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Simultaneous determination of procaine and *para*-aminobenzoic acid by LC–MS/MS method

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Abstract

A sensitive high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method has been developed for simultaneous determination of procaine and its metabolite *p*-aminobenzoic acid (PABA). *N*-Acetylprocainamide (NAPA) was used as an internal standard for procaine and PABA analysis. This assay method has also been validated in terms of linearity, lower limit of detection, lower limit of quantitation, accuracy and precision as per ICH guidelines. Chromatography was carried out on an XTerra™ MS C_{18} column and mass spectrometric analysis was performed using a Quattro Micro™ mass spectrometer working with electro-spray ionization (ESI) source in the positive ion mode. Enhanced selectivity was achieved using multiple reaction monitoring (MRM) functions, m/z 237 → 100, m/z 138 → 120, and m/z 278 → 205 for procaine, PABA and NAPA, respectively. Retention times for PABA, procaine and NAPA were 4.0, 4.7 and 5.8 min, respectively. Linearity for each calibration curve was observed across a range from 100 nM to 5000 nM for PABA, and from 10 nM to 5000 nM for procaine. The intra- and inter-day relative standard deviations (RSD) were <5%.

Keywords: Esters; LC–MS/MS; Local anesthetics; NAPA; PABA; Procaine

1. Introduction

Procaine, 4-aminobenzoic acid 2-(diethylamino) ethyl ester, is a local anesthetic [1] which is marketed as its hydrochloride salt under a variety of names such as novocaine or neocaine. It is administered by injection because of its poor penetration of mucous membranes after which it is metabolized primarily by plasma butyrylcholinesterase and secondarily by liver esterases to produce *p*-aminobenzoic acid (PABA) and diethylaminoethanol [2]. Procaine hydrochloride is also the active substance in the Romanian drugs Gerovital H3 and Aslavital, both of which are used in the treatment of aging and trophy disturbances [1]. PABA is a key building block in the enzymatic synthesis of dihydrofolic acid [3–5] and is found in plant and animal tissues [3]. It has also been administered as a therapeutic, namely as a treatment for typhus and other rickettsial diseases [3], as well as used as a common ingredient in sunscreen agents [6].

The determination of PABA as an impurity in drugs containing procaine or procaine raw materials has been previously performed by using UV and HPLC methods [7,8]. Several other methods have also been reported in the literature for the determination of PABA based on colorimetric [9], spectrometric [10,11], chromatographic [12,13] and electrochemical [14,15] detection. Procaine has likewise been detected by various methods including polarimetry [16], HPLC [17,18], IR [19], fluorimetric [20] and Raman spectroscopy [21]. A few methods for the simultaneous detection of procaine and PABA have been reported in multi-target screening analysis using HPLC [22–24]. These methods require a long analysis time [23], lack high sensitivity in biological matrices [23], or require amine additives to improve peak shapes [24]. Finally, given the antiarrhythmic properties of *N*-acetylprocainamide (NAPA), methods for its determination have also been developed [25–27]. The chemical structures of procaine, PABA and NAPA are shown in Fig. 1.

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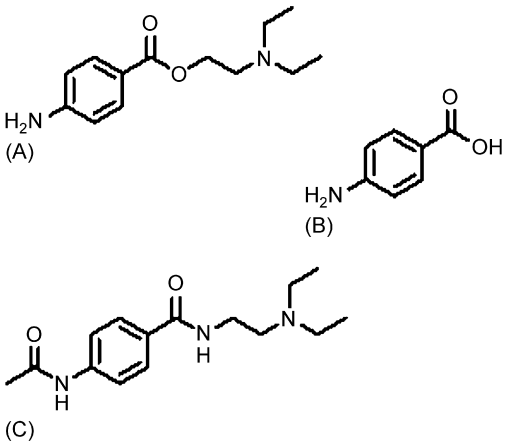


Fig. 1. Chemical structures of: (A) procaine; (B) PABA; and (C) NAPA.

