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Research Article

# Determination of Articaine in Human Blood by Gas and Liquid Chromatography and its Application in a Preliminary Pharmacokinetic Study

Manda. C.V[1,*], Popescu. S.M[2], Baniceru M[1]

[1]*Faculty of Pharmacy, University of Medicine and Pharmacy, 4 Petru Rares, 200349 Craiova, Romania, Postal Code 200638*
[2]*Faculty of Dental Medicine, University of Medicine and Pharmacy, 4 Petru Rares, 200349 Craiova, Romania, Postal Code 200638*

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**ABSTRACT**
Two simple, sensitive and specific gas and liquid chromatographic methods are developed and validated for quantification of articaine in human whole blood. Liquid-liquid extraction with n-hexane-isoamyl alcohol 90:10 (v/v) is used for sample preparation in both methods. GC-FID (gas chromatographic-flame ionization detection) analysis is performed with a gas chromatograph equipped with a split-splitless injector, flame-ionization detector and HP INNOwax capillary column. Reverse phase LC-PDA (liquid chromatographic-photo diode array detection) analysis is carried out using a C18 Hypersil GOLD column with two mobile phases: acetonitrile-water 10 mM ammonium acetate 50:50 (v/v), pH 7, and acetonitrile-buffer solution sodium acetate 10 mM and acetic acid 10 mM 50:50 (v/v), pH 4.7, respectively. A comparison between GC-FID and LC-PDA is performed, as well as between internal and external standard as quantification methods in LC. The best results in terms of accuracy (as recovery) and precision (as relative standard deviation) are obtained in GC (95-98% and 5.5-8.2%, respectively) with lidocaine internal standard and in LC (96-102% and 4.2-6.1%, respectively) with external standard and mobile phase pH 4.7. This method is applied in a preliminary pharmacokinetic study to three healthy volunteers to whom anesthesia with articaine is performed.

**Keywords:** Articaine, Lidocaine, Human Blood, GC, HPLC.

## INTRODUCTION

Articaine hydrochloride [4-methyl-3(2-propylaminopropion-amido) thiophene-2-carboxylic acid methyl ester hydrochloride] is the most widely used local anesthetic agent in dentistry in a number of European countries[1]. Due to ester group, articaine is rapidly hydrolyzed by the action of pseudocholinesterase in blood, unlike amide type anesthetics that are mainly metabolized by the liver microsomes. Therefore, plasma elimination half-life of articaine is about 20 minutes, whereas elimination half-life of other amide-type local anesthetic is about 100 minutes[2]. Because its main metabolite (articainic acid) is inactive, articaine has low central nervous system toxicity[2]. In dentistry, a 4% articaine solution with 1:200000 epinephrine is widely used for infiltration and conduction anesthesia. The addition of epinephrine is required to produce vasoconstriction that leads to local anesthesia duration extension and bleeding limitation. Unfortunately, epinephrine also brings about undesirable effects, such as an increase in blood pressure and tachycardia[3]. For children, some authors recommend that dosage limits of local anesthetics should be smaller than for adults[4].

The ester-type local anesthetics in biological samples were analyzed by gas chromatography[6] and liquid chromatography[1-3,7-16]. These methods include liquid-liquid extraction (LLE) of the sample with t-butyl-ethyl ether[8], diethyl ether[9], cyclohexane[14], and solid-phase extraction (SPE) with polystyrenedivinylbenzene copolymer (SDB-RPS)[1], SDB-XC[10] and extrelut[6]. In most of the works, the sample was directly introduced in LC column after the precipitation with perchloric acid[2,3,15]. The blocking of pseudocholinesterase activity was achieved with neostigmine[6] or echothiophate[15,16], while in some works this activity was not blocked[9-13]. In this work articaine is analyzed by GC-FID and LC-PDA and LLE from whole blood. We consider this as the most appropriate sample preparation method since in order to inhibit cholinesterase activity, before acquiring the blood sample in heparin tube, a neostigmine aqueous solution (or another inhibitor) must be introduced in the tube, and causing hemolysis. Because of hemolysis, this approach makes impossible a subsequent separation of serum or plasma from blood. When the blood is added first, articaine is hydrolyzed. With this work, a comparison between GC-FID and LC-PDA is being performed, as well as between internal standard (IS) and external

**Author for Correspondence*

standard (ES) as quantification methods in LC. The method is applied in a dentistry clinic to three healthy volunteers in a preliminary pharmacokinetic study.

