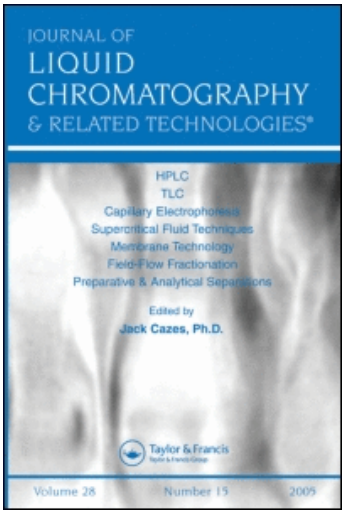

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AN HPLC METHOD FOR DETERMINATION OF ATROPINE IN HUMAN PLASMA

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ABSTRACT

A development of a high performance liquid chromatographic method for the determination of atropine in human plasma is presented. Atropine is extracted from plasma basified with 0.1N sodium hydroxide using chloroform, subsequently subjected to base hydrolysis, followed by derivatization of the generated tropic acid with 4-bromomethyl-7-methoxycoumarin (Br-Mmc). The derivative produced has a strong blue fluorescence at excitation wavelength of 328 nm and emission cutoff filter of 389 nm. *dl*-Mandelic acid as internal standard (I.S.) was added after hydrolysis. The chromatographic separation was achieved on a reversed phase ODS column with a mobile phase of 33% acetonitrile in 0.01M ammonium phosphate buffer (pH 5). The minimum quantitative limit was 125 ng/ml of plasma.

INTRODUCTION

Atropine, alpha-(hydroxymethyl)benzeneacetic acid 8-methyl-8-azabicyclo[3.2.1]Oct-3-yl ester(1), is a classical antimuscarinic agent. It acts as a competitive antagonist at cholinergic nerve ending to block the effects of acetylcholine or muscarinic agonists and decrease the activity of smooth muscle and exocrine glands(2).

Despite the long history and well known therapeutic action of atropine, relatively little is known of the relationship between the doses, resulting plasma concentrations and clinical effects. Methods reported for determination of atropine in biological fluids include phosphorimetry(3), gas chromatography-mass spectrometry (GC-MS)(4), and radio-immunoassay(5,6,7). These methods either lack sensitivity or deal with specific manipulation or radioactive materials. The GC-MS method is based on measuring free tropine base, therefore the volatilization of free tropine would be a problem.

Atropine has natural fluorescence which is too weak to be used for the determination of plasma levels. Therefore, derivatization before chromatography was considered in order to increase the sensitivity. 4-Bromomethyl-7-methoxycoumarin as a fluorescence labelling reagent reacts with fatty acids and other organic acids(8,9,10,11). Atropine can easily undergo hydrolysis under basic conditions(12) to tropic acid and tropine base. Tropic acid reacts with Br-Mmc in the presence of potassium compounds and a crown ether catalyst in aprotic solution. The derivative produced has strong fluorescence (13).

This paper describes atropine determination in human plasma by extraction, hydrolysis, tropic acid derivatization, and reversed phase HPLC on ODS column using fluorescence detection. The method is specific, has sufficient sensitivity, and is applicable for monitoring plasma levels.

MATERIALS AND METHODS

Instrumentation

A hewlett Parkard HPLC Model 1090 equipped with an automatic injector, a Dupont 5 micron Octadecyl-silane (ODS) column (15cm x

4.6mm), and a Schoeffl FS 970 spectrofluoro detector at excitation wavelength of 328 nm and emission cutoff filter of 389 nm. The column temperature was maintained at 40°C. The degassed mobile phase was pumped at 2.0 ml/min. The signal was recorded using 3392A HP integrator at a chart speed of 0.2 cm/min.

Chemicals and Standards

Atropine (free base), tropic acid, 4-bromomethyl-7-methoxy-coumarin, and 18-crown-6 were obtained from Sigma Chemical Company. Ammonium phosphate and potassium bicarbonate (anhydrous) are AR grade. Methylene chloride and acetonitrile are HPLC grade. *dl*-Mandelic acid (I.S.) was obtained from Eastman Kodak Co.

