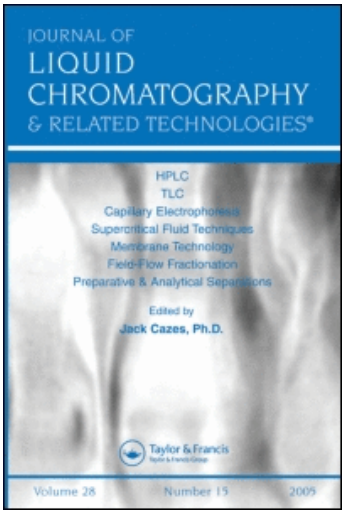

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HPLC Determination of the Cardiotonics, Dopamine and 4-Methyl-2-aminopyridine, in Serum Following Fluorescamine Derivatization

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Abstract: A method for the simultaneous determination of the cardiotonics dopamine and 4-methyl-2-aminopyridine in serum, previously derivatized with fluorescamine, by high performance liquid chromatography coupled with a fluorescence detector is presented. Chromatographic and detection conditions were optimized; a LiChrospher 100 RP-18 reversed phase column (250×4 mm; 10 μm) and the mobile phase of methanol:water, in gradient mode, were found as the most suitable. The derivatives were eluted in 10 min with good reproducibility at a flow rate of 0.9 mL/min, being 20 μL the volume of sample injected. The detection was done at 490 nm using borax buffer solution (pH 8) for both cardiotonics. Relative standard deviations ($n = 10$) of 6.7% and 5.9% (concentration) and 2.4% and 3.05% (retention time), were obtained for dopamine and 4-methyl-2-aminopyridine, respectively. Recoveries of the spiked female rabbit serum ranged from 90% to 110%, and detection limits of 122 ng/mL for dopamine and 108 ng/mL for 4-methyl-2-aminopyridine, were obtained.

Keywords: Cardiotonics, Dopamine, Fluorescamine, HPLC, 4-Methyl-2-aminopyridine

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INTRODUCTION

Dopamine hydrochloride (4-(2-aminoethyl)-1,2 benzenediol or 4-(2-aminoethyl) pyrocatechol) is a drug indicated for its adrenergic effects, chemically it is known as dopamine. The drug is official in the U. S. P., acts on the sympathetic nervous system, producing effects such as increased heart rate and blood pressure, however, because dopamine cannot cross the blood brain barrier, dopamine given as a drug does not directly affect the central nervous system.[1] In this manual is described a non aqueous potentiometric titration for the assay of raw material and an HPLC technique for the injections. Different spectrofluorimetric methods,[2–5] ion exchange column chromatographic technique,[6] reverse phase ion pair liquid chromatographic technique,[7–9] gas chromatography,[10–12] spectrophotometric methods[13] with stopped flow[14] or FIA system,[15] been described by means of HPLC.[16–19]

4-Methyl-2-aminopyridine (4M2AP) exhibits positive cardiotonic activities such as dopamine, which belong to a family of isomers being the most effectively marked, increase the contractility of cardiac muscle by causing the release of catecholamines from sympathetic nerve ending or other storage sites.[20–22] No analytical methods have been found in the literature in reference to this cardiotonic.

Udenfriend[23] introduces fluorescamine as a labeling reagent to determine primary amines; this is superior to dansyl chloride because both the reagent and its hydrolysis products are non-fluorescent and permit homogeneous fluorogenic labelling. Such an approach has proved its usefulness in numerous analytical applications.[24–28] Considerable efforts have been made to develop highly selective and sensitive derivatization reagents for use in liquid chromatography with fluorescence detection. Several excellent reagents are currently available for the main functional groups, e.g., hydroxy, amino, thiol, carbonyl, and carboxyl groups. The most useful application of fluorescamine in high performance liquid chromatography derivatization is its ability to hydrolyze the reagent excess after the derivatization process, releasing non-fluorescent products. Thus, the fluorimetric detection is blind to the fluorescamine excess. This is especially useful in pre-column derivatization HPLC methods.

In this work, the optimum experimental conditions for the spectrofluorimetric determination of dopamine and 4M2AP based on the fluorophore generation by derivatization with fluorescamine (FC) were investigated obtaining better R.S.D. and better recovery. Reversed phase LC determination of dopamine and 4M2AP with precolumn derivatization was also carried out and applied to spiked female rabbit serum.

EXPERIMENTAL

Chemicals

Dopamine (purity 99.9%) and 4M2AP (98%) were purchased from Sigma (St. Louis, USA). Methanol and acetone were of LiChrosolv gradient grade (Merck). Borax were obtained from Panreac (Barcelona, Spain), sodium hydrogen carbonate from Merck (Darmstadt, Germany), potassium phosphate dibasic from Codex (Milano, Italy), potassium phosphate monobasic from Probus (Barcelona, Spain), HCl from Merck and fluorescamine (FC) (98%) from Aldrich (Milwaukee, USA). The solvents were previously sonicated for 30 min and filtered through 0.2 μm nylon membrane filters.