## MATERIALS AND METHODS

Articaine and lidocaine (IS) free bases were purchased from Sigma-Aldrich, and acetonitrile, triethylamine, ammonium acetate, sodium acetate, acetic acid, n-hexane and isoamyl alcohol from Merck. Purified water was prepared on a Milli-Q Waters purification system. Stock solution of articaine and lidocaine (IS) free bases, containing 1 mg/mL was prepared in acetonitrile and stored at 4ºC. Working solutions (20 µg/mL) were made by further dilution with acetonitrile.

For calibration curve heparinized blood (1 mL) was successively spiked with 0.1; 0.2; 0.3; 0.6; 1.2; 2.4; 4.8 µg/mL articaine. For a recovery and precision test, blood was spiked with 0.5; 1 and 2 µg/mL articaine. In IS method 0.6 µg/mL lidocaine (30 µL working solution) were added. To block esterases activity in the blood neostigmine methylsulfate was mixed before the addition of articaine. A posterior superior alveolar nerve block with articaine hydrochloride 4% 1.7 mL (68 mg) with adrenaline hydrochloride 0.017 mg (Ubistesin forte, 3 M ESPE, Germany) was performed. On the test day, the patients were not allowed to eat, to drink alcohol, coffee, cola, tea or grapefruit juice. Venous blood was collected in heparinized tubes (in which neostigmine methylsulfate was previously introduced) at 5; 10; 15; 20; 30; 45; 60; 75; 90 min after administration of drug. Regarding liquid-liquid extraction, all the samples were identically treated, as follows. To 1 mL heparinized blood, 500 µL 0.3 mM neostigmine methylsulfate, 30 µL solution lidocaine (0.6 µg) and 300 µL solution $Na_2CO_3$ 1 M (pH 10) were added. The mixture was twice extracted with 1 mL solvent (n-hexane: isoamyl alcohol 90:10 (v/v), each time). The sample was gently shaken for 5 min and centrifuged for 10 min at 3000 x g. Then, the organic layer was transferred into conical tubes end evaporated to dryness under a gentle stream of nitrogen at ambient temperature. For GC analysis the residue was dissolved in 200 µL acetonitrile, and 1 µL was injected into GC injection source; for LC analysis the residue was dissolved in 200 µL mobile phase, and 20 µL were manually injected into LC injection source. GC analysis was performed with Agilent Technologies model HP 6890 gas chromatograph equipped with a split-splitless injector and flame-ionization detector. Compounds were separated on a 30 m x 0.25 mm i.d. x 0.25 µm film thickness HP INNOwax capillary column purchased from Agilent. The carrier gas was hydrogen at a flow rate of 2 mL/min. Samples (1 µL) were injected in splitless mode. Injector and detector temperatures were 240ºC. The oven temperature was maintained at 80ºC for 1 min after injection then programmed at 20ºC/min to 150ºC, then 5ºC/min to 240 ºC, which was maintained for 10 min. The detector gas (nitrogen) make up flow was 35 mL/min.

LC analysis was performed with a Thermo Finnigan Surveyor HPLC System equipped with photodiode array detector and Thermo Finnigan Xcalibur data system. Separation was achieved using a $C_{18}$ reversed phase column, (Thermo Scientific) Hypersil GOLD, 250 mm x 4.6 mm I.D. particle size 5 µm. Compounds were subsequently eluted isocratically at a flow rate of 1 mL/min with two mobile phases: acetonitrile-water 10 mM ammonium acetate 50:50 (v/v), pH 7, and acetonitrile-buffer solution sodium acetate 10 mM and acetic acid 10 mM 50:50 (v/v), pH 4.7. Column eluates were monitored at 274 nm (for articaine) and 235 nm (for lidocaine).