Stock Solutions

A 100.0 mcg/ml stock solution of atropine was prepared in 0.1N hydrochloride acid. A 100.0 mcg/ml stock solution of dl-mandelic acid, and a 150.0 mcg/ml stock solution of 18-crown-6 were prepared in acetonitrile. A 250.0 mcg/ml stock solution of 4-bromomethyl-7-methoxycoumarin was prepared in acetonitrile monthly and protected form light.

Working Solutions

250.0, 500.0, 750.0, 1000.0, 1500.0, 2000.0, and 3000.0 ng/ml working solutions were prepared by diluting atropine stock solution with 0.1N HCl. A 10.0 mcg/ml mandelic acid working solution, a 50.0 mcg/ml Br-Mmc working solution, and a 15.0 mcg/ml 18-crown-6 working solution were prepared by diluting their stock solutions with acetonitrile.

Mobile Phase

0.01M Ammonium phosphate solution was prepared in deionized distilled water and the pH was adjusted to 5.0. The mobile phase

consisted of 33% acetonitrile and 67% buffer (v/v). The effects of changes in pH of the buffer, buffer molarity, and percent acetonitrile on the capacity factor were studied.

Preparation of Plasma Standard

To 2.0 ml of plasma in a 60-ml separation funnel fitted with glass stopper, aliquots of atropine working solution were added to simulate drug concentrations (125.0 - 1500.0 ng/ml of plasma). The spiked plasma was then basified with 0.1 ml of 1N NaOH and extracted with 10.0 ml chloroform by shaking for 30 seconds. An aliquot of 9 ml of organic phase was carefully transferred to centrifuge tubes. The atropine was re-extracted into 1ml of 0.1N HCl. An aliquot of 0.9 ml of aqueous phase was transferred into clean tubes each containing 0.1 ml of 5N NaOH. Atropine hydrolysis was carried out for three hours at 38^0C. After acidification with 5N HCl, the liberated tropic acid was extracted into 8.0 ml methylene chloride by vortexing for two minutes followed by centrifugation. The organic phase was evaporated to dryness under a gentle stream of nitrogen at 40^0-45^0C. To each tube containing tropic acid residue, 3 mg of solid potassium bicarbonate, 0.1ml of internal standard working solution, 0.3ml of Br-Mmc working solution, and 0.1ml of crown ehter working solutions were added. The tubes were capped, vortexed for 15 seconds, and then kept at 70^0C for 45 min. The derivatized solution was transferred to a glass HPLC vial, and a 25 mcl aliquot was injected onto the column.

Quantitation

By monitoring the fluorescence, the peak heights were determined, and the ratios of tropic acid and internal standard derivatives were calculated and plotted against atropine concentration in nanograms per milliliter of plasma.

Recovery

Plasma samples containing a known concentration of atropine were carried through the extraction and hydrolysis procedures described. Equivalent amounts of tropic acid were placed into other tubes. The content of all the tubes were derivatized simultaneously and injected onto the column. The ratios of tropic acid derivative to internal standard derivative from plasma samples were compared with the ratios of derivatives without extraction and hydrolysis.

RESULTS AND DISCUSSION

Atropine is easily degraded to tropic acid and tropine under basic conditions. 4-Bromomethyl-7-methoxycoumarin can react with liberated tropic acid to produce a derivative with strong blue fluorescence. The choice of chromatographic conditions was based on resolution, minimum interferences, and reasonable retention times. Sufficient sensitivity for the determination of tropic acid derivative was achieved by setting the fluorescence detector at excitation wavelength of 328 nm and emission cutoff filter of 389 nm. The sensetivity limit of the assay was 1 ng tropic acid on column and 108 ng atropine/ml of plasma.

