH at the same concentration (n = 3).

3.4.9. Stability/Recovery with Alkaline Digestion

Extraction of hair with various solvents following alkaline digestion has been widely mentioned in many publications [11,19-21]. In order to investigate whether the proposed method has any advantages over the alkaline digestion method, the first step was to check how stable the analytes would be during the incubation/extraction with NaOH/MTBE. Twenty five milligrams of blank/washed hair was incubated for 3 hours at 50°C with the analytes at a concentration of 1 ng/mg and mixed with 0.5 mL 1 M NaOH. Extraction was with 2 mL of MTBE on an orbital shaker for 30 minutes followed with back extraction with 150 µL of 1% formic acid. Twenty microlitres were injected into the LCMSMS system.

The stability results shown in **Table 5** indicate that almost all the analytes are stable under the extraction/incubation conditions of the methanolic method. The least stable analyte was oxazepam with 56.1% recovery. The average recovery was 89.6% with coefficient of variation of 4.2%. While stability of analytes under the alkaline condition was remarkably lower with an average of 51.7%. The results are also demonstrated in **Figure 1**.

3.5. Real Samples (Methanolic vs. Alkaline Extraction)

The methanolic method was applied to 10 cases, one of them postmortem. Hair samples were taken from the posterior vertex region of the scalp. Nine of these cases were patients taking doses of different classes of basic drugs. Seven of the nine were addicts under rehabilitation.

About 0.6 cm segment from the root end (proximal) of each sample was decontaminated and extracted by the methanolic method. However, there was an additional step added to the analysis of those cases. After full incubation and extraction steps, hair sample remained in the tubes were not discarded. Instead they were re-extracted with the alkaline digestion method described earlier. Results are shown in **Table 6** and **Figure 2** and discussed in the following section. Some of the real samples chromatograms are shown in **Figure 3**.

4. Discussion and Conclusion

The development of the proposed basic drugs extraction method was based on previous studies showing that methanolic and aqueous solution is capable of extracting drugs from hair samples [2]. However, a comparison between this extraction solution and the digestion with

Table 5. Stability (%) of the analytes after sonication/incubation at 50°C/18h (n = 3) and after extraction and digestion with 1 M NaOH at 50°C/3h (n = 2).

Analyte	Methanolic Extraction	%CV	NaOH Digestion	%CV
7-Aminoclonazepam	98.3	3	19.7	35.9
Alprazolam	97.5	2	67.2	7.7
Amisulpride	93.6	4	25.0	40.5
Carbamazepine	101.8	3	66.3	2.1
Chlordiazepoxide	70.3	9	83.6	19.4
Citalopram	101.3	7	28.3	37.0
Clobazam	104.5	4	61.3	7.0
Clonazepam	92.3	0	50.4	7.3
Desmethyldiazepam	76.3	4	76.4	28.1
Diazepam	98.5	1	51.1	15.9
Lorazepam	64.2	4	52.6	19.1
Lormetazepam	98.2	1	60.7	13.0
Medazepam	100.6	6	30.5	5.3
Midazolam	100.5	4	32.6	11.5
Nitrazepam	73.3	6	49.2	14.2
Oxazepam	56.1	8	54.4	29.8
Phenazepam	86.1	5	75.6	40.9
Temazepam	81.1	1	60.4	19.9
Zaleplon	97.7	7	36.7	11.6
Zolpidem	98.9	4	52.0	14.6
Mean	89.6	4.2	51.7	19.04

NaOH was not probably suggested in this specified way, particularly with general basic drugs screening. Therefore, the mixture of methanol, formic acid and acetonitrile (methanolic solution) was proposed to extract basic drugs. Methanol and 0.1% formic acid are protic solvents and capable of swelling the hair and attracting/ionizing basic drugs entrapped inside the hair shaft, at least theoretically. Acetonitrile was added in a small amount (10%) as it was recommended by some articles [15,22].

The first early recovery experiments with the methanolic method produced extracts that were turbid in colour which brought about the necessity of adding a filtration step to the method and checking the effect of these filters on the drugs' recovery. Mini-UniPrep filters were chosen as they are syringeless, simple and fast. As these filters come in different materials, a comparison study was carried out and showed that PTFE based filters gave the highest recoveries for the analytes.