Mass spectrometry has been increasingly perceived to be an essential tool in the drug discovery process including lead identification, assessment of compound purity, quality control of bulk drug substance, and toxicology and pharmacokinetics [28]. Advantages of using mass spectrometry for detection includes selectivity and sensitivity, especially when combined with separation techniques such as gel electrophoresis, gas chromatography (GC) or liquid chromatography (LC). PABA has been detected as its ethyl-esterified derivatives by using gas chromatography–mass spectrometry [29–32], these methods also being applicable to analyses of folates from human whole blood [29–32]. While methods for detection of PABA using a nitrogen-phosphorus detector with an open tubular column have been met with difficulty [33], detection of procaine and other local anesthetics from human plasma and urine have been successfully reported by using GC–MS with electron impact ionization [34–36].

In comparison to GC methods, LC methods have advantages such as reduced sample preparation time because there is typically no need for derivatization [32]. Liquid chromatography coupled with single quadrupole mass spectrometry (LC–MS) offers high sensitivity. However, insufficient selectivity often complicates the unequivocal determination of analytes from biological matrices, sewages [37] and plant crude extracts [38]. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) offers both high sensitivity and selectivity for the unambiguous determinations of trace-level concentrations of analytes even in complex matrices. LC–MS/MS detection of PABA has been reported from plants [38] but, in this case, a long analysis time was required. The closest report to the present study pertains to a LC–MS/MS method for determination of *p*-amino methyl benzoate from human whole blood [32]. Procaine has also been detected using LC–MS/MS methods in solutions [39–41] and from biological matrices [42,43] as one of the drugs in multi-target analysis. However, in these cases, PABA, the metabolite was not detected.

From this backdrop, it was interesting to find that no LC–MS/MS method appears to be available for simultaneous detection of procaine and PABA in a single run. Thus, there remains a need for an analytical method capable of selective, sensitive, rapid and reliable simultaneous determination of procaine and PABA. Such a method would particularly be useful for the determination of PABA as an impurity in drugs containing procaine and procaine raw materials, quality control studies, and in vitro enzyme assays where procaine and PABA need to be monitored simultaneously. We have developed such a method and, with minor modification, it can also be deployed across other anesthetics that fall within this category. To our knowledge, this is the first study for the simultaneous detection and quantitation of procaine and PABA by using an LC–MS/MS method. Linearity, lower limit of detection, lower limit of quantitation, precision and accuracy were assessed according to ICH guidelines [44].

2. Experimental

2.1. Materials

Procaine (>97% purity; lot #114K0569), *p*-aminobenzoic acid (99% purity; lot #03711DO), *N*-acetyl-procainamide (>99% purity; lot #10209JQ) and ammonium acetate (98% purity; lot #044K3443) were purchased from Sigma–Aldrich chemicals (St. Louis, MO). Assessment of the procaine impurities by LC–MS showed that they were not related to PABA and that they did not interfere with the determinations of either procaine or PABA. Methanol, acetonitrile and formic acid (FA) were of HPLC grade and were purchased from Fisher Scientific (New Jersey, USA). HPLC grade water was obtained using a Milli Q system. A 10 mM solution of ammonium acetate was prepared using HPLC water and the pH was adjusted to 4.0 using acetic acid. Nitrogen gas was produced from liquid nitrogen by Dewar. Liquid nitrogen and argon gas were purchased in high purity (99.998%) from Linde gas (Toledo, OH, USA).

2.2. Instrumentation

An Alliance® HT liquid chromatograph (model 2795) equipped with a quaternary pump, a degasser, an auto sampler/injector (syringe volume = 250 μl) and a column oven from Waters corporation (Milford, MA, USA) were used. Mass spectrometric analysis was performed using a Quattro Micro™ (triple–quadrupole) instrument from Micromass (Manchester, UK) equipped with an ESCi™ Multi mode ionization source. MassLynx (version 4.01) software from Micromass was used for data acquisition and handling.

