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Determination of atropine in pharmaceutical preparations by liquid chromatography with fluorescence detection

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Abstract

A method is described for the determination of atropine in pharmaceutical preparations by precolumn derivatization and high-performance liquid chromatography (HPLC). Atropine is derivatized by reacting with 1-anthroylnitrile (1-AN) in acetone. The 1-AN derivative of atropine is then used for quantitative HPLC analysis on a reversed-phase column with fluorescence detection. The calibration graph was linear over a sample concentration range from 0.05 to 1.0 μg/ml; the limit of detection was reached at a sample concentration of 0.01 μg/ml. The proposed method was satisfactorily applied to the determination of atropine in pharmaceutical preparations. © 1997 Elsevier Science B.V.

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1. Introduction

Atropine, an alkaloid obtained as the main component from *Atropa belladonna* L. and in much smaller quantities from *Datura stramonium* L. and other Solanaceae is composed of approximately equal amounts of *d*- and *l*-hyoscyamine [1]. Atropine and related alkaloids have been used for the treatment of gastrointestinal diseases, cardiopathy and Parkinson's disease. The dosage levels are generally very low because belladonna alkaloids are very potent and very toxic. For example, the Japanese Pharmacopoeia describes the total alkaloid content, calculated as the free base, as being 0.90–1.09% of the scopolia extract [2]. Sometimes, the untreated belladonna extract is incorporated into commercial tablets; in such instances, the US Pharmacopoeia (USP) requires that the total alkaloid content, calculated as the free base, is 1.15–1.35% of the belladonna extract [3]. The analysis of belladonna alkaloids is a subject of interest because of the extensive use of atropine and scopolamine in pharmaceutical preparations.

Some of the published methods for the determination of belladonna alkaloids involve the formation of colored [4,5] or fluorescent derivatives [6,7]. However, these methods are not specific for belladonna alkaloids, because other compounds, especially organic bases, react in a similar manner. Gas chromatography (GC) is used to separate atropine from scopolamine and generally to quantify one or both alkaloids [8,9]. Quantitative thin-layer chromatography (TLC) [10,11] and, more recently, liquid chromatography (LC) with reversed-phase columns and ultraviolet (UV) absorption detection [12–15] have also been applied to the determination of

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atropine or scopolamine. As described, these methods appear to lack the sensitivity required for measuring atropine at low levels in pharmaceutical preparations, because atropine does not have an adequate UV chromophore. It must be measured at a low wavelength when direct UV detection is used. Therefore, other methods for the determination of atropine are required that are not only extremely sensitive but are also highly selective.

Recently, a new fluorescence labelling reagent that had a carbonyl nitrile group was developed for use with the high-performance liquid chromatographic (HPLC) analysis of hydroxy compounds [16–19]. This reagent is not only extremely fluorescent but is also reactive with hydroxy groups under mild conditions. We found that atropine was derivatized quantitatively into a fluorescent compound by 1-anthroylnitrile (1-AN).

In this paper, we describe the precolumn reaction and fluorimetric detection of atropine with 1-AN and its separation by reversed-phase HPLC. The proposed method was applied to the determination of small amounts of atropine in pharmaceutical preparations.

2. Experimental

2.1. Reagents and standards

dl-Atropine was obtained from Sigma (St. Louis, MO, USA). A stock standard solution was prepared by dissolving atropine in acetone to give a concentration of 100 μg/ml. Working standard solutions were prepared by diluting the stock standard solution with acetone. 1-AN (used as the reagent for fluorescence labelling) was purchased from Wako (Osaka, Japan). The fluorescent reagent was prepared using 2 mg of 1-AN per ml of acetone. Quinuclidine, HPLC-grade acetonitrile and acetone were obtained from Nacalai Tesque (Kyoto, Japan). The quinuclidine solution was prepared by dissolution in acetone to a final concentration of approximately 4 mg/ml. Acetonitrile and reagents and solvents were of analytical grade. Water was purified using a Milli-Q II water purification unit (Nihon Millipore, Tokyo, Japan).