Solutions

Stock standard solutions of dopamine (6.5×10^{-3} M) and 4M2AP (9.2×10^{-3} M) were prepared by dissolving the compounds in water and stored at 4°C. Working standard solutions were prepared by dilution with water. Fluorescamine (3.59×10^{-3} M) was dissolved in acetone. Solutions of sodium hydrogen carbonate (0.1 M), borax (0.05 M) and phosphate buffer (0.1 M) were prepared in doubly deionised water. The solutions were filtered through 0.2 μm nylon membrane filters.

Instrumentation

A Merck-Hitachi (Darmstadt, Germany) liquid chromatograph was used which consisted of an L-6200 pump, an AS-4000 autosampler, a F-8000 fluorescence detector, and a D-6000 interface. Integration was carried out with a PC/AT computer and the instrumental parameters were controlled by Hitachi-Merck HM software. All the derivatization steps are performed automatically by the AS-4000 auto sampler.

Fluorescence measurements were made with a Perkin Elmer LS50 Spectrofluorimeter (Beaconsfield, U.K.) and equipped with a Xenon discharge lamp and two monochromators. Fluorescence Data Manager (FLDM) Software and a RS232C interface were used to send information to an external computer.

Derivatization

Aliquots of 100 μL of aqueous standard solutions of dopamine (16–48 ng) and 4M2AP (16–48 ng) were introduced in a 1.5 mL flask, and then

Table 1. Mobile phase composition

Time (min)	Methanol (%)	Water (%)	Flow (mL/min)
0.0	50	50	0.9
6.0	30	70	0.9
10	50	50	0.9

135 μL of a 3.6×10^{-3} M acetone solution of fluorescamine and 300 μL of pH 8 buffer solution were added. The mixture was diluted in water up to 1.5 mL. After each addition of the reagent the mixture was agitated. A volume of 20 μL of this solution was injected into the chromatograph and analyzed. All these operations were automatically performed by the auto sampler.[29]

LC Operating Conditions

The cardiotonics samples were analyzed using a LiChrospher 100 RP-18 reversed phase column (25 cm × 4 mm I.D.; 10 μm particle size) from Merck. The injection volume was 20 μL for the standard aqueous solutions and samples. The mobile phase composition is detailed (Table 1), and the flow rate was 0.9 mL/min. The peak-area response was measured at the retention times of dopamine (3.79 min) and 4M2AP (7.10 min), calibration graphs were constructed using the responses.

Recovery Test

Female rabbit serum was used to prepare three samples with known levels. Dopamine and 4M2AP (20 ng of dopamine + 20 ng of 4M2AP, 30 ng of dopamine + 30 ng of 4M2AP, and 40 ng of dopamine and 40 ng of 4M2AP), and 1 mL methanol are added to 0.5 mL of serum. The mixtures were vortexed for 15 s and centrifuged for 10 min. The supernatant was saved for analysis;[30] 0.5 mL of the solutions was diluted with doubly deionised water to a final volume of 10 mL. These solutions were used for analysis.

RESULTS AND DISCUSSION

Dopamine and 4M2AP, its molecular structures are shown in Figure 1, react with FC to form two fluorophors with similar spectral profiles. The excitation and emission spectra of the FC derivatives of dopamine and 4M2AP under the final experimental conditions are shown in

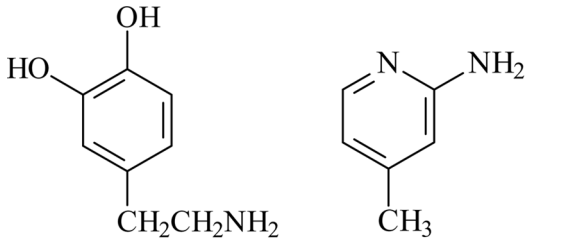

Figure 1. Molecular structures of the cardiotonics studied.

Figure 2. As expected, the spectral parameters for both compounds are similar. Each compound is characterized by its well resolved excitation maximum (390 nm) and its single emission peak (480 nm).

The operating parameters for the individual compounds were optimized to give an analytical method for each. Consequently, after fixing the individual optimum conditions in order to determine isolated dopamine and 4M2AP, a new set of conditions was selected to obtain good emission signals for each compound before carrying out the analysis of mixtures of dopamine and 4M2AP by high performance liquid chromatography.