To obtain good chromatographic separation of tropic acid and internal standard derivatives, the pH, molarity, and percentage of organic modifier in the mobile phase were varied independently to find their effects on the capacity factor. The changes of pH and molarity in mobile phase produced slight effects (Fig 1 and 2), while the changes of percentage of acetonitrile affected the capacity factor the most (Fig 3). The mobile phase which provided the best resolution and reasonable retention times for the separation of tropic acid and internal standard derivatives on ODS

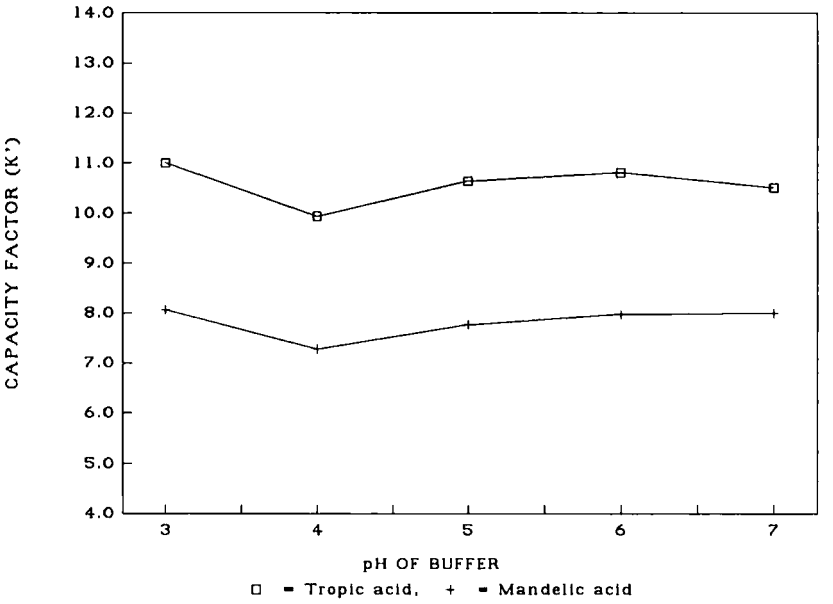

FIGURE 1. Effect of the pH of the buffer (0.01M ammonium phosphate) in mobile phase on capacity factor

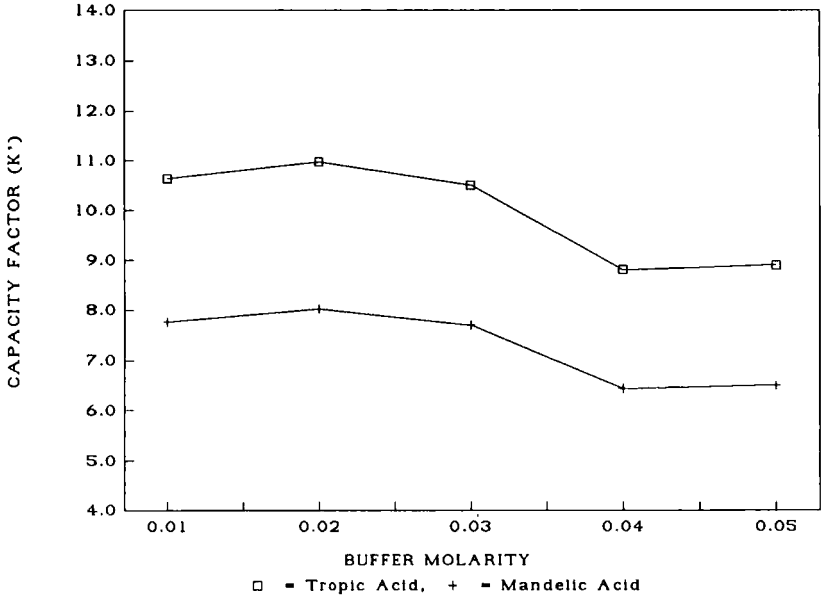

FIGURE 2. Effect of buffer molarity (pH5 ammonium phosphate) in mobile phase on capacity factor