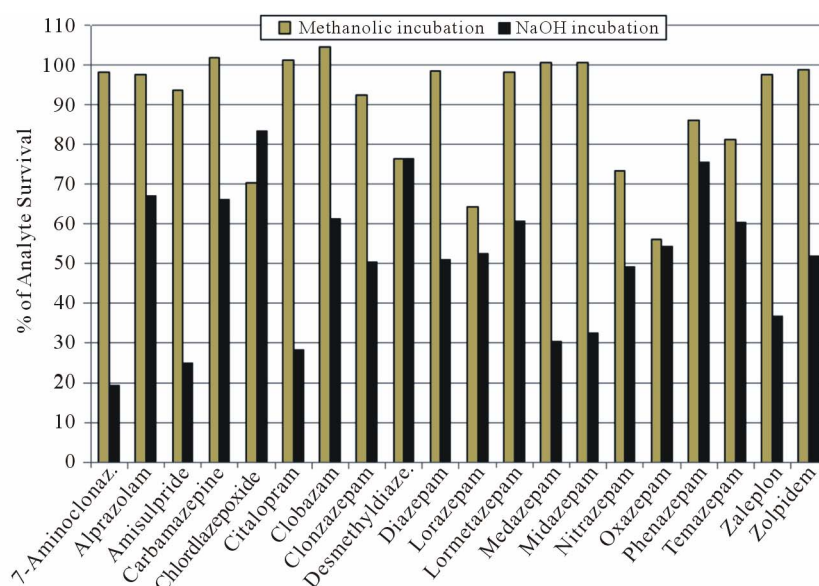


Figure 1. Stability of analytes during methanolic and alkaline conditions.

Table 6. Real samples: comparison of results.

Case	Hair Colour	Hair Segments	Methanolic Incubation	NaOH Digestion
1	Black	20.3 mg (0.6 cm)	0.134 ng/mg Codeine 2.53 ng/mg Quetiapine	0.763 ng/mg Codeine 31.7 ng/mg Quetiapine
2	Black	17.5 mg (0.6 cm)	42.3 ng/mg Amfetamine	0.173 ng/mg Amfetamine
3	Dark Brown	22.6 mg (0.6 cm)	19.1 ng/mg Amfetamine 0.88 ng/mg Sulpride 3.41 ng/mg Quetiapine 13.5 ng/mg Amfetamine	0.123 ng/mg Amfetamine Negative Sulpride 11.46 ng/mg Quetiapine 0.036 ng/mg Amfetamine
4	Black	17.4 mg (0.6 cm)	0.019 ng/mg Mirtazapine 0.039 ng/mg OH-Risperidone 0.059 ng/mg Risperidone	0.010 ng/mg Mirtazapine 0.035 ng/mg OH-Risperidone 0.18 ng/mg Risperidone
5	Black	24.5 mg (0.6 cm)	16 ng/mg Mirtazapine 0.138 ng/mg OH-Risperidone 1.37 ng/mg Risperidone	40 ng/mg Mirtazapine 0.134 ng/mg OH-Risperidone 4.1 ng/mg Risperidone
6	Black	19.9 mg (1.5 cm)	14.8 ng/mg Mirtazapine 1.26 pg/mg Risperidone	43.2 ng/mg Mirtazapine 0.022 ng/mg Risperidone
7	Black	33.6 mg (0.6 cm)	6.58 ng/mg Quetiapine 0.08 ng/mg Mirtazapine 0.016 ng/mg Amfetamine	33 ng/mg Quetiapine 0.3 ng/mg Mirtazapine Negative Amfetamine
8	Black	33.4 mg (0.6 cm)	3.7 ng/mg Amfetamine 0.067 ng/mg Codeine 18.9 ng/mg Mirtazapine	0.015 ng/mg Amfetamine 1.225 ng/mg Codeine 41.4 ng/mg Mirtazapine
9	Grey	80 mg (~1.5 cm)	Negative Aripiprazole Negative DehydroAripiprazole 0.015 ng/mg Amlodipine	Positive Aripiprazole Positive DehydroAripiprazole Negative Amlodipine
10*	Dark Brown	27 mg (1 cm) 1st	4.52 ng/mg Amfetamine 6.46 ng/mg MDMA 0.258 ng/mg MDA 0.143 ng/mg MDEA 2.68 ng/mg Cocaine 28.42 ng/mg Benzoyllecognine	
		35 mg (2 cm) 2nd	0.644 ng/mg Ecognine methyl ester 4.75 ng/mg Amfetamine 6.72 ng/mg MDMA 0.272 ng/mg MDA 0.147 ng/mg MDEA 1.434 ng/mg Cocaine 27.83 ng/mg Benzoyllecognine 0.165 ng/mg Ecognine methyl ester	

