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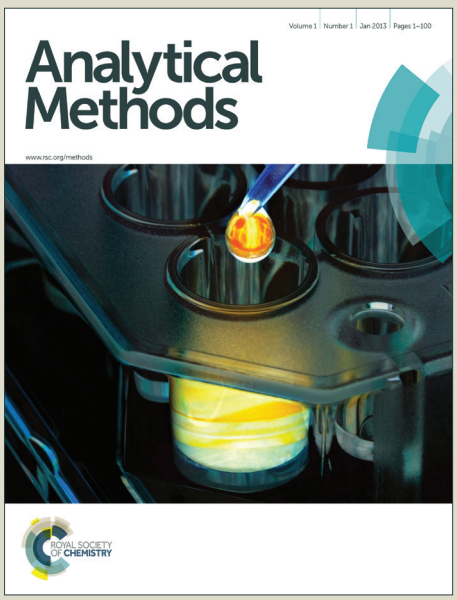

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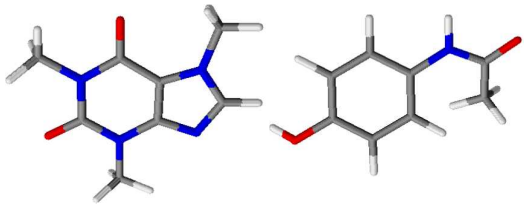

A LC-MS/MS method for quantification of caffeine and acetaminophen in amniotic fluid has been developed and validated.

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LC-MS/MS ANALYS OF ACETAMINOPHEN AND CAFFEINE IN AMNIOTIC FLUID.

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The intake of several substances by pregnant women could be hazardous to the fetus and mother health: many substances can cross the placenta and reach the fetal compartment, causing adverse outcomes. Consequently, to accurately measure the presence of xenobiotics in fetal matrices, sensitive and specific bioanalytical methods are necessary: this would allow to assess fetal exposure to substances which, although licit, can be dangerous for the fetal and child's growth. The aim of this study was to develop and validate a liquid chromatography tandem mass spectrometry method for the simultaneous determination and quantitation of caffeine and acetaminophen in amniotic fluid. Amniotic fluid is a quite complex biological matrix and, as such, it requires a purification step prior to analysis.

The extraction method has been optimized by comparing three different commercially available SPE cartridges (Supel^{TM} Select HLB, Phenomenex Strata C18-E, and Agilent ABS Elut-NEXUS), and a liquid/liquid extraction with acetonitrile. A reverse-phase HPLC with a C18 column and gradient elution program was used. MS detection was carried out in MRM mode. Quantitation was performed using the internal standard method. Validation parameters were very satisfactory. The high selectivity and sensitivity of the method (LOQ < 9.5 ng/ml, and LOD < 3.3 ng/ml) allow to determine target analytes even in small amounts. Precision, matrix effect, and stability were also evaluated.

The whole validated method has finally been applied to the analysis of 194 real samples of human amniotic fluid obtained from pregnant women (15-21 weeks of gestation) in order to monitor the effective intake of target analytes: 96% of examined women consumed caffeine during pregnancy while a lower percentage (20%) showed acetaminophen intake. The whole procedure is simple and easy to perform with minimal sample preparation and short analysis time.

Introduction

The intake of several substances by pregnant women could be hazardous to the fetus and mother health: many substances can cross the placenta and reach the fetal compartment, causing adverse outcomes[1]. Consequently, to accurately measure the presence of xenobiotics in fetal matrices, sensitive and specific bioanalytical methods are necessary: this would allow to assess fetal exposure to substances which, although licit, can be dangerous for the fetal and child's growth. The diagnosis of prenatal exposure is based on a positive maternal history or screening, and identification of a drug or its metabolites in either maternal or neonatal specimen. In order to evaluate the fetal exposure, several biological matrices are available, such as meconium, umbilical cord tissue, and amniotic fluid.