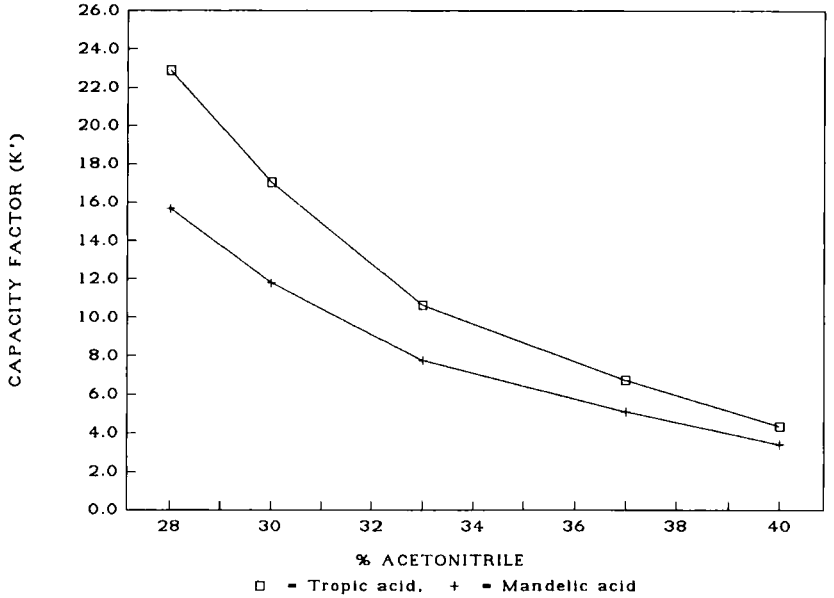

FIGURE 3. Effect of percent organic modifier on capacity factor (buffer: 0.01M ammonium phosphate pH 5)

column consisted of 33% acetonitrile and 67% 0.01M ammonium phosphate buffer (pH5) at 2.0 ml/min flow rate.

dl-Mandelic acid was chosen as the internal standard because it can react with 4-bromomethyl-7-methoxycoumarin under the same conditions as tropic acid and its derivative can be detected at the same wavelength.

Atropine can be extracted from basified plasma (pH>9) using chloroform in a single extraction step. Complete hydrolysis of atropine was carried out under alkaline conditions for three hours at 38^0C(Fig 4). Following acidification, the generated tropic acid can be extracted into Methylene chloride in a single step. The choices of extraction and hydrolysis conditions were based on obtaining a high recovery with minimum interferences. The

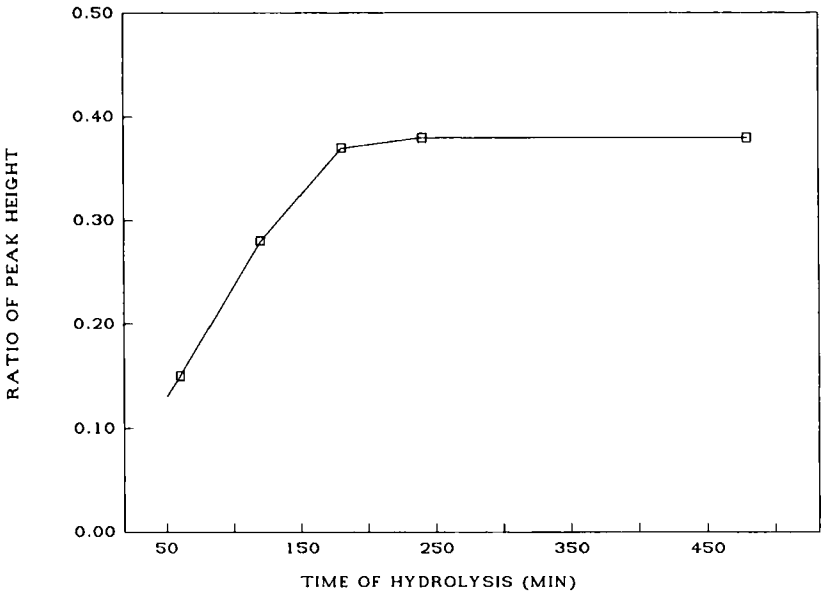

FIGURE 4. Effect of time on hydrolysis of atropine under basic conditions

extraction of mandelic acid failed under the extraction conditions of tropic acid. The possibility of extracting any of the metabolites of atropine and interferences from other drugs were eliminated by basic extraction of atropine from plasma and subsequent extraction of tropic acid after hydrolysis.