## RESULTS AND DISCUSSION

Calibration curve was achived by preparing standard samples at seven concentration levels (0.1- 4.8 µg/mL). Standard samples were three times prepared and analyzed. The linear regression analysis for articaine was obtained by plotting the peak area ratio (area articaine/area lidocaine) against the concentration of articaine (in IS method) and the peak area of articaine against the concentration of articaine (in ES method). The concentration of IS was the same for all samples. The linearity domain for the methods was found between 0.1 and 4.8 µg/mL blood. The mean regression equations are presented in Table 1. As solvents extraction, cyclohexane, n-hexane, n-hexane- i-propanol 80:20 (v/v), cyclohexane-isoamyl alcohol 80:20 (v/v), diethyl ether and n-hexane-isoamyl alcohol were tested. The best results in terms of articaine recovery have been obtained with n-hexane-isoamyl alcohol 90:10 (v/v). Combination between an aprotic solvent (n-hexane) and a hydrogen-bonding solvent (isoamyl alcohol) is appropriate for extraction of articaine and lidocaine from blood. GC separation of some local anesthetics (mepivacaine and lidocaine) on an INNOwax (cross-linked PEG) capillary column has been previously presented[17]. In that case, symmetrical peaks have been obtained in spite of the fact that both stationary phase and the analytes had a polar character. As can be noticed in Figure 1, there are many peaks in the chromatogram because there are many endogenous compounds in the samples. The flame ionization detector is not selective and it generates a signal for all compounds and the peak area is proportional with the quantity of the analyte and the number of carbon atoms in the molecule. Therefore, the GC chromatogram contains many peaks which are not related with the analytes. The retention time was used for identification of the target compounds, by spiking the samples with articaine and lidocaine followed by GC analysis. The spiked samples presented increased areas for the peaks at 17.6 and 23.1 min, corresponding to lidocaine and articaine. The compounds have been thermally stable at injector, column and detector temperatures. LC separation was performed on a Hypersil GOLD C18 column. The factors that determine the separation and analysis in LC, in the rank of their importance are: pH of mobile phase, solvent type, column type, % organic solvent, buffer concentration and type[18]. The solute retention changes with pH only when the pH of the mobile phase is within ± 1.5 units of the $pK_a$ value of the solute. The retention time will not modify if pH of the mobile phase is lower or

Table 1: Results of calibration curves (n = 3)

| Method | Range (µg/ml) | Regression equation | $r^2$ |
|---|---|---|---|
| GC, IS | 0.1 – 4.8 | y = 0.10 + 1.45 x | 0.9995 |
| LC, IS mobile - phase pH 7 | 0.1 – 4.8 | y = 0.23 + 12.35 x | 0.9994 |
| LC, ES mobile - phase pH 7 | 0.1 – 4.8 | y = – 0.01 + 8.8 x | 0.9992 |
| LC, IS mobile - phase pH 4,7 | 0.1 – 4.8 | y = – 0.63 + 12.39 x | 0.9960 |
| LC, ES Mobile - phase pH 4,7 | 0.1 – 4.8 | y = 0.57 + 6.84 x | 0.9990 |

higher than this three units interval. Because $pK_a$ values for articaine and lidocaine were 7.8 and 7.9, respectively, we choose pH 7 for buffer solution-mobile phase. The effect of acetonitrile ratio was tested in the range from 30 to 60%, v/v. Large differences of recovery (98% versus 85 %) were observed when comparing GC and LC methods - both with IS quantification - at 1 µg/mL spiked blood with articaine and 0.6 µg/mL lidocaine. That determined us to change both the working conditions and quantification method in LC. In acidic media, compounds with alkaline properties are fully ionized, but at some degree still retained on the lipophilic stationary phase. It is important that the vast majority of the molecules of solute be in the same form, ionized or non-ionized. With a mobile phase acetonitrile-buffer solution sodium acetate 10 mM and acetic acid 10 mM, pH 4.7, articaine and lidocaine are fully ionized. The retention times have been decreased from 6.4 min for articaine and 9.7 min for