*Results of this subject are from combined method (methanolic and alkaline extraction).

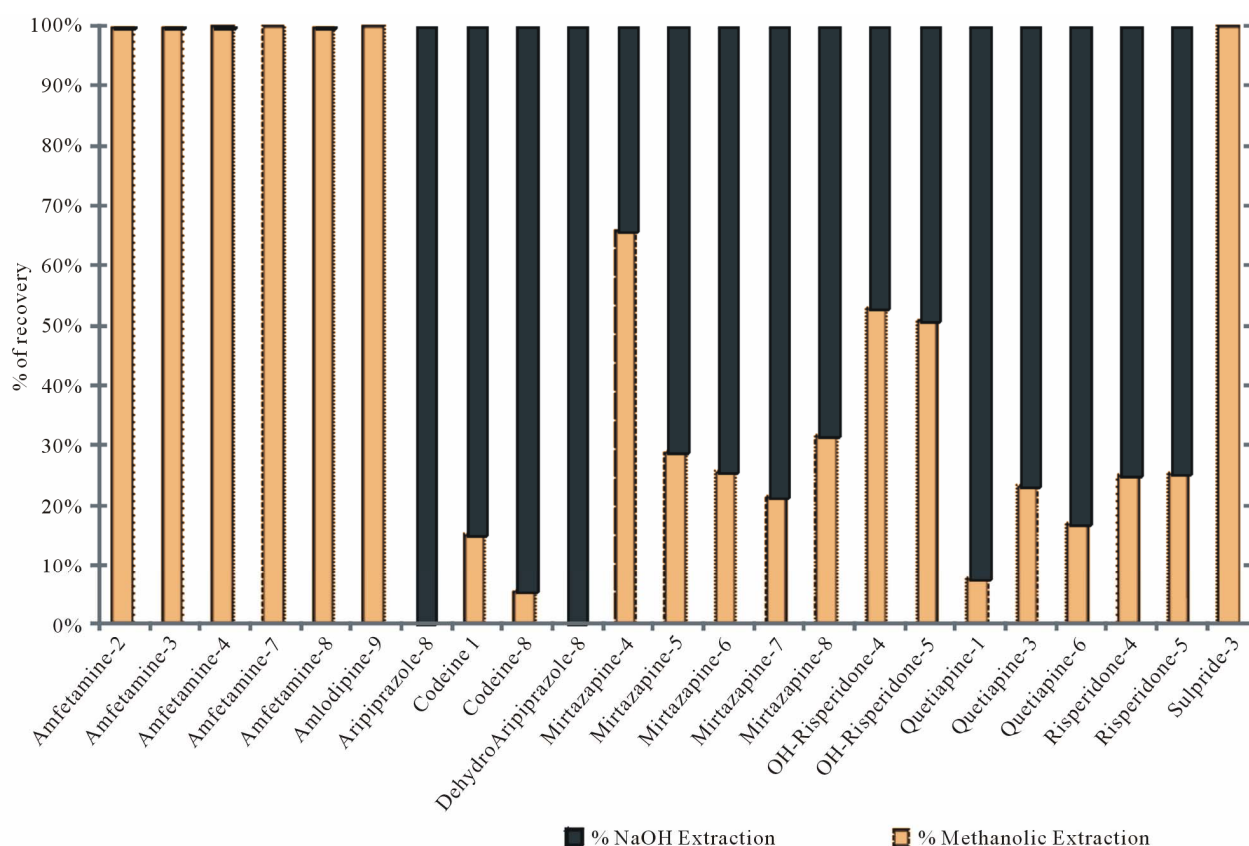


Figure 2. Real samples: comparison of results for methanolic and alkaline extraction (Drug-Case No.).