Amniotic fluid (AF) consists of a filtrate of maternal blood and it acts as a fetal excretion reservoir, accumulating drugs through gestation. The fetus is potentially re-exposed to drugs excreted in urine due to continuous swallowing of amniotic fluid. The major disadvantage of AF testing is the highly invasive collection. AF can only be non-invasively collected at birth, or as excess specimen from other medical procedure such as amniocentesis, usually performed to detect fetal genetic abnormalities. The aim of this study was to develop and validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination and quantitation in AF of acetaminophen (ACE) and caffeine (CAF) (Figure 1), components of pharmaceutical preparations commonly consumed also by pregnant women.

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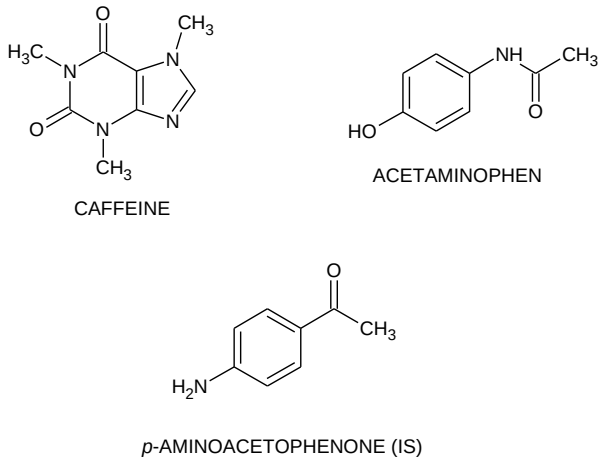
Fig. 1 Chemical structures of target analytes and internal standard



The prenatal exposure to these substances may contribute to the occurrence of serious pathologies. Clinical investigation and animal experiment demonstrate that caffeine ingestion by pregnant women results in reproductive and embryo toxicities[2,3]. Whereas the danger of caffeine is a controversial topic, acetaminophen is considered one of the safest drugs among those taken during pregnancy. Despite the extensive use, until recently, data on potential adverse effects in children prenatally exposed to this drug were lacking. Moreover, in the last years, the potential danger has been highlighted in several paper, suggesting that its use could be associated with risk of hyperkinetic disorders[4], wheeze[5] and asthma[6]. These hypotheses made interesting to focus our investigations on the presence of ACE in AF. However further investigations about the real harmfulness of ACE are certainly needed.

AF is a quite complex biological matrix and, as such, it requires a purification step prior to analysis. To develop a clean-up procedure, three commercially available SPE cartridges (Supel^{TM} Select HLB, Phenomenex Strata C18-E and Agilent ABS Elut-NEXUS) have been compared with a liquid-liquid extraction to evaluate their retention capacity toward the target analytes.

In literature some methods for the quantitation of CAF and ACE in plasma[7-8], wastewater[9] and other matrices[10,11] are reported; sensitivity is comparable or worse than that reported in our method. To the best of our knowledge this is the first report to evaluate CAF and ACE concentration in amniotic fluid. A simple and rapid LC-MS/MS method has been developed and validated for tested compunds, using *p*-aminoacetophenone as internal standard (IS). The validated method has finally been applied to the analysis of real samples of human amniotic fluid obtained from pregnant women (15-21 weeks of gestation) in order to monitor the effective intake of CAF and ACE in women during pregnancy.

Experimental

Chemicals and reagents

Caffeine (CAF), acetaminophen (ACE), and *p*-aminoacetophenone (IS) were purchased from Sigma Aldrich (Milan, Italy). MS grade methanol, acetonitrile, and formic acid were purchased from Sigma (Sigma-Aldrich, Milano, Italy). Deionised and distilled water was purified through a Milli Q water system (Millipore, Billerica, MA, USA). Other reagents and solvents used were of the highest quality commercially available. Supel^{TM}-Select HLB SPE columns (30mg/1ml) were purchased from Supelco (Bellafonte, PA); Strata C18-E (100 mg/1ml) SPE columns were purchased from Phenomenex (Torrance, CA, USA); ABS Elut- NEXUS (30mg/1ml) were purchased from Agilent Technologies (Palo Alto, CA). SPE columns were mounted on a VacElut vacuum manifold (Sigma Aldrich, Milan, Italy). Individual stock solutions (1.0 mg/ml each) were prepared in methanol and were used to prepare working solution, by appropriate dilution. Calibration samples containing all analytes at six working concentrations (10, 50, 100, 250, 500 and 1000 ng/ml) and 100 ng/ml of IS were prepared with blank AF. Drug-free AF specimens were collected to make blank and spiked samples containing the analytes. The AF samples were kept frozen at -20°C until analysed.