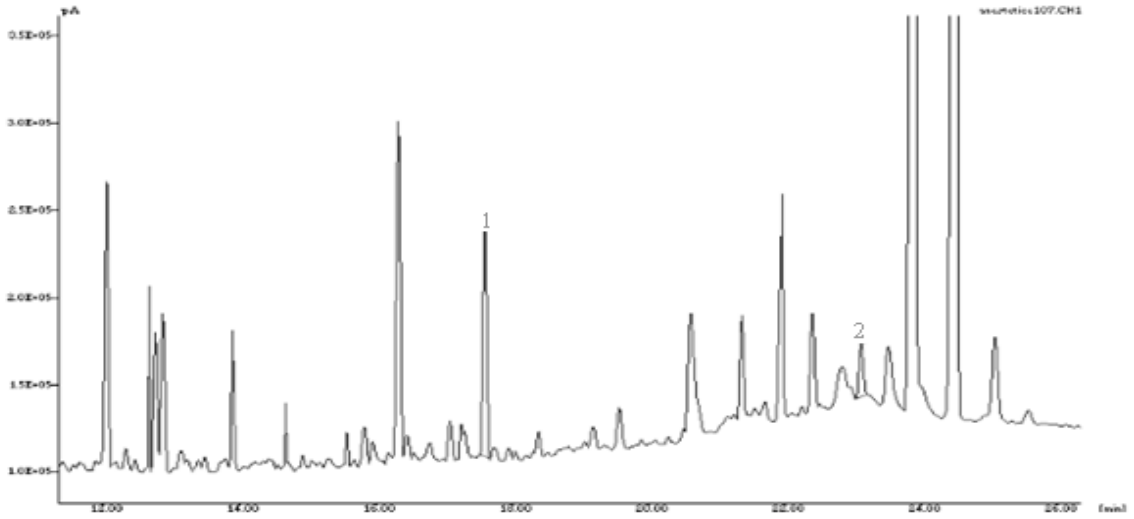


Fig. 1: GC chromatogram of a blood sample at 20 min after the administration of articaine to a patient. Peaks: 1 – lidocaine (IS), 2 – articaine

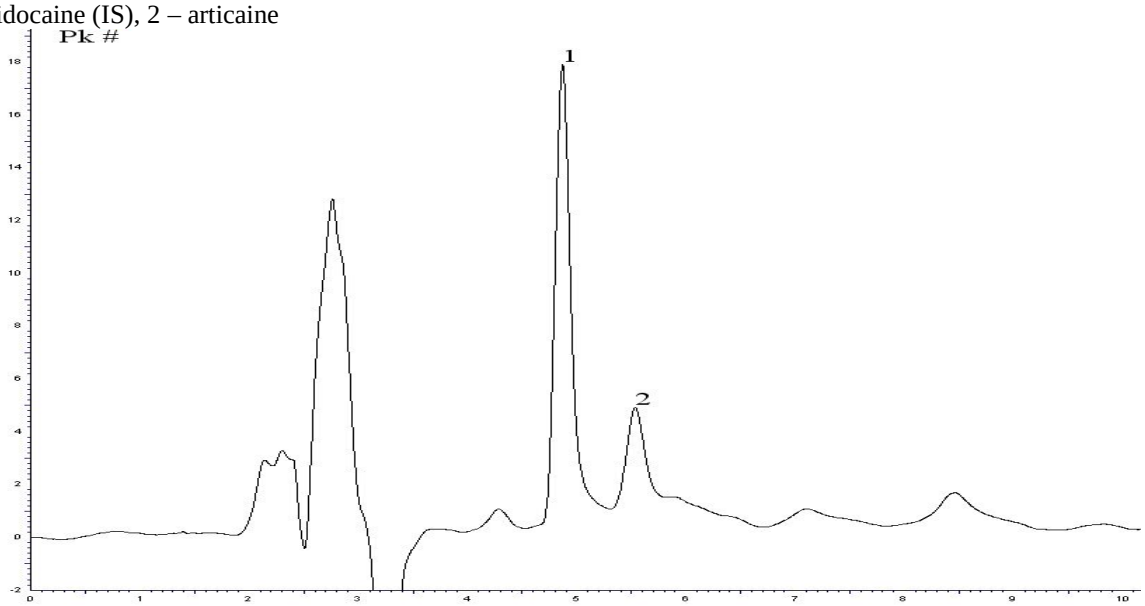


Fig. 2: LC chromatogram of a blood sample at 15 min after administration of articaine to a patient, at mobile phase pH 4.7 and 235 nm wavelength. Peaks: 1 – articaine, 2 – lidocaine (IS)

Table 2: The precision and accuracy of the assay for articaine in human blood (n = 4)