2.3. LC conditions

An XTerra™ MS C_{18} analytical column (2.1 mm × 150 mm, 5 μm) and a guard column (2.1 mm × 10 mm, 5 μm) from Waters Corporation (Milford, MA, USA) were used for the chromatographic separation of procaine, PABA and NAPA. A security guard column (filter size: 0.2 μm) from MAC MOD (Chadds Ford, PA, USA) was also used for each analytical run. Chromatography was carried out via a gradient system with a flow rate of 400 μl/min after an injection volume of 10 μl. The mobile phase consisted of eluent A, 10 mM ammonium acetate

at pH 4.00 ± 0.05 and eluent B, 0.1% FA in methanol. The starting eluent was 95% A and 5% B after which the proportion of eluent B was increased linearly to 10% in 1.00 min, 15% in 4.00 min, 50% in 4.50 min, 90% in 4.75 min, returned to initial composition of eluent A (95%) and B (5%) in 5.00 min and then held for 3.00 min in order to re-equilibrate the column. A 0.2% FA in methanol/water mixture (50:50, v/v) was used as the needle wash solvent; 10 mM ammonium acetate as the purge solvent and 100% methanol was used as a seal wash solvent. The column and samples were kept at 35 ± 5 °C and 4 ± 5 °C, respectively.

2.4. MS conditions

A Micromass® Quattro Micro™ triple–quadrupole instrument was used for mass spectrometric detection of procaine, PABA and NAPA using an electro-spray ionisation (ESI) source in the positive mode. The source conditions for procaine, PABA and NAPA were set as follows: dissolution gas (N_2) and cone gas (N_2) flow rates were 600 l/h and 25 l/h, respectively; the source and dissolution gas temperatures were 130 °C and 350 °C, respectively. The ESI source tip (capillary) voltage was 0.5 kV, extractor was 2 V; and, ion energy for MS1 was 0.2 and for MS2 was 1.0 V. The LC–MS/MS was operated in the MRM mode under unit mass resolution (10% valley definition) in both the Q1 and Q3 mass analyzers. Argon gas was used as the collision gas. The argon gas cell pressure was approximately 3.1×10^{-3} mbar. The dwell time of each compound was 200 ms. Data acquisition and processing were carried out using software MassLynx version 4.01.

2.5. Sample preparation

Parent stock solutions of procaine, PABA and NAPA were prepared at concentrations of 1 mM in methanol/water (50:50, v/v) in separate glass vials. Two subsequent 1:10 dilutions of each parent solution were made in methanol/water (50:50, v/v) to give three 10 μM stock solutions. A combined stock solution of 5 μM procaine and PABA was obtained by adding equal volumes of 10 μM stock solutions of both procaine and PABA, from which a series of working standards were prepared by the appropriate dilutions to obtain concentrations across a range of 5 nM to 5000 nM. The 10 μM NAPA solution was also serially diluted to make working standards for the concentration range of 50 nM to 1000 nM. Combined quality control samples in four different concentrations, 10 nM (LQC, low quality control); 100 nM and 500 nM (MQC, medium quality control); and 1000 nM (HQC, high quality control) were also prepared in a similar manner with separate weightings. All parent stock solutions, working standards and quality control standards were immediately stored at −20 °C.

2.6. Calibration curve

The standard calibration curves were constructed using the combined working standard solutions ranging from 10 nM to 5000 nM for procaine and PABA, and 50 nM to 1000 nM for NAPA. The linear regression analysis for NAPA was obtained by plotting the peak areas (y) against the concentration of NAPA with $1/x$ fit weighting; and, for procaine and PABA were obtained from the peak area ratio (Area × (IS Conc/IS Area)) against the concentrations of procaine and PABA with $1/x$ fit weighting. The firm relationship between peak areas and concentrations (linearity) was demonstrated.

2.7. LLOD and LLOQ determinations

Lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) can be determined by several approaches according to FDA guidelines [44]: (i) visual evaluations; (ii) signal-to-noise ratio (s/n) wherein, in general, s/n between 3:1 is considered as an acceptable range for detection limit and an s/n of 10:1 for quantitation limit; and (iii) based on standard deviation of the response and the slope associated with the calibration curve, expressed as LLOD = (SD × 3.3)/slope for lower limit of detection and LLOQ = (10 × SD)/slope for lower limit of quantitation. In this assay, the lower levels of detection and quantitation limit are calculated based on signal to noise ratio.