Specimen collection

Women (n = 194) undergoing routine (15-21 weeks gestation) amniocentesis for age-related genetic testing in our university hospital were invited to participate in this study. A signed consent from each participant allowed collection of the surplus AF not used during genetic testing. Samples were aliquoted (1000 µl) and stored at −80 °C until analyzed.

Blank specimen have been collected from women did not consume neither CAF and ACE.

All experiments were conducted according to the institutional guidelines, and were approved by the Local Ethical Committee.

Specimen preparation

Three commercially available SPE cartridges (Supel^{TM} Select HLB, Phenomenex Strata C18-E and ABS Elut-NEXUS) with liquid-liquid extraction were compared. SPE extractions were performed according to the manufacturer instructions:

Supel^{TM}-Select HLB: the sample (0.5 ml of AF + 0.5 ml of ammonium acetate buffer 25 mM, pH 10) was applied to a SPE column (30 mg/ml), previously activated and conditioned with 1 ml of methanol and 1 ml of ammonium acetate buffer (25 mM, pH 10). After application of the sample, the column was washed with 1 ml of Milli-Q water and 1 ml of 5 % methanol in water and dried by passing a stream of air for 5 min. The analytes were then eluted with 1 ml of methanol/acetonitrile 50:50 and the eluate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.1 ml of IS standard solution (100 ng/ml).

Phenomenex Strata C18-E: the sample (0.5 ml of AF + 0.5 ml of sodium hydrogen carbonate buffer 100 mM, pH 10) was applied to a SPE column, previously activated and conditioned

with 1 ml of methanol and 1 ml of sodium hydrogen carbonate buffer (100 mM, pH 10). After application of the sample, the column was washed with 2 ml of Milli Q water and dried by passing a stream of air for 5 min. The analytes were then eluted with 1 ml of methanol, and the eluate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.1 ml of IS standard solution (100 ng/ml).

ABS Elut-NEXUS: the sample (0.5 ml of AF + 0.5 ml of ammonium acetate buffer 25 mM, pH 10) was applied to a SPE column without activation and conditioning. After application of the sample, the column was washed with 2 ml of Milli Q water and dried by passing a stream of air for 5 min. The analytes were then eluted with 1 ml of methanol/acetonitrile 50:50 and the eluate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.1 ml of IS standard solution (100 ng/ml).

LLE extraction: 0.5 ml of AF were extracted with 1 ml of acetonitrile in order to deproteinize AF samples. The mixture was vortexed briefly and centrifuged at 1500 g for 15 min. The organic layer was transferred to clean tubes and evaporated to dryness under nitrogen. The dry residue was reconstituted in in 0.1 ml of IS standard solution (100 ng/ml).

Liquid Chromatography-Tandem Mass Spectrometry

Liquid chromatography was performed using a Shimadzu LC-20AD$_{XR}$ (Shimadzu Italia, Milano, Italy). Chromatographic separation was carried out on a Synergi C18 column (50 × 2.0 mm, 4 µm, Phenomenex, Torrance, CA, USA) fitted with a 3 µm C18 security guard cartridge (4 × 2.1 mm i.d.) (Phenomenex). A sample volume of 5 µL was injected into the LC-MS/MS system.

Two solvents were used for gradient elution: (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile. The gradient program was: 0 min - 10% B; 4 min - 90% B; 5.5 min - 90% B; 5.6 min - 10% B; 9.0 min - 10% B. The flow rate was 0.3 ml/min. The retention time of IS was 1.9 min. Each run was replicated three times.