| Method | Target concentration (μg/ml) | Mean found (μg/ml) | | RSD % | | Recovery % | |
|---|---|---|---|---|---|---|---|
| | | Intra-day | Inter-day | Intra-day | Inter-day | Intra-day | Inter-day |
| GC, IS | 0.5 | 0.48± 0.03 | 0.47± 0.03 | 7.92 | 8.2 | 96 | 95 |
| | 1 | 0.98± 0.07 | 0.97± 0.06 | 7.22 | 6.90 | 98 | 97 |
| | 2 | 1.94± 0.12 | 1.96± 0.10 | 6.15 | 5.50 | 97 | 98 |
| LC,IS mobile phase, pH 7 | 0.5 | 0.42± 0.05 | 0.42± 0.05 | 13.20 | 12.82 | 84 | 83 |
| | 1 | 0.85± 0.10 | 0.86± 0.10 | 12.33 | 11.98 | 85 | 86 |
| | 2 | 1.68± 0.20 | 1.64± 0.21 | 12.02 | 13.01 | 84 | 82 |
| LC,ES mobile phase, pH 7 | 0.5 | 0.45± 0.02 | 0.43± 0.02 | 5.28 | 6.25 | 89 | 87 |
| | 1 | 0.91± 0.05 | 0.90± 0.05 | 6.29 | 5.82 | 91 | 90 |
| | 2 | 1.84± 0.12 | 1.78± 0.09 | 6.82 | 5.51 | 92 | 89 |
| LC,IS mobile phase, pH 4,7 | 0.5 | 0.48± 0.05 | 0.48± 0.06 | 11.89 | 14.52 | 96 | 95 |
| | 1 | 0.98± 0.10 | 1.07± 0.12 | 10.20 | 12.72 | 98 | 107 |
| | 2 | 1.98± 0.18 | 1.92± 0.19 | 9.48 | 10.25 | 99 | 96 |
| LC,ES mobile phase, pH 4,7 | 0.5 | 0.48± 0.02 | 0.49± 0.02 | 5.10 | 6.02 | 96 | 98 |
| | 1 | 0.97± 0.04 | 1.02± 0.05 | 4.20 | 5.04 | 97 | 102 |
| | 2 | 1.96± 0.09 | 1.94± 0.11 | 4.88 | 6.12 | 98 | 97 |

Table 3: Mean concentration articaine in patient blood at different times after administration (n=3)

| Time (min) | GC, IS (μg/ml) | LC, ES mobile phase pH 4.7 (μg/ml) |
|---|---|---|
| 5 | 0.140 | 0.150 |
| 10 | 0.350 | 0.345 |
| 15 | 1.620 | 1.770 |
| 20 | 2.390 | 2.340 |
| 30 | 0.410 | 0.427 |
| 45 | 0.330 | 0.345 |
| 60 | 0.325 | 0.324 |
| 75 | 0.215 | 0.216 |
| 90 | 0.200 | 0.202 |

lidocaine to 4.7 min and 5.3 min, respectively (Fig. 2). Calibration curves for articaine in human blood showed good linearity over the concentration range studied (Table 1). The limit of detection in both GC-FID and LC with ES was 20 ng/mL blood. Accuracy and precision results are presented in Table 2. Accuracy was determined as 100 x (mean found concentration/spiked concentration), and precision - RSD – as 100 x (standard deviation/mean found concentration). With LC methods, from Table 2 there are at least two obvious remarks that can be noticed. Better recoveries have been obtained with the mobile phase pH 4.7. That might be due to lower solubility of articaine and lidocaine in mobile phase pH 7. Better precisions have also been obtained with ES against IS. The sensitivity of PDA detector varies from compound to compound due to their different absorptivities that depend both of the compound structure and the wavelength the absorbance is measured. The maximum absorbance of articaine is at 274 nm, and the one of lidocaine at 220 nm. Due to absorption of mobile phase at 220 nm we measured the absorbance of lidocaine at 235 nm. Small errors on the wavelengths can cause substantial errors on the absorbance. For these reasons, in this case, we recommend the use of ES method in LC. Additionally, articaine has a much higher absorptivity than lidocaine and, consequently, a better limit of detection. If in GC with manual injection the use of IS is mandatory, in LC the injection of repeatable volumes can be easier performed by means of precise and reliable injection systems (loop valves). The best results in terms of accuracy (as recovery) and precision (as relative standard deviation) have been obtained in GC – FID (95-98% and 5.5-8.2%, respectively) with lidocaine IS and in LC (96-102% and 4.2-6.1%, respectively) with ES and mobile phase pH 4.7. These methods have been successfully applied to three healthy volunteers for quantitative analysis of articaine in human whole blood. From Table 3 one can notice that the maximum concentration of articaine in blood is 2.34 μg/mL (GC) and 2.39 μg/mL (LC) at 20 min after administration. With the similar administered quantity of anesthetic T.B. Vree and M.J.M. Gielen[19] obtained 2.1±1.3 μg/mL after 12.5±2.5 min but R.Oertel et al.[20] obtained 0.4 μg/mL with eprinephrine and 0.58 μg/mL without eprinephine, after 10–15 min. The concentrations may differ in a small extent from patient to patient, but the differences are mainly due to the methods of analysis utilized.

## CONCLUSION

GC and LC methods have been validated to determine articaine in human whole blood.

The best recovery and precision have been obtained by GC-FID (internal standard) and LC-PDA (external standard). Validation results have qualified these methods as suitable to appropriately quantitate articaine and also other structurally similar local anesthetics in pharmacokinetic studies.

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