Journal of Chromatography B, 867 (2008) 247–252

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid and sensitive HPLC assay for simultaneous determination of procaine and *para*-aminobenzoic acid from human and rat liver tissue extracts

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ARTICLE INFO

Article history:
Received 19 February 2008
Accepted 11 April 2008
Available online 29 April 2008

Keywords:
Esters
HPLC
Local anesthetics
Liver extracts
Intestine extracts
Procaine
PABA

ABSTRACT

A sensitive and rapid high-performance liquid chromatography method has been developed for simultaneous determination of procaine and its metabolite *p*-aminobenzoic acid (PABA) from human and rat liver tissue extracts. The method has been validated according to ICH guidelines in terms of selectivity, linearity, lower limit of detection, lower limit of quantitation, accuracy, precision and recovery from human and rat liver tissue extracts. Chromatography was carried out on a Discovery® C_{18} column using 10 mM ammonium acetate at pH 4.0 and acetonitrile as mobile phase. Retention times for procaine and PABA were 6.6 and 5.3 min, respectively. Linearity for each calibration curve in both tissue extracts was observed across a range from 10 μM to 750 μM for procaine and PABA. The lower limit of detection for both procaine and PABA was 5 μM and the lower limit of quantitation was 10 μM in both tissue extracts. The intra- and inter-day relative standard deviations (R.S.D.) for both procaine and PABA were <6%. Recoveries of procaine and PABA from human and rat liver tissue extracts were determined by two different methods with a single-step protein precipitation technique being employed in both methods. Recoveries for both procaine and PABA were greater than 80% from both human and rat liver tissue extracts.

1. Introduction

Carboxylesterases structurally belong to a super-family of α/β-fold proteins, which consist of alternate α-helix and β-sheets connected by loops with a varying length [1]. It comprises a multi-gene family, the gene products of which are localized in the endoplasmic reticulum (ER) and cytosol of many tissues. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing compounds, drugs and prodrugs to the respective free acids. They are also involved in detoxification or metabolic activation of various drugs, environmental toxicants, and carcinogens [2]. Several studies have shown that different isozymes of carboxylesterases are present in a wide variety of organs and tissues of many mammalian species. Humans also express carboxylesterase in the liver, plasma, small intestine, brain, stomach, colon, macrophage, and monocytes [2]. Of all the tissues studied, the highest hydrolase activity occurs in liver tissues [3–5].

Procaine is a local anesthetic drug of the amino ester group [6] and it is metabolized to *p*-aminobenzoic acid (PABA) and *N*,*N*-diethyl-2-aminoethanol [7] by esterases in liver and by butyrylcholinesterase in plasma. The chemical or esterase hydrolysis of procaine to PABA and *N*,*N*-diethyl-2-aminoethanol is shown in Fig. 1. We have previously reported a highly sensitive LC–MS/MS method to assess the purity and integrity of procaine when accompanied by its hydrolytic products PABA in solid or aqueous solution samples [8]. Wanting to extend this method for general use with various biological matrices, we also appreciated that LC–MS/MS is a relatively expensive technology that is not always readily available in academic research laboratories. While published HPLC methods for the independent determination of procaine or PABA from biological matrices are reasonably sensitive, they are time-consuming and require tedious extraction procedures [9,10]. We describe herein a convenient and rapid HPLC method for the simultaneous determination of procaine and PABA from human and rat liver tissue extracts. The assay has a short HPLC run time and sample preparation involves a simple protein precipitation step. Selectivity, linearity, lower limit of detection, lower limit of quantitation, recovery, precision and accuracy are delineated according to ICH guidelines [11]. This method should be particularly useful for *in vitro* and *in vivo*

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doi:10.1016/j.jchromb.2008.04.014

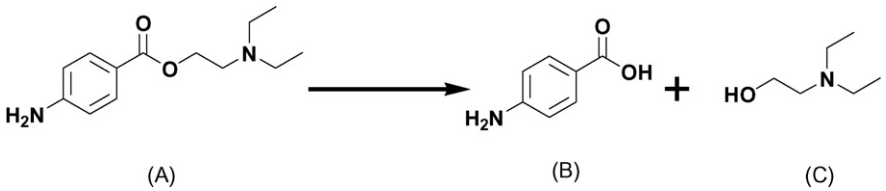

Fig. 1. Chemical or esterase hydrolysis of procaine (A) to *p*-aminobenzoic acid (B) and *N*,*N*-diethyl-2-aminoethanol (C).

pharmacology and toxicology studies when procaine and PABA need to be monitored simultaneously. To our knowledge, this is the first study for the simultaneous determination of procaine and PABA from biological tissue extracts by using an HPLC assay method.