MS detection was performed with an Applied Biosystem MDS Sciex (Concord, Ontario, Canada) API 2000 triple quadrupole mass spectrometer equipped with an electrospray ionization interface (ESI). Infusion experiments by syringe pump method were carried out for each analyte and IS (10 µg/ml) in both negative and positive electrospray ionization modes, to optimize ESI parameters. The quadrupole full-scan spectra were finally acquired in the positive mode, since the signal intensities were more significant. Mass spectra revealed intense protonated molecular ion peaks $[M+H]^+$, that were selected as precursor ion and fragmented to obtain product ions. From product ions spectra, the prominent product ions were selected for each analyte. Thus, a multiple reaction monitoring (MRM) method was developed. For each compound two transition were selected: one transition was taken as confirmation and the other one as quantitation (Table 1). Data acquisition and processing were accomplished using the Applied Biosystem Analyst version 1.6 software. Qualitative analysis was performed according to retention times and two MRM transitions. Quantitation was performed using the Applied Biosystem MultiQuant version 2.1 software, using the internal standard method.

Validation

The method was performed according to the accepted guidelines[12-14]. The parameters determined were selectivity, sensitivity, matrix effect, linearity, precision, recovery and stability. Selectivity was assessed by comparing the chromatograms of 9 different batches of blank AF. The method was also assessed to determine matrix effect on ion suppression or enhancement[15,16]. Matrix effect was determined with the post extraction addition method[17]. 9 drug-free AF samples from different sources were extracted as described above and subsequently fortified with analytes at three different concentrations (10, 400 and 800 ng/ml). Peak areas obtained from the extracts were compared with the corresponding peak areas produced by the reference solutions at the same concentration. The matrix effect percentage was calculate according to the following equation:

$$\text{Matrix effect (\%)} = \frac{AF - RS}{RS} \times 100$$

where AF is the "mean peak area of reconstituted AF extract" and RS is the "mean peak area of the reference solution".

For construction of calibration curves, blank AF samples, fortified with appropriate amounts of the tested drugs at concentrations ranging from LOQ to 1000 ng/ml, were extracted and analyzed as described above. The linearity of the compound-to-IS peak ratio versus the theoretical concentration was verified in plasma by using a 1/*x* weighted linear regression. The correlation coefficients (*r*) and the curvature were tested on a set of five calibration curves. The limits of detection (LODs) and the limits of quantitation (LOQs) were calculated using the signal-to-noise criteria of 3 and 10, respectively. The precision of the method was evaluated at three concentrations over the linear dynamic range (10, 400 and 800 ng/ml). Precision was expressed as the percent relative standard deviation (RSD), where the sample standard deviation (SD) was calculated for five replicates for each level for the intraday precision and over 5 consecutive days for the interday precision. Recoveries were determined at three concentrations (10, 400 and 800 ng/ml) for each compound and each extraction method. Nine blank samples were fortified with the appropriate amounts of mixed standard solution. The recoveries were calculated by comparing the peak areas obtained from the extracts of the spiked AF samples with those obtained by direct injection of standard solutions at the same concentration.

The freeze-and-thaw stability of the analytes was determined after performing three freeze-and-thaw cycles of AF samples at two concentration levels (10 and 400 ng/ml for all compounds). Three AF samples were spiked with the appropriate amounts of each compound, frozen for 24 h and then thawed for 2 h at room temperature. This cycle was performed three times. After this process, three fresh AF samples at 10 and 400 ng/ml were

prepared. The stability samples and the fresh samples were extracted as described above.

Results and discussion

Qualitative analysis was performed according to retention times and two MRM transitions (Table 1). Selection of the quantifier and qualifier transitions was based on transitions from the molecular ion to the most and second-most predominant fragment ions, respectively. For positive identification of each analyte the ratio of the qualifier transition to the quantifier transition was required to be within ± 30% of the average measured ratio from standards[18].