3. Results and discussion

Several criteria associated with assay validation were undertaken according to ICH guidelines [44] so as to define the method's linearity, lower limit of detection, lower limit of quantitation, precision and accuracy.

3.1. Assay development

3.1.1. LC optimization

For the present study, we tested several combinations of reverse phase columns and different mobile phases at different pH ranges for the chromatographic separation of procaine and its metabolite, PABA. The best result was obtained with the XTerra™ MS C_{18} analytical column (2.1 mm × 150 mm, 5 μm) with 10 mM ammonium acetate at pH 4.0. This combination provided high resolution and excellent peak shape compared to other analytical columns and systems that were used. We also tested several structurally related compounds including procainamide, *p*-hydroxyl-benzoic acid and *N*-acetyl-procainamide as internal standards for procaine and PABA. Among those tested, NAPA was found to be a suitable internal standard for both procaine and PABA, eluting at 5.8 min without overlapping either of the procaine and PABA peaks.

3.1.2. MS/MS optimization

In this study, electro-spray ionization was chosen as the ionization source. The highest signal intensity for procaine, PABA and NAPA were found when using the ESI source in a positive ionization mode. The maximum abundance of the parent and product-ions for procaine, PABA and NAPA were obtained by optimizing the mass spectrometric parameters. Full-scan mass spectra of procaine, PABA and NAPA were obtained by direct infusion of standard aqueous methanolic solutions (5 μM) into the ESI source of the mass spectrometer at a flow rate of 10 μl/min. Full-scan spectra produce protonated molecular

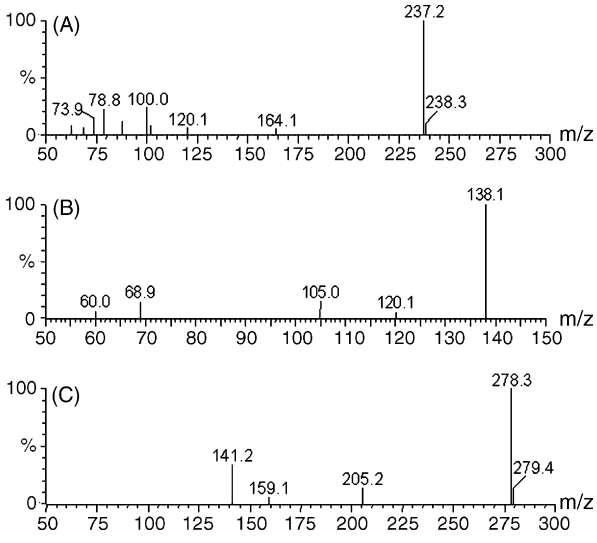


Fig. 2. Positive-ion ESI mass spectra: (A) procaine; (B) PABA; and (C) NAPA. These parent ions spectra were obtained by direct infusion of 5 μM of aqueous methanolic solutions of procaine, PABA and NAPA into the ESI source of the mass spectrometer at a flow rate of 10 μl/min.