2. Experimental

2.1. Materials

Procaine (>97% purity, lot #114K0569), *p*-aminobenzoic acid (99% purity, lot #03711DO), and ammonium acetate (98% purity, lot #044K3443) were purchased from Sigma–Aldrich chemicals (St. Louis, MO, USA). Methanol, acetonitrile and glacial acetic acid were of HPLC grade and were purchased from Fisher Scientific (Pittsburg, PA, USA). HPLC grade water was obtained using a Millipore Q system (Billerica, MA, USA). Human liver tissues were obtained from the Asterand human tissue bank (Detroit, MI, USA). Rat liver tissues were collected from euthanized rats at University of Toledo's animal facility. A 10 mM ammonium acetate solution was prepared in HPLC water and the pH adjusted to 4.0 with glacial acetic acid using an Accumet pH meter (model XL15) with Accumet pH electrode from Fisher Scientific. A Bradford assay kit was purchased from Bio-Rad (Hercules, CA, USA) to determine total protein content in biological media. A Mettler analytical balance (model XS205) was used to weight solids and a routine calibration and study calibration were carried out before and after each weighing.

2.2. Instrumentation

An Alliance® liquid chromatography system (model 2695) equipped with a photodiode array detector (model 2996), a quaternary pump, a degasser, an auto sampler/injector (syringe volume = 500 μl) and a column oven from Waters corporation (Milford, MA, USA) were used. A Discovery® C_{18} analytical column (4.6 mm × 150 mm; 5 μm) with a guard column (4.0 mm × 20 mm, 5 μm) from SupelCo (Bellefonte, PA, USA) and a security guard column (filter size: 0.2 μm) from MAC MOD (Chadds Ford, PA, USA) were used for the chromatographic separation of procaine and PABA. Chromatography was carried out in a gradient system with a flow rate of 1.0 ml/min. The mobile phase consisted of eluent A (10 mM ammonium acetate at pH 4.00 ± 0.05) and eluent B (100% acetonitrile). The starting eluent was 95% A and 5% B after which the proportion of eluent B was increased linearly to 10% in 3.5 min, 15% in 5.0 min, and then returned to initial composition of eluent A (95%) and B (5%) in 5.5 min followed by another 2.0 min to re-equilibrate the column. The detector wavelength was set at 290 nm. A 10 μl injection volume was used for each analytical run. The column and samples were kept at 35 ± 2 °C and 4 ± 2 °C, respectively. A methanol/water mixture (50:50, v/v) was used as the needle wash solvent. Empower (Version 5.00) software from Waters corporation was used for data acquisition and handling.

2.3. Sample preparation

Parent stock solutions of 10 mM procaine in water and 10 mM PABA in DMSO/water (10:90, v/v) were prepared in glass vials. Both solutions were diluted with water to 5 mM. A combined stock solution of 1 mM procaine plus 1 mM PABA was obtained from the 5 mM stock solutions. A series of working standards were prepared by appropriate dilutions to obtain concentrations across a range of 5–750 μM in human and rat liver tissue extracts, separately. All parent stock solutions and working standards were immediately stored at −20 °C. Combined quality control samples in three different concentrations, 10 μM (LQC, low quality control), 100 μM (MQC, medium quality control), and 500 μM (HQC, high quality control), were also freshly prepared in a similar manner in separate weighings by using human and rat liver tissue extracts.

2.4. Tissue homogenization

Homogenized tissue extracts were prepared according to reported method [12]. Frozen tissues collected from euthanized rats or obtained from a human tissue bank were thawed on ice, weighed and homogenized at 4 °C in 4 volumes of either 50 mM Tris HCl buffer containing 0.15 M KCl at pH 7.4 or 10 mM KH_2PO_4 buffer containing 1.0 mM 2-mercaptoethanol at pH 7.4 by using a Brinkman Polytron homogenizer. To avoid over-heating, tissues were homogenized three times for 1 min each with 1 min between. Homogenates were then transferred to 50 ml conical tubes and centrifuged at 9000 × *g* for 15 min while at 4 °C. The supernatants were transferred to new tubes and stored at −80 °C. The supernatants were used as a source of crude tissue extract that is rich in esterase enzymes. Total protein content was determined by using a Bradford assay kit.