Table 1 Tandem mass spectrometric conditions

Compound	Parent ion (*m/z*)	Fragment ions (*m/z*)	DP (V)	CE (V)	CXP (V)
CAF	195.2	138.2 [a] 110.2 [b]	35	30 34	5 4
ACE	152.3	110.3 [a] 93.3 [b]	30	27 32	4 3
IS	136.2	94.3	20	20	4

[a] Fragment ion used for quantitation; [b] fragment ion used for confirmation
DP: declustering potential; CXP: collision cell exit potential; CE: collision energy.
Entrance Potential (EP): 10; Focusing Potential (FP): 200; Ion source temperature: 400 °C; Ion spray voltage: 5 000 V; Curtain gas: 6 arbitrary units; Collision gas: 6 arbitrary units.

Preliminary experiments were carried out by comparing three commercially available SPE cartridges (Supel™ Select HLB, Phenomenex Strata C18-E and Agilent ABS Elut-NEXUS) with a liquid-liquid extraction to evaluate their retention capacity toward the target analytes. In Table 2 average recoveries (from three concentrations 10, 400 and 800 ng/ml) for each extraction method are compared.

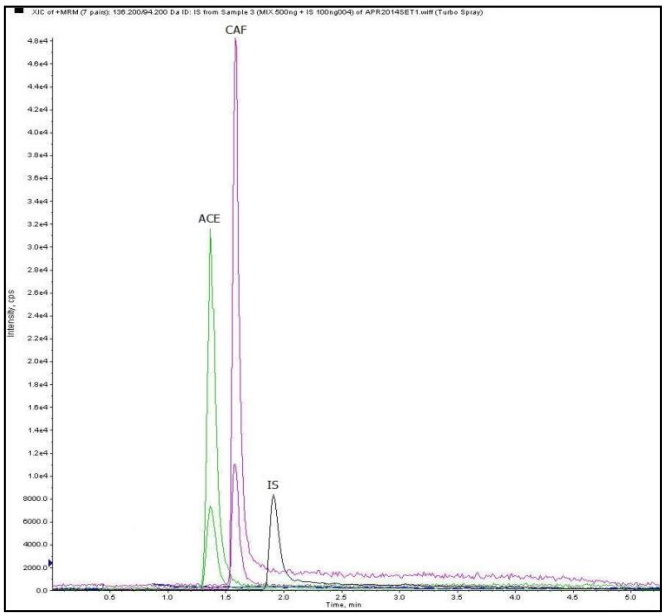
Table 2 Recoveries from Supel™ Select HLB, Phenomenex Strata C18-E, ABS Elut-NEXUS, and liquid/liquid extraction (LLE)

	HLB	C18	NEXUS	LLE
ACE	80%	20%	30%	56%
CAF	88%	96%	35%	49%

As shown in the table, the most suitable recoveries for both ACE and CAF were achieved with the HLB cartridges. Indeed, Strata C18-E cartridges gave the best recovery for CAF, while were totally unsatisfactory for ACE.

HLB columns are characterized by hydrophilic modifications in the styrene-based polymer backbone; it allows to obtain excellent retention and consequently to have good recoveries for a broad range of compounds, including polar drugs; in fact this SPE cartridge proved to be suitable for both CAF and ACE, which are characterized by significant differences in terms of polarity. Instead, the mechanism of retention of Strata C18-E cartridge is primarily based on hydrophobic interaction, leading to good recoveries especially for non-polar drugs, such as CAF. ABS Elut-NEXUS cartridge should extract both polar and non-polar analytes, but it was inefficient in our specific case.
An exemplifying chromatogram of SPE extract (HLB cartridges) of AF sample spiked with 500 ng/ml of ACE, CAF and IS (100 ng/ml) is shown in Figure 2.

Fig. 2 AF sample spiked with ACE, CAF, and IS



The analytes retention times are given in Table 3. The whole validation has been developed with the HLB cartridges.
Regression equations were linear over the tested concentration range (from LOQ to 1000 ng/ml) with good correlation coefficients for all compounds, that exceeded 0.998 (Table 3). The limits of detection and quantitation were 3.3 and 9.5 ng/ml, and 2.4 and 7.6 ng/ml for ACE and CAF, and, respectively.