The influence of all derivatizatin conditions was studied. These conditions include the choices of solvent and reactants, the amounts of Br-Mmc, potassium compounds, and catalyst, derivatization temperature, and reaction time. Acetonitrile was chosen in the study as the solvent for both tropic acid and Br-Mmc as it gave less interferences on column than other aprotic solvents. 18-Crown-6 as phase transfer catalyst complexes potassium ion within the cavity and creates unhided anion, therefore

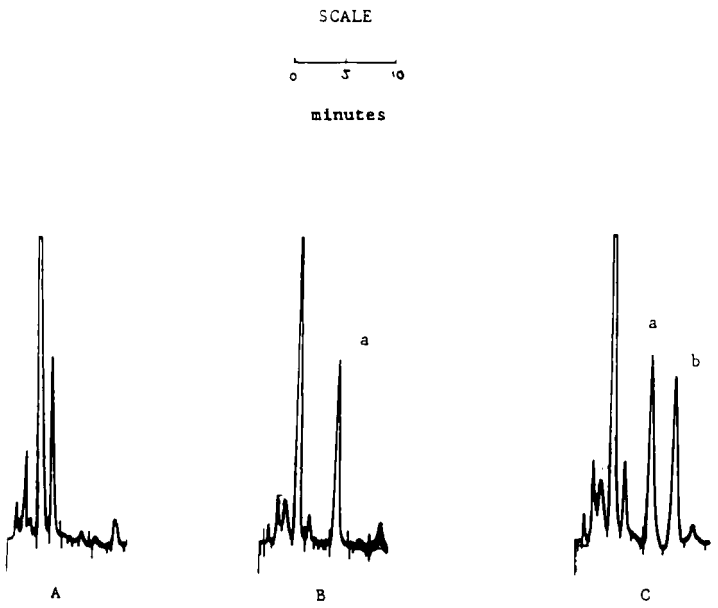

FIGURE 5. Typical chromatograms of atropine plasma assay. key: a, internal standard derivative; b, tropic acid derivative; A, blank plasma; B, plasma extract and internal standard derivative; C, extract containing 750.0 ng atropine/ml of plasma

TABLE 1

Linearity and Precision of the Atropine Plasma Assay

Theoretical Conc. (ng/ml plasma)	Found Conc.* (ng/ml plasma)	CV%
125.0	125.4 ± 14.7	11.7
250.0	268.4 ± 47.9	17.8
375.0	393.5 ± 38.1	9.7
500.0	493.9 ± 49.4	10.0
750.0	717.2 ± 99.6	13.9
1000.0	912.4 ± 93.2	10.2
1500.0	1559.2 ± 87.5	5.6

* Mean Concentration ± standard deviation
Correlation coefficient = 0.9863, n = 38
Regression Model: Y = aX + b, slope(a) = 1.2270, Intercept(b) = -0.00318

TABLE 2.

Recovery of Atropine Plasma Assay

Atropine Conc. (ng/ml plasma)	Recovery% (Mean)	± Standard Deviation	Number of Replicates
125.0	62.41	7.37	4
250.0	65.69	5.09	4
500.0	63.97	2.07	4
750.0	62.32	7.17	4
1000.0	64.34	8.45	4

accelerating the derivatization. The ratio of 1:10 of 18-crown-6 to Br-Mmc was used for tropic acid derivatization. Because of the presence of unknown amounts of atropine in plasma, excess reagent was required for derivatization. However, broad peak and loss of resolution was noticed when large amounts of Br-Mmc were used probably due to interaction. Based on the separation and sensitivity, 15.0 mcg of Br-Mmc was used in the study. Either $KHCO_3$ or K_2CO_3 was suitable as condensating agent in the reaction.

Although the derivatization was not complete under the described conditions, a linear relationship exists between the yield of tropic acid derivative and the amount of tropic acid present in aprotic solution, and consequently the amount of atropine present in plasma. A standard curve was constructed, which is a plot of the ratio of peak height of tropic acid derivative to peak height of mandelic acid derivative versus the amount of atropine in plasma. Reproducibility and linearity were indicated by data analysis using linear regression in the range of 125.0 - 1500.0 ng/ml of plasma (Table 1). Typical chromatograms are shown in Figure 5. The percentage of recovery from plasma samples in the range of 125.0 - 1000.0 ng/ml of plasma for the method described are shown in Table 2.

CONCLUSION

The described method required single extraction, base hydrolysis, and derivatization prior to injection on the chromatographic column. HPLC analysis using ODS column under isocratic elution conditions gave good separation of tropic acid and internal standard derivatives. Quantitation was successful by monitoring the effluent using fluorescence detector. The method needs less manipulation and could be recommended for clinical analysis of plasma levels.

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