2.5. Calibration curve

Standard calibration curves were constructed from the working standard solutions ranging from 10 μM to 750 μM for procaine and PABA in human and rat liver tissue extracts. A linear regression analysis was carried out by plotting the peak areas (y) of each compound against their respective concentrations (x). Linearity was demonstrated by a correlation coefficient (r^2) value greater than 0.997.

2.6. LLOD and LLOQ determinations

The lower limit of detection (LLOD) and the lower limit of quantitation (LLOQ) were calculated according to ICH guidelines [11]. Several different approaches can be used to ascertain LLOD and LLOQ [8,11]. We used the approach that is "based on the standard deviation (S.D.) of the response and the slope (σ) associated with the calibration curve" to calculate both LLOD and LLOQ [13,14] wherein LLOD = (S.D. × 3.3)/σ; and LLOQ = (S.D. × 10)/σ. The calculated LLOD and LLOQ were further substantiated by six consecutive injections at these concentrations so that their accuracy and precision became additionally confirmed in an experimental manner.

2.7. Recovery, precision and accuracy

The absolute recoveries of freshly prepared combined solutions of procaine and PABA from human and rat liver tissue extracts were evaluated at three different concentrations in triplicate. The recoveries were determined by two different methods. The first method involved spiking the combined solutions of procaine and PABA at three concentrations into human liver tissue extract containing 300 μg total protein in 50 mM Tris and 0.15 M KCl buffer at pH 7.4 in a total volume of 100 μl, followed by the addition of 100 μl of 0.25% trifluoroacetic acid in acetonitrile to yield final concentrations of procaine and PABA of 10, 100 and 500 μM. In the second method, human liver tissue extract solutions containing 300 μg total protein extract in 50 mM Tris and 0.15 M KCl buffer at pH 7.4 were first inactivated by adding 100 μl of 0.25% trifluoroacetic acid in acetonitrile and then the combined solutions of procaine and PABA were added to yield final concentrations of 10, 100 and 500 μM. In both cases, samples were vortexed and centrifuged at 16,000 × *g* for 15 min at 4 °C, and then 200 μl of clear supernatant was transferred into a micro vial for HPLC analysis. One set of blank extracts without procaine and PABA, and another set of positive control QC standards of procaine and PABA at all three concentrations in buffer without tissue extracts, were also processed in parallel. A similar pair of extraction methods was deployed to evaluate the recovery of procaine and PABA from rat liver tissue extracts. Precision and accuracy were evaluated by calculating the percent relative standard deviation (%R.S.D.) and percent accuracy for each set of biological media.

3. Results and discussion

3.1. HPLC assay development

Procaine and PABA exhibit two UV absorption maxima, namely at approximately 225 and 290 nm for procaine, and at 220 and 285 nm for PABA. In both cases, the first absorption maxima have lower intensity than the second absorption maxima, and significant portions of the absorption maxima were found to overlap near 225 and 280 nm. While there was little change in absorption intensity when measuring PABA either at 280 nm or 290 nm, a more significant change in absorption intensity was observed for procaine when measured at 290 nm compared to 280 nm. Hence, a wavelength of 290 nm was chosen to accommodate the simultaneous detection of procaine and PABA. The diode-array derived UV–vis spectra for both procaine and PABA are shown in Fig. 2.

HPLC columns are typically selected on the basis of hydrophobic and hydrophilic properties of the analytes. PABA is more hydrophilic compared to procaine and hence PABA will elute faster than procaine in a typical reversed-phase column using a mobile phase containing lower organic content. As part of the assay development process, several columns were tested including typical reversed-phase (SunFire C_{18} and XTerra™ MS C_{18}) and polar-embedded reversed-phase (symmetry Shield RP8 and Discovery C_{18}) columns. The best result was obtained with the Discovery C_{18} analytical column (15 cm × 4.6 mm, 5 μm) using 10 mM ammonium acetate at pH 4.0 and acetonitrile. Convenient retention times with well-resolved peak shape and good reproducibility were obtained when compared to the other analytical columns that were explored. Discovery C_{18} packing materials also provide good column stability toward more acidic and slightly basic mobile phase conditions. Another advantage of using a polar-embedded reversed-phase column is that a 100% aqueous system can be used as the mobile phase and this can be advantageous for the separation hydrophilic endogenous components that are present in the matrices. Thus, a polar-embedded reversed-phase column, Discovery C_{18}, was selected with 10 mM ammonium acetate at pH 4.0 as the mobile phase and the separation was monitored at 290 nm. Under the stated conditions delineated in the experimental section, the retention times for procaine and PABA were 6.6 and 5.5 min, respectively.