Table 3 Retention times, calibration curves, limits of detection (LODs) and limits of quantitation (LOQs)

Compound	t_R (min)	*r*	*y*=a*x*+b	LOQ (ng/ml)	LOD (ng/ml)
CAF	1.7	0.998	*y*=0.007*x*+0.045	7.6	2.4
ACE	1.3	0.998	*y*=0.005*x*+0.039	9.5	3.3

t_R: retention time; SD: standard deviation; LOQ: limit of quantitation; LOD: limit of determination

Precision was evaluated by analyzing AF samples with three different concentrations (10, 400 and 800 ng/ml) as shown in Table 4. The intraday and interday precision results showed RSD values all within acceptable limits. Selectivity was satisfactory, because no interfering endogenous substances at the retention times of the test compounds and IS were observed when nine blank AF samples were analysed.

Table 4 Intraday and interday precision for the method (n=5)

Compound	Concentration added (ng/ml)	Intraday precision (RSD)	Interday precision (RSD)
ACE	10	4.5	12.1
	400	7.4	9.7
	800	11.3	10.6
CAF	10	9.5	11.1
	400	11.0	13.1
	800	8.1	9.4

RSD (%): Relative Standard Deviation (%)

Recoveries and matrix effect for each analytes were assessed at three concentrations (10, 400 and 800 ng/ml); average recoveries ranged from 77 to 93% (Table 5). The matrix effect, expressed as ion enhancement, was always lower than 8% (Table 5). Analyte recoveries in the stability experiments were within the permitted variability range. No significant loss or deterioration was observed after three freeze and thaw cycles for 3 days for any of the compounds of interest with differences from the initial concentration less than 10 % (data not shown).
The validated method has been applied to the analysis of 194 human AF samples. Data obtained showed that the 96% of examined women consumed CAF during pregnancy, with found concentrations in AF samples ranging from 30 to 6010 ng/ml. A lower percentage (20%) showed ACE intake with found concentrations from traces (<LOQ) to 1390 ng/ml.

Table 5 Recoveries from AF samples, and the matrix effect

Compound	Spiked concentration (ng/ml)	Recovery (%) (n=9)	Matrix effect (%) (n=9)
ACE	10	81.9	7.45
	400	76.9	6.86
	800	80.5	2.78
CAF	10	82.2	6.92
	400	87.5	4.86
	800	93.2	2.87

It is evident that the high percentage of samples positive to caffeine can not be exclusively due to the consumption of pharmaceutical preparations. The positivity of most of the samples is probably due to the fact that in Italy the consumption of caffeinated beverages is a widespread practice even among pregnant women.

Conclusions

To the best of our knowledge, this is the first report to validate a method for the analysis of ACE and CAF in AF specimens. Simultaneous detection of the target analytes was carried out by LC-MS-MS, using SPE as clean-up step. Validation parameters were very satisfactory. The high selectivity and sensitivity of the method (LOQ < 9.5 ng/ml, and LOD < 3.3 ng/ml) allow to determine target analytes even in small amounts. The whole procedure is simple and easy to perform with minimal sample preparation and short analysis time. The method has been successfully applied to the analysis of real AF samples: the 96% of examined women consumed CAF during pregnancy while a lower percentage (20%) showed ACE intake.
These results show that also if the current recommendation is to either eliminate or limit caffeine intake during pregnancy, the consumption of caffeinated beverages by pregnant women is widespread practice[19]. High intakes of caffeine are even more probable taking into account new caffeine sources such as bottled water, energy drinks, and herbal supplements, which often do not report caffeine content and may therefore covertly increase caffeine consumption during pregnancy[20]. Moreover, in Italy, caffeine positivity of most of the samples is likely due to the Italian common practice to drink espresso coffee, even among pregnant women. It is well known that drugs should not be taken in pregnancy, especially in the first trimester because of the increased risk of miscarriage, notwithstanding our results show ACE intake[21]. Our method has proved to be a reliable, accurate and sensitive bioanalytical procedure, that allowed to identify and quantify target analytes. It could be useful in order to determinate the in utero drug exposure, and to connect it to toxicological outcomes.

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