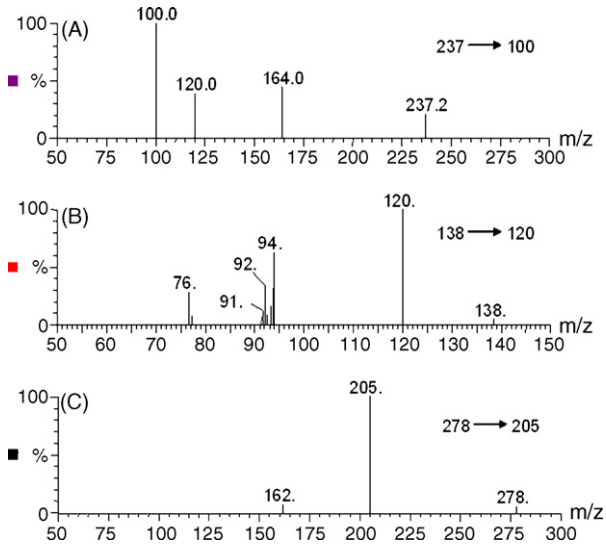
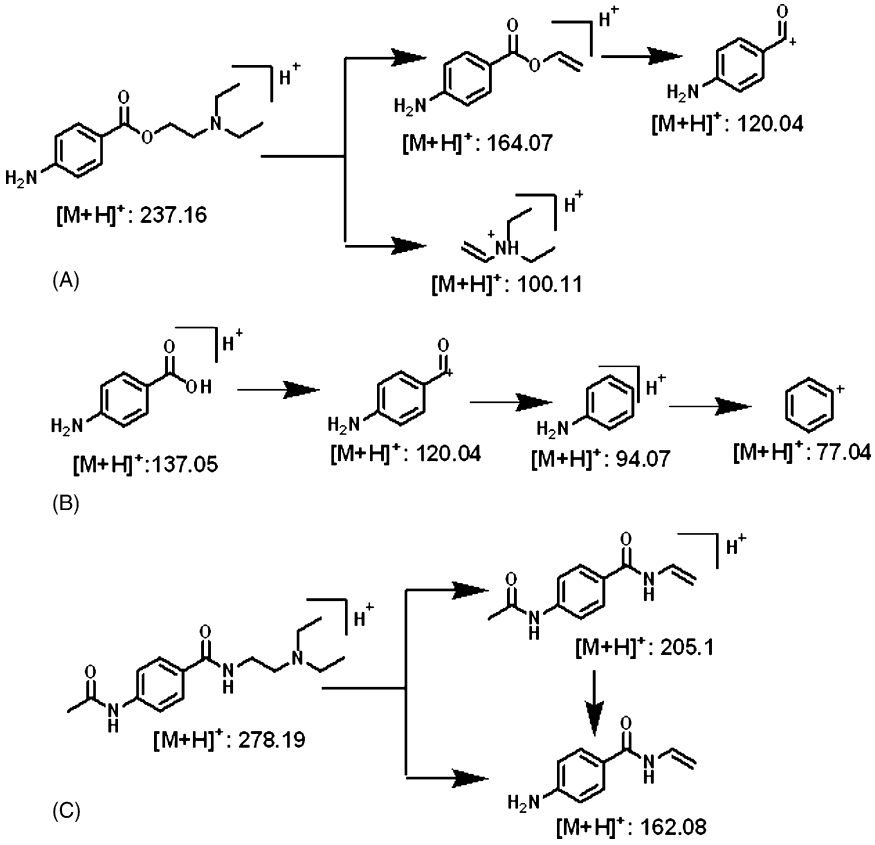


Fig. 3. MS/MS product-ion spectra: (A) procaine; (B) PABA; and (C) NAPA. These parent ions spectra were obtained by direct infusion of 5 μM of aqueous methanolic solutions of procaine, PABA and NAPA into the ESI source of the mass spectrometer at a flow rate of 10 μl/min.

ions (parent-ions), $[M+H]^+$, at *m/z* 237 for procaine, *m/z* 138 for PABA and *m/z* 278 for NAPA. The full-scan spectra are shown in Fig. 2. These parent-ions were selected using the first quadrupole analyzer (Q1) and then collisionally-activated dissociation (CAD) was carried out using suitable collision energy to produce product-ions within the second quadrupole (Q2) using an rf-only mode (collision cell, Q2). The most abundant product-ions were investigated by applying various collision energies. Increases in the collision energy caused marked increases in the fragmentation processes. The most abundant product-ion was found at *m/z* 100 for procaine, *m/z* 120 for PABA and *m/z* 205 for NAPA by applying suitable collision energies. The product-ions spectra are shown in Fig. 3. The compound specific parameters such as collision energies and cone voltages are listed in Table 1.



Scheme 1. MS/MS fragmentation pathways: (A) procaine (B) PABA; and (C) NAPA.

Table 1
Compound-specific parameters for procaine, PABA and NAPA

Compound	Parent-ions (*m*/*z*)	Product-ions (*m*/*z*)	Cone voltage (V)	Collision energy (V)
Procaine	237	100	25	19
PABA	138	120	25	17
NAPA	278	205	28	18

Scheme 1 shows the proposed dissociation pathways for the protonated procaine [39], PABA [37] and NAPA. Under these LC and MS/MS conditions, the retention times of procaine, PABA and NAPA were 4.7, 4.0 and 5.8 min, respectively. Representative LC–MS/MS chromatograms of procaine, PABA and NAPA are shown in Fig. 4.