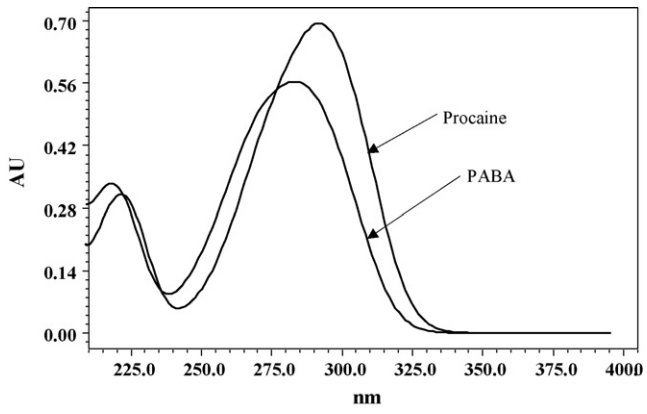

Fig. 2. The diode-array derived UV–vis spectra of procaine and its metabolite, PABA.

3.2. Assay validation

Selectivity, linearity, lower limit of detection, lower limit of quantitation, precision, accuracy and recovery from tissue extracts were investigated to validate this bioanalytical assay method. Validation was undertaken according to ICH guidelines [11].

3.3. Selectivity

The principal objective when establishing an assay's 'selectivity' is to be able to accurately measure the analyte with no interference from other components in the sample. Interferences can be from the endogenous components derived from the bio-matrices, the analytes' own metabolites and degradants, or any other compounds within the sample or introduced as part of sample preparation and assay. Selectivity was demonstrated for procaine and PABA in both human and rat liver tissue extract samples. Fig. 5B shows chromatographic base-line separation of procaine and PABA, and Figs. 5C and 6C further confirm that no interferences were found at the retention times of procaine and PABA due to endogenous constituents present in human and rat liver tissue extract samples after precipitation.

3.4. Linearity and lower limit of detection and quantitation

A standard calibration curve illustrates the relationship between the instrument response (peak area or peak height) and the concentration of the target compound. These should be directly proportional to each other within the desired assay range. According to ICH guidelines, a 15% deviation from nominal concentration will be acceptable for non-zero standards and a 20% deviation for lower limit of quantitation sample. Reliable responses were established for both procaine and PABA on the basis of six standards covering a concentration range of 10–750 μM. The standard calibration curves for procaine and PABA are shown in Figs. 3 and 4 in human liver and rat liver tissue extracts, respectively, which represent the mean of the three corresponding replicate standards. The correlation coefficients (r^2) were greater than 0.997 and the %R.S.D for each concentration studied were less than 6%. The quantification of peaks was carried out by deploying an external standard and using measurement of peak areas relative to the calibration curves of procaine and PABA.

Table 1
Intra- and inter-day precision and accuracies of procaine and PABA in human and rat liver tissue extracts ($n = 3$ in all cases)

	Intra-day			Inter-day		
	Mean ± S.D.	%R.S.D.	%Accuracy	Mean ± S.D.	%R.S.D.	%Accuracy
Human liver tissue extract						
Procaine (μM)						
10	10.16 ± 0.32	3.16	101.62	10.31 ± 0.54	5.26	103.11
100	100.23 ± 1.15	1.15	100.23	105.70 ± 0.45	0.42	105.70
500	487.96 ± 7.04	1.14	97.59	492.93 ± 0.01	0.01	98.58
PABA (μM)						
10	9.56 ± 0.07	0.76	98.62	9.86 ± 0.01	0.03	98.56
100	104.90 ± 0.13	0.13	104.90	105.49 ± 0.69	0.65	105.49
500	479.15 ± 5.33	1.11	95.83	465.00 ± 2.00	0.43	93.00
Rat liver tissue extract						
Procaine (μM)						
10	9.92 ± 0.10	1.05	91.97	8.78 ± 0.03	0.26	87.83
100	97.61 ± 0.62	0.63	97.61	96.58 ± 0.61	0.64	96.58
500	516.26 ± 0.62	0.12	103.25	521.96 ± 0.11	0.02	104.39
PABA (μM)						
10	8.87 ± 0.03	0.29	88.78	8.90 ± 0.07	0.78	89.03
100	99.93 ± 0.54	0.54	99.93	98.08 ± 0.30	0.30	98.08
500	485.39 ± 7.04	1.44	97.08	494.65 ± 4.21	0.85	98.93