2.2. Derivatization

A 1.0-ml volume of atropine solution (0.05–1.0 μg/ml) was placed in a 10-ml reaction vial and the solvent was evaporated to dryness at 40°C under reduced pressure in a rotary evaporator. To the residue, 0.5 ml of quinuclidine solution and 0.5 ml of an acetone solution containing 1-AN (2 mg/ml) were added, in a 10-ml volumetric flask, and the mixture was kept at 30°C for 10 min. After reacting, a 1.0-ml volume of a 2% phosphoric acid solution was added. Also, after cooling to room temperature, the mixture was made up to a volume of 10 ml with acetone. An aliquot (10 μl) of this solution was injected into the HPLC system. The reaction solution was then analyzed by HPLC under the conditions described.

A peak-area calibration graph for atropine was established over the sample concentration range of 0.05–1.0 μg/ml. All standard solutions were analyzed in triplicate, for construction of the calibration graph.

2.3. Chromatography

The HPLC apparatus consisted of a JASCO Model 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Rheodyne injector, Model 7125, equipped with a 10-μl loop (Rheodyne, Berkeley, CA, USA), a JASCO Model 860-CO column oven, a JASCO Model 821-FP fluorescence detector set at excitation and emission wavelengths of 255 and 474 nm, respectively, and a Chromatopac CR-3A digital integrator (Shimadzu, Kyoto, Japan). The HPLC column used was a Cosmocil 5C18-MS (Nacalai Tesque, Tokyo, Japan) (150×4.6 mm I.D.; 5 μm). The mobile phase was acetonitrile–0.02 *M* sodium dodecyl sulfate (adjusted to pH 3.5 with phosphoric acid) (60:40, v/v). Before use, it was filtered through a Millipore membrane filter (0.45 μm; Millipore, Bedford, MA, USA) followed by degassing using sonication under vacuum. The eluent was pumped at a flow-rate of 1.0 ml/min and with a column oven temperature of 40°C.

2.4. Sample preparation

In order to dissolve a sufficient quantity of the

atropine preparation, an amount equivalent to about 25 μg of atropine and two additional 10 ml volumes of 1.3 *M* ammonium chloride buffer (adjusted to pH 10) were collected in a 50-ml separator. If the dosage form was tablet composites, a representative number of tablets (usually twenty) were accurately weighed and finely powdered. They were extracted with 40 ml of $CHCl_3$. The $CHCl_3$ layer was drawn off into a 50-ml volumetric flask using a filter. The filter was washed with $CHCl_3$ and the wash was collected in the flask. $CHCl_3$ was added to the required volume and the solution was mixed. The final atropine concentration was 0.5 μg/ml. A 1.0-ml volume of this solution was subjected to derivatization and then HPLC analysis was performed, as described above.

3. Results and discussion

Three fluorescence labelling reagents, each having a carbonyl nitrile group (pyrene-1-carbonyl cyanide, 9-anthronyl cyanide, 1-AN) were tested for the determination of atropine. The extent of each reaction product using these labelling reagents was measured by HPLC with fluorescence detection. The following relative fluorescence intensities (%) were obtained: 1-AN, 100; pyrene-1-carbonyl cyanide, 80 and 9-anthronyl cyanide, 10. Hence, the best sensitivity was obtained using 1-AN. The postulated reaction is shown in Fig. 1.

The optimum conditions for the fluorescent derivatization of atropine with 1-AN were determined.

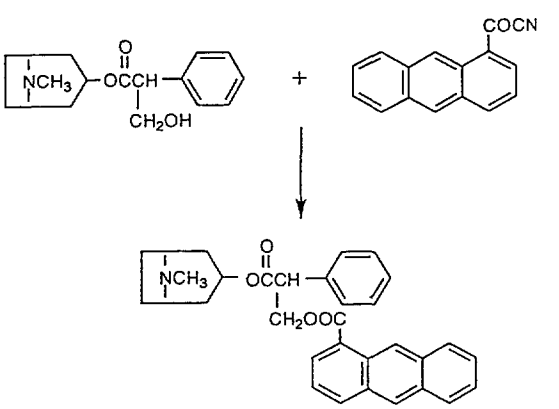

Fig. 1. Fluorescence derivatization of atropine with 1-AN.