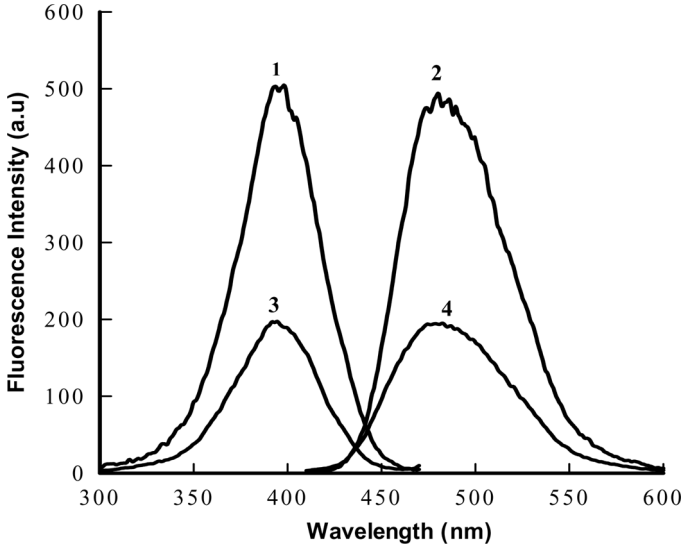

Figure 2. Excitation and emission spectra of FC derivatives (1, 2) 4M2AP (1.44×10^{-4} M) and (3, 4) dopamine (1.8×10^{-4} M) with [FC] = 3.24×10^{-4} M (pH 8).

As a fluorigenic reagent for amino compounds, FC lacks selectivity, which emphasizes the need for more detailed information about the effect of the main reaction conditions, so that the fluorescence yield might be improved to permit the selective analysis of mixtures of fluorophors with FC.

Influence of Reaction Variables

The effect of pH on fluorescence intensity was explored by carrying out several assays of solutions, in 5 mL volumetric flasks containing 2 μg/mL of dopamine (or 2 μg/mL of 4M2AP) and 500 mL of different buffer solutions that covered the pH range 5–10, together with 250 μL of FC standard solution and leveling up to volume with water.[24] The maximum fluorescence of the dopamine fluorophore occurred at pH 7 and that of 4M2AP at pH 8 (Figure 3). Dopamine fluorescence profile shows two other minor maxima at pH 8 and pH 9.5. In both instances, the narrow range in which the fluorescence intensity was maximal suggests careful control of the pH solution is required. On the other hand, to obtain good yields in the labeling reactions of mixtures of both compounds, the pH setting must be a compromise and in this work, pH 8 appeared to be the optimum.

FC reacts very quickly with primary amines ($t_{1/2} = 100$–500 ms), but frequently a great excess of FC is needed to produce good thermodynamic equilibrium conditions.[31] The effect of FC concentration on

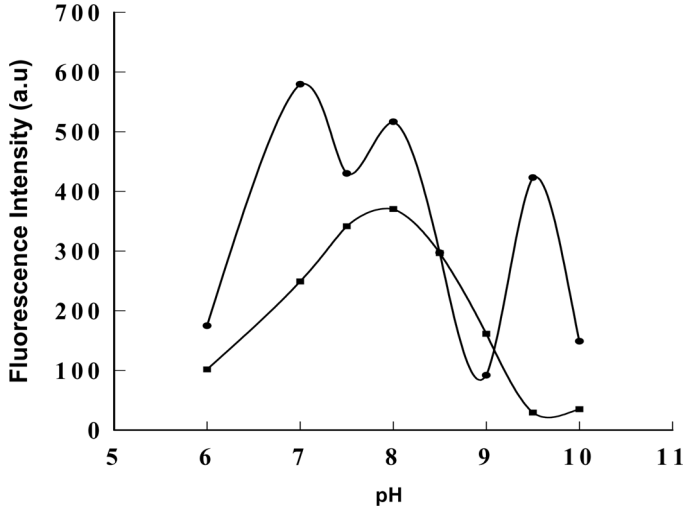

Figure 3. Influence of pH on the relative fluorescence intensity of (•) Dopamine and (■) 4M2AP. Dopamine (1.8×10^{-4} M) and 4M2AP (1.44×10^{-4} M); [FC] = 3.24×10^{-4} M.