Table 2
Percent recoveries of procaine and PABA from human and rat liver tissue extracts ($n = 3$ in all cases)

	Method 1		Method 2	
	Human liver extract (mean ± S.D.)	Rat liver extract (mean ± S.D.)	Human liver extract (mean ± S.D.)	Rat liver extract (mean ± S.D.)
Procaine (μM)				
10	88.64 ± 0.54	87.13 ± 0.28	90.45 ± 0.34	89.56 ± 2.06
100	94.32 ± 6.43	82.87 ± 2.93	96.89 ± 4.71	89.82 ± 4.68
500	101.13 ± 5.67	93.67 ± 3.87	99.49 ± 0.04	99.62 ± 4.72
PABA (μM)				
10	89.47 ± 0.43	90.28 ± 0.18	90.40 ± 0.59	87.52 ± 1.14
100	90.96 ± 6.25	81.90 ± 3.15	93.38 ± 4.59	89.19 ± 4.58
500	97.56 ± 5.51	93.24 ± 4.06	96.51 ± 0.87	99.95 ± 4.68

LLOD is defined as the lowest analyte concentration that can be detected, but not necessarily quantitated, under the stated experimental conditions. LLOQ is defined as the lowest analyte concentration that can be determined with acceptable precision and accuracy under the stated experimental conditions. The analyte's response at the LLOQ should be identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80–120%. In this assay method, the lower limit of detection for both procaine and PABA was 5 μM in both extracts since the peak area for this concentration was clearly distinguishable from the response given by blank samples. The relative mean errors (RME) from six consecutive injections of 5 μM were less than 9% for procaine and less than 10% for PABA in both tissue extracts. Procaine and PABA can be reliably quantified at 10 μM in both extracts with relative standard deviation of the mean (R.S.D.) less than 8% and relative

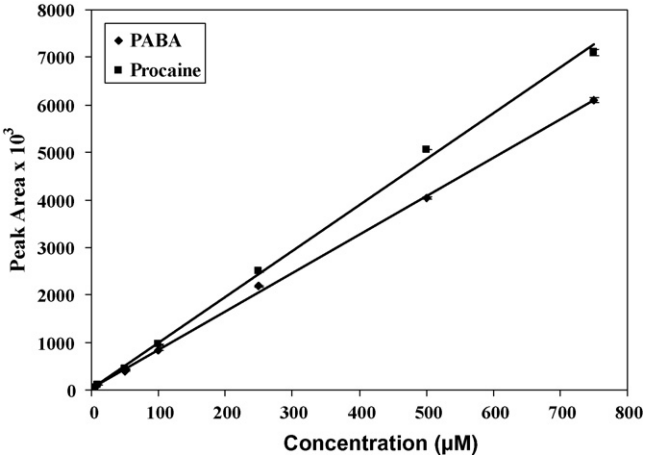

Fig. 3. Standard calibration curves for procaine and PABA in human liver tissue extract. Each curve is based on six calibration standards and three quality control standard with triplicate injections for each error bars represent above 95% confident limits.

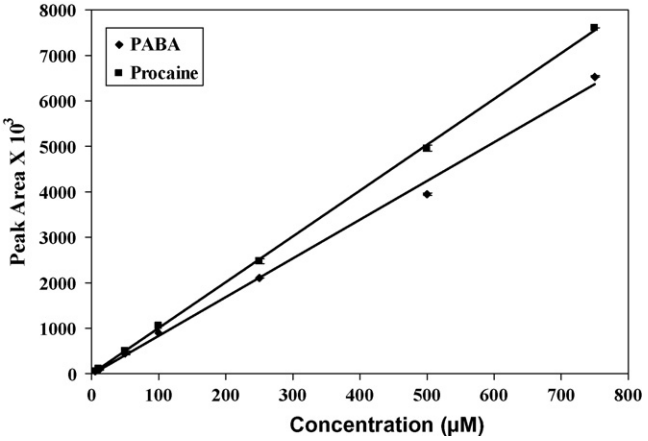

Fig. 4. Standard calibration curves for procaine and PABA in rat liver tissue extract. Each curve is based on six calibration standards and three quality control standard with triplicate injections. Each error bars represent above 95% confident limits.

mean error (RME) within 5% variation for six consecutive injections; hence the lower limit of quantification was 10 μM for both procaine and PABA in both human and rat liver tissue extracts. The R.S.D. and RME values are indicators for precision and accuracy, respectively.