It was seen from the stability results that digestion with NaOH reduces the stability of most of the analytes whereas methanolic incubation has no such effect on stability as the great majority of drugs showed >90% stability with or without blank hair.

Some method validation steps have been carried out on the methanolic method, such as recovery, LOD/LOQ, matrix effect and selectivity. Absolute recovery experiments showed that all the analytes have a recovery ranging between 58.4% and 119.3% with a CV from 0.2 to 13.4% after excluding the results of two drugs. Amisulpride and citalopram gave low recoveries (12.9% - 29.8%, CV 2.5% - 34.7%). During the matrix effect study five different sources of hair have been incorporated (different colours and ethnicities). Results showed that all the drugs have ME ranging between 91.4 and 115.6% (CV 3.2% - 11.1%) except 7-aminoclonazepam and amisulpride (ME 77% and 49.1%, CV 18.4% and 26.8%, respectively). The selectivity study also showed no major problem affecting the method selectivity as no analyte eluted in the same retention time and window of the other analytes and no false positive from blank hair.

Last but not least, analysis of authentic hair samples from a group of subjects taking basic or weak basic drugs revealed the usefulness of the proposed method at least

for the detection of basic drugs in hair. To further examine the extent of extraction the same samples were digested with 1 M NaOH after the methanolic extraction to compare the extractability of both methods on the administered drugs in a way by using the exact same samples. Results revealed that the methanolic method was able to extract most of the investigated drugs from hair samples, but the downside about it is the fact that it only extracts part of the incorporated drug in hair. In addition, this part or this percentage of the incorporated drug varied between the investigated compounds. Results showed that 99% to 100% of detected amfetamine from authentic hair samples was extracted by the methanolic method as shown in **Figure 3**, while less than 1% was revealed by the alkaline digestion. Amlodipine and sulpiride were only detected by the methanolic extraction method. On the contrary, aripiprazole and its active metabolite (dehydroaripiprazole) were revealed only with the alkaline digestion. Codeine showed a weak response with the methanolic method as only 5% - 15% of the incorporated drug revealed, while the 85% - 95% of it was detected with the alkaline method. Mirtazapine was also recovered better with the alkaline digestion (69% - 79%) except in one sample (35%). Similarly, risperidone was recovered better with the alkaline digestion (75%) while