3.2. Method validation

3.2.1. Linearity and lower limit of detection and quantitation

A range of reliable responses was established on the basis of 11 triplicate standards in methanol/water (50/50, v/v) covering concentrations from 10 nM to 5000 nM of combined procaine and PABA solution in the presence of 500 nM of internal standard, NAPA. The regression equations with back-calculated precision (RSD) and accuracy (RME) and correlation coefficients are given in Table 2 wherein each calibration equation represents the mean of the three corresponding replicate standards. The standard calibration plots for procaine and PABA are shown in Fig. 5.

The lower limit of detection was 1 nM and 50 nM for procaine and PABA, respectively, with the peak area for those concentrations being very distinguishable from the response given by blank sample. 98.5% accuracy was found from six consecutive injections of 1 nM of procaine with 500 nM NAPA and 96.3%

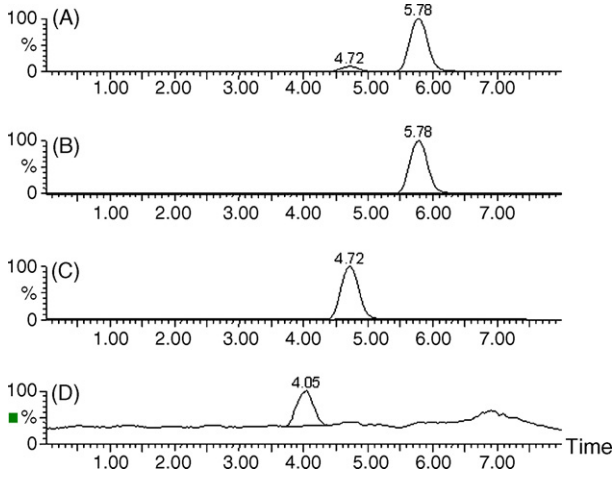


Fig. 4. Representative MRM chromatograms of procaine, PABA and NAPA: (A) total-ion chromatogram of procaine, PABA and NAPA; (B) individual MRM channel of NAPA; (C) individual MRM channel of procaine; and (D) individual MRM channel of PABA. These chromatograms were obtained from 10 μl injection of a combined standard solution that contains 100 nM of procaine, 100 nM of PABA and 500 nM of NAPA.

Table 2
Back-calculated calibration standards of procaine and PABA

	STD A 10 nM	STD B 25 nM	STD C 50 nM	STD D 100 nM	STD E 250 nM	STD F 500 nM	STD G 750 nM	STD H 1000 nM	STD I 2000 nM	STD J 3000 nM	STD K 5000 nM	Regression equation, Y	r^2
Procaine													
Mean ($n=3$)	9.0	24.9	49.8	103.8	252.7	544.5	783.3	932.4	1983.8	2993.2	5007.6	$0.471x-0.0214$	0.999
SD	0.1	0.3	0.6	0.7	2.3	4.5	2.5	6.3	11.5	15.4	12.1		
%RSD	1.0	1.2	1.1	0.7	0.9	0.8	0.3	0.7	0.6	0.5	02		
%RME	−9.6	−0.5	−0.5	3.8	1.1	8.9	4.4	−6.8	−0.8	−0.2	0.2		
PABA													
Mean ($n=3$)				101.9	277.2	534.8	777.7	1014.0	1977.8	2883.0	5044.3	$0.00181x+0.0129$	0.998
SD				7.6	1.6	7.7	5.2	35.6	43.2	48.4	47.8		
%RSD				7.5	0.6	1.4	0.7	3.5	2.2	1.7	0.9		
%RME				1.9	10.9	7.0	3.7	1.4	−1.1	−3.9	0.9		