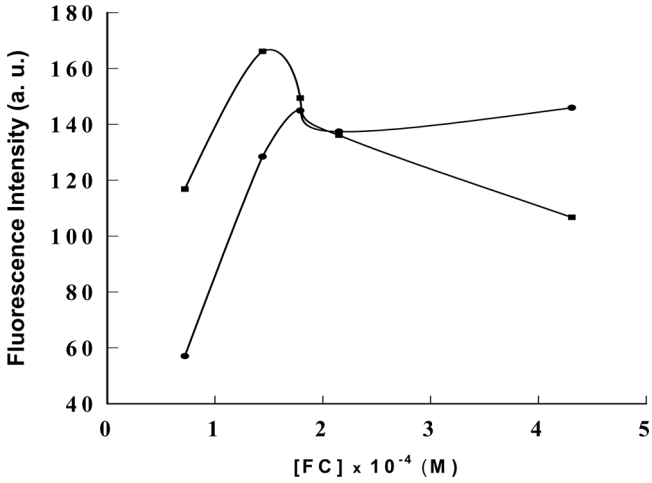

Figure 4. Influence of the [FC] on the relative fluorescence intensity of (•) dopamine (pH 8) and (■) 4M2AP (pH = 8).

fluorophore formation was observed by measuring the fluorescence intensity for each compound at different FC concentrations, while all other experimental conditions were kept constant at the optimum values. Figure 4 shows that the maximum response was obtained when the FC concentration was 1.80×10^{-4} M in the reaction with dopamine and 1.44×10^{-4} M in the reaction with 2-amino-4-picoline. For the simultaneous determination of the two compounds a [FC] = 3.24×10^{-4} M was selected.

The calibration graphs are linear between 16–48 ng for dopamine and 4M2AP. The lower limit of the linear dynamic range is determined by the quantification (C_Q) limit. Typical relative standard deviations (R.D.S.) are between 5.9−6.7%. A linear regression analysis gave the following fits:

$$\text{Dopamine: Area} = 55298.68\ [\text{Dopamine}] - 117242.59;\quad r = 0.990\ (n = 6)$$

$$\text{4M2AP: Area} = 89335.02\ [\text{4M2AP}] + 233523.91;\ r = 0.990\ (n = 7)$$

where [Dopamine] and [4M2AP] is the injected concentrations in ng/mL, respectively.

Determination of Dopamine and 4-Methyl-2-aminopyridine in Serum

Three different plasma samples were spiked, prior to the extraction, with a mixture of the dopamine and 4M2AP prepared in doubly deionised water after checking for the absence of the cardiotonic under study. After extraction, the samples were subjected to the LC procedure. The chroma-

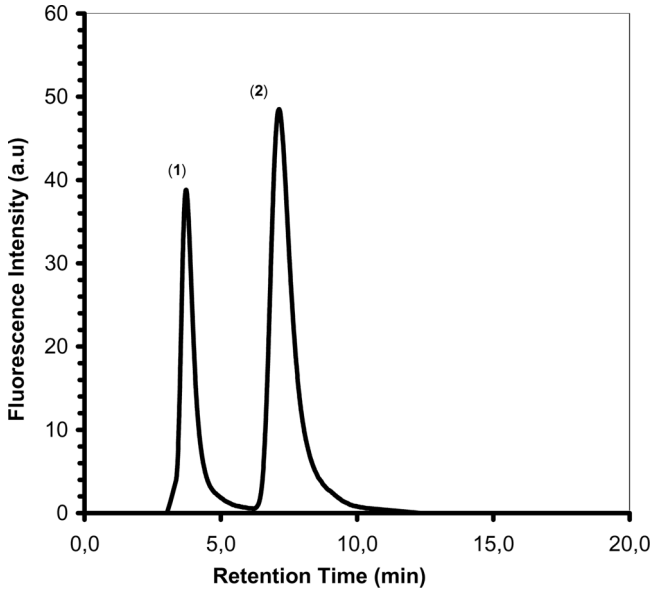

Figure 5. Chromatogram of spiked serum. (1) 1.2 μg/mL dopamine and (2) 1.2 μg/mL 4M2AP.

togram of plasma extracts is reported in Figure 5. Table 2 presents the results obtained in the determination of dopamine and 4M2AP in serum by applying the LC method. As can be seen, recoveries are within 90–110%. The results obtained demonstrate the effectiveness of the proposed methods in determining the analytes assayed in this type of samples.

Table 2. Recovery of cardiotonic from spiked serum

Compound	Taken/ Found(ng/mL)	R.S.D.[a] (%)	D_L[b] (ng/mL)	C_Q[c] (ng/mL)	Recovery (%)
Dopamine			122	405	
	1.000/1.075	1.40			107.50
	1.500/1.536	3.30			102.40
	2.000/2.090	3.50			104.50
2-Amino-4-picoline			108	360	
	1.000/1.040	7.60			104.00
	1.500/1.410	4.40			94.00
	2.000/2.102	1.75			105.10

[a] n = 3.
[b] Detection limit for a signal-to-noise ratio = 3.
[c] Quantification limit for signal-to-noise ratio = 10.

CONCLUSION

Fluorescamine derivatization of two cardiotonics, primary amines (aliphatic and aromatic), has been performed with the goal of developing a chromatographic method with fluorescent detection. Good results were obtained with speed, precision, accuracy, and recovery values over synthetic and spiked rabbit serum samples.

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