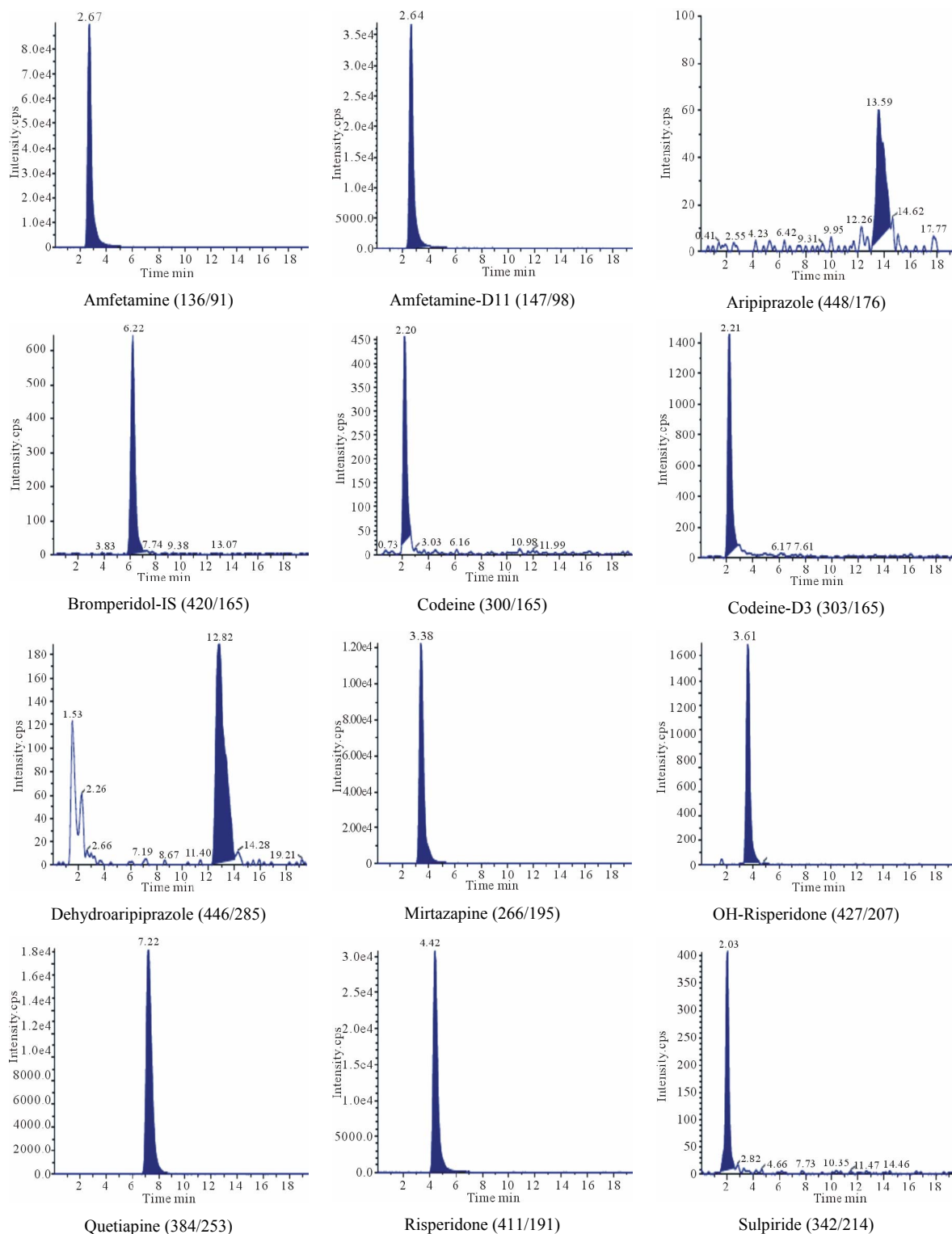


Figure 3. Some of the real sample chromatograms, between brackets are precursor ion/product ion.

its metabolite showed a 50/50 detection in both methods. Quetiapine as well showed like pattern to risperidone and mirtazapine with a 77% - 93% found with the alkaline

digestion method.

These results suggest that the incorporated drugs in hair need either complete digestion of the hair samples or

maybe powdering the samples to ensure complete release of the entrapped compounds. Although some drugs have been extracted or revealed only by methanolic method without digestion, like sulphiride and amlodipine, it does not mean that the percentage of recovery was really 100%. It is likely that these drugs were not stable under NaOH digestion which may have led to their degradation and disappearance. High pH possibly has caused hydrolysis to both amlodipine's ester moiety and sulphiride's amide type link.

In conclusion, the methanolic method is capable of extracting most basic drugs in hair samples but only part of the total incorporated drug. As all the drug in the real samples were detected by the methanolic method except one drug, aripiprazole. Therefore, these results suggest that a combination of both methods (methanolic and alkaline extractions) in hair sample processing for the detection of basic and weak basic drugs seems to provide better extraction yield than either method alone. However, not all basic drugs are suitable for alkaline digestion. A further study with more real samples is needed and preferably in comparison with other hair extraction techniques, such as powdering the hair and other digestion methods.

4.1. Limitations

Ideally, the method should be applied to more samples especially from subjects taking the same drugs as the drugs used in the method development. Unfortunately, only those patients who were studied agreed to participate. Also, the access time on the LCMSMS was very limited and optimization of every variable was not feasible. Moreover, the method is intended to analyse basic drugs in general and basic drugs are very common so it is almost impossible to include all of them. Alkaline digestion has limited application to only analytes stable under basic conditions, while methanolic extraction showed partial extraction ability even after 18 hours of incubation.

4.2. Ethical Notes

Collection of hair samples from patients was performed after receiving the written approval of the health research ethics committee in ministry of health, Kuwait and informed consent was also signed by patients after explanation of the study and its aim.

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