3.5. Accuracy and precision

The accuracy of the method is defined as the closeness of observed results to the true value as determined by replicate analyses of samples that contain known amounts of the analyte. It is reported as the deviation of the mean from the true value. The ICH guidelines recommend that the mean value be within 15% of the actual value, except at the LLOQ, where 20% is acceptable. The intra- and inter-day accuracy of the method was determined from the analysis of standards in three different concentrations at 10, 100 and 500 μM with triplicate analyses. Intra- and inter-day accuracies for procaine range from 97.59–101.62% to 98.58–105.70%, respectively, in human liver tissue extracts; and 91.97–103.25% to 87.83–104.39%, respectively, in rat liver tissue extracts. Intra- and inter-day accuracies for PABA range from 95.83–104.90% to 93.00–105.49%, respectively, in human liver tissue extracts; and, 88.78–99.93% to 89.03–98.93%, respectively, in rat liver tissue extracts. All values of accuracy were found within recommended limits and the results are summarized in Table 1.

The precision of a method is defined as the measure of the amount of agreement among the observed results when the method is applied repeatedly to multiple samples. It is normally expressed as the percent relative standard deviation (%R.S.D.; also referred to as the coefficient of variation). ICH guidelines recommend that the precision measured at each concentration should not exceed 15% of the coefficient of variation, except for the LLOQ which should not exceed 20% of the coefficient of variation. The intra- and inter-day precision of the method was determined from the analysis of standards at three different concentrations at 10, 100 and 500 μM with triplicate analyses. Intra-day deviation was less than 3.5% for procaine and less than 2% for PABA in human liver tissue extract and less than 2% for both procaine and PABA in rat liver tissue extract; and, inter-day deviation was less than 6% for procaine and less than 1% for PABA in human liver tissue extract and less than 1% for both procaine and PABA in rat liver tissue extract. All values of precision were found within recommended limits and the results are summarized in Table 1.

3.6. Recovery

The recovery or extraction efficiency can be determined by comparing the response from a sample extracted from the matrix relative to a reference standard for the same initial concentration. ICH guidelines recommend that it should be determined at least three concentrations by comparing the results of extracted samples against reference standards that represent 100% recovery. Guidelines also indicate that while the recovery of the analyte does not need to be 100%, it must be consistent, precise, reproducible and quantitative. In this assay method, recoveries were calculated by two different methods using three different concentrations of 10, 100 and 500 μM in triplicate. Peak areas obtained from tissue extract samples were compared with those obtained by direct injection of standards at the same concentrations. A similar range of recoveries of procaine and PABA were found in both methods from human and rat liver tissue extracts. The recoveries of procaine and PABA were found to be greater than 80% from both human and rat liver tissue extract. The overall mean recoveries from both methods at different concentrations of procaine and PABA are given in

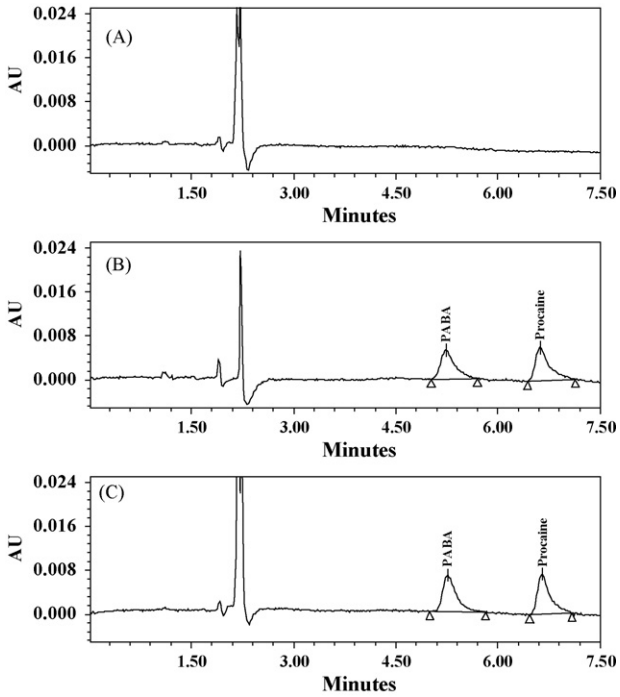

Fig. 5. Representative HPLC chromatograms obtained from the analysis of procaine and PABA in human liver tissue extract. (A) Blank human liver tissue extract; (B) 10 μM combined standard solution of procaine and PABA in buffer solution; and (C) 10 μM combined standard solution of procaine and PABA spiked in human liver tissue extract.