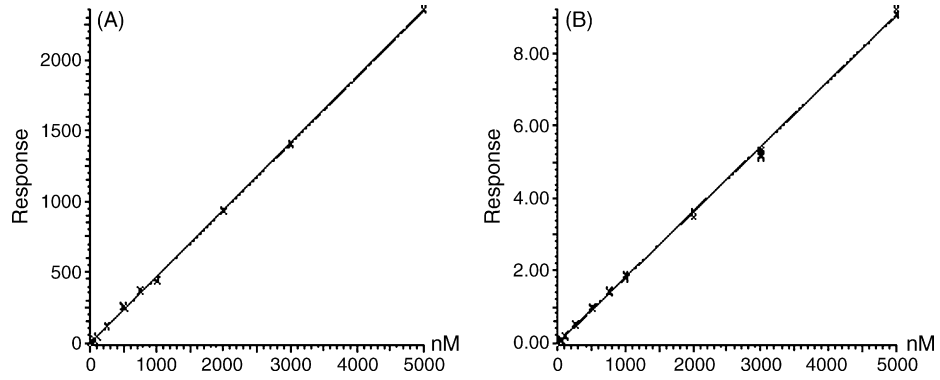


Fig. 5. Standard calibration plots: (A) procaine and (B) PABA. Each calibration line represents the mean of the three corresponding replicate standards.

Table 3
Intra- and inter-day accuracy and precision of procaine and PABA ($n = 3$ in all cases)

	Intra-day			Inter-day		
	Mean ± SD	%RSD	%Accuracy	Mean ± SD	%RSD	%Accuracy
Procaine (nM)						
10	9.36 ± 0.1	1.5	93.600	9.04 ± 0.1	1.0	94.400
100	103.82 ± 0.7	0.7	103.820	103.00 ± 0.6	0.5	10.3.000
500	487.25 ± 3.1	0.6	97.450	492.27 ± 1.3	0.3	98.454
1000	962.00 ± 6.1	0.6	96.200	970.00 ± 3.6	0.4	97.000
PBA (nM)						
100	100.25 ± 4.7	4.7	100.250	105.12 ± 0.4	0.3	105.120
500	508.24 ± 4.2	0.8	101.648	512.76 ± 3.8	0.7	102.552
1000	1020.50 ± 3.5	0.4	102.050	1018 ± 7.1	0.7	101.800

accuracy was found from six consecutive injections 50 nM of PABA with 500 nM NAPA. However, it is interesting to note that under the same stated experimental conditions and without changing the retention times of procaine, PABA and NAPA, the sensitivity of lower detection limit for PABA was decreased from 50 nM to 250 nM when one of the mobile phases was changed from 0.1% formic acid in methanol to 100% methanol, whereas the same detection limit was observed for procaine.

The lower limit of quantitation was 10 nM for procaine and 100 nM for PABA, at which procaine and PABA can be reliably quantified with both relative standard deviation of the mean <5% (%RSD) and relative mean error <2% for six consecutive injections. The RSD and RME values are indicators for precision and accuracy, respectively.

3.2.2. Precision and accuracy

The intra- and inter-day precision (%RSD) and accuracy of the method were determined from the analysis of quality control samples at four different concentrations with triplicate injections and the results are summarized in Table 3. All values of accuracy and precision were found within recommended limits. Intra- and inter-day precisions (%RSD) when measured at concentrations of 10, 100, 500 and 1000 nM were less than 2% for procaine and for concentrations of 100, 500 and 1000 nM were less than 5% for PABA. Intra- and inter-day accuracies measured at similar concentrations were between 94 and 104% for procaine, and between 100 and 105% for PABA.

4. Conclusion

A new LC–MS/MS method has been developed for simultaneous determination of procaine and PABA. The assay has high sensitivity and reliability, and provides a linear response across a wide range of concentrations. Because the determination is simultaneous for procaine and PABA, this method should be helpful in quality control laboratories to assess the purity and integrity of procaine. The assay affords the sensitivity, accuracy and precision needed for quantitative measurements of procaine and PABA. Preliminary findings from studies involving the simultaneous determination of procaine and PABA from biological media such as mouse plasma and urine further suggest that the method should be readily applicable to these types of matrices when preceded by routine sample preparation protocols.

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