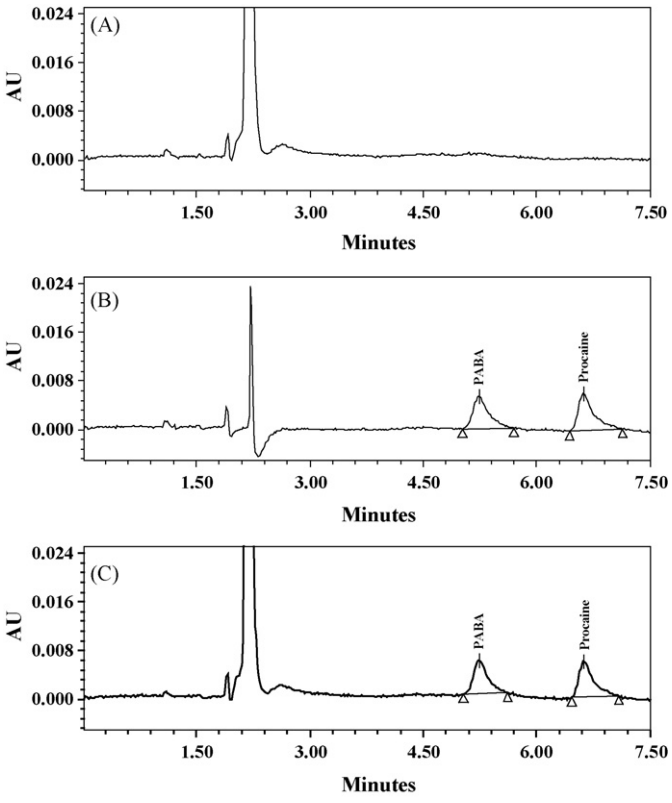

Fig. 6. Representative HPLC chromatograms obtained from the analysis of procaine and PABA in rat liver tissue extract. (A) Blank rat liver tissue extract; (B) 10 μM combined standard solution of procaine and PABA in buffer solution; and (C) 10 μM combined standard solution of procaine and PABA spiked in rat liver tissue extract.

Table 2. Figs. 5 and 6 show a representative chromatogram of blank tissue samples after extraction (Figs. 5A and 6A), combined standards of procaine and PABA but without tissue extract (Figs. 5B and 6B), and combined standard of procaine and PABA spiked in tissue extracts after extraction (Figs. 5C and 6C). It is also important to note that the retention times of procaine and PABA within each of the tissue extracts were exactly the same as those obtained from standards in non-tissue extract samples.

4. Conclusion

A sensitive and rapid HPLC assay method has been developed for the simultaneous determination of procaine and PABA. The method has been validated according to ICH guidelines in terms of selectivity, linearity, lower limit of detection, lower limit of quantitation, precision, accuracy and recovery from human and rat liver tissue extracts. The assay provides a linear response across a wide range of concentrations. The assay is fast, accurate and precise for the simultaneous quantitation of procaine and PABA in both human and rat liver tissue extracts. A simple and rapid protein precipitation method was employed to clean-up the tissue extract samples and it provided excellent recoveries of procaine and PABA. Because the determination is simultaneous for procaine and PABA, this method should be helpful for further pharmacokinetic research in clinical laboratory settings and during therapeutic drug monitoring as well as for *in vitro* enzyme assays where procaine and PABA need to be monitored simultaneously. Like the LC–MS/MS assay, it can also be used in quality control laboratories to determine PABA as an impurity in drugs containing procaine and procaine raw materials. Preliminary findings from studies involving the simultaneous determination of procaine and PABA from biological media such as mouse plasma and urine also suggest that the method should be readily applicable to these types of matrices when preceded by routine sample preparation protocols.

Acknowledgment

The authors would like to thank Pfizer Inc. for funding this research project.

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