First, seven organic solvents (acetone, acetonitrile, dimethyl sulfoxide, chloroform, hexane, tetrahydrofuran and N,N-dimethylformamide) were tested and the results obtained for each reaction system with each solvent were compared. The following relative fluorescence intensities (%) were obtained: acetone, 100; acetonitrile, 96; dimethyl sulfoxide, 56; chloroform, 0.6; tetrahydrofuran, 0.3; N,N-dimethylformamide, 0.1 and hexane, 0.01. Acetone was selected as the solvent of choice, because it gave the highest fluorescence intensity.

The effect of the concentration of 1-AN was studied in the range of 0.01 to 1.5 mg per 0.5 μg of atropine. Constant peak areas were obtained above 0.75 mg of 1-AN. A 1-mg amount of 1-AN was chosen for subsequent experiments. The 1-AN was stable for more than one month at ordinary temperatures and when shielded from light.

Fig. 2 shows the effects of reaction temperature and reaction time on the production of atropine–1-AN derivatives. The derivatization reagent, 1-AN, tended to decompose at high temperatures and with long reaction times. The optimum reaction temperature was found to be 30°C and a reaction time of 10 min was optimal.

In this study, the atropine ester was separated by reversed-phase (C_{18}) HPLC with a mobile phase consisting of acetonitrile and a sodium dodecyl sulfate solution. The composition of the mobile phase had a strong influence on the separation of the

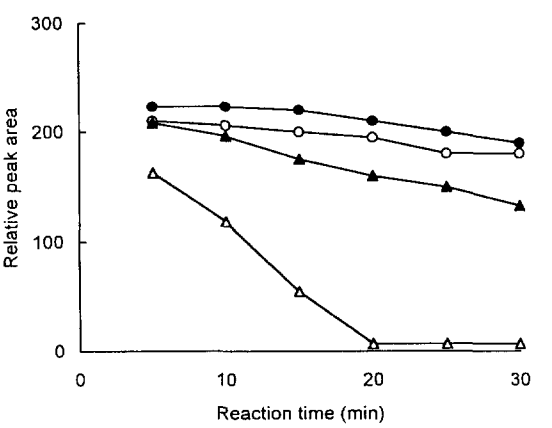

Fig. 2. Effect of reaction time and reaction temperature on the reaction yield of atropine–1-AN derivatives. (○) at 20°C, (●) at 30°C, (▲) at 50°C and (△) at 70°C.

atropine ester. The optimum mobile phase composition was chosen for each condition (percentage of water and salt concentration) to give the best separation of the atropine ester from impurities from the derivatization. The influence of acetonitrile concentration, sodium dodecyl sulfate concentration and the pH of the mobile phase on the chromatographic characteristics of the atropine ester was examined systematically.

The capacity factor increased with decreasing concentration of acetonitrile and was not sensitive in the pH range of 2.0–5.0. The relationship between the concentration of sodium dodecyl sulfate and the capacity factor was examined by increasing the concentration from 0.005 to 0.5 *M*. The capacity factor rapidly increased with increasing concentrations of sodium dodecyl sulfate, to about 0.2 *M*, however, thereafter, the capacity factor began to decrease with increasing concentrations of sodium dodecyl sulfate [20]. The optimum concentration of sodium dodecyl sulfate for separating the atropine ester was determined to be 0.02 *M*. From the results, the solvent system finally chosen was acetonitrile–0.02 *M* sodium dodecyl sulfate (adjusted to pH 3.5 with phosphoric acid; 60:40, v/v). Fig. 3 shows plots of the capacity factor of atropine–1-AN versus the acetonitrile concentration.

When the atropine ester was eluted in the flow-through cell of the detector, the flow-rate of the mobile phase was stopped and both the excitation and emission spectra were measured (Fig. 4). The maximum excitation and emission wavelengths were found to be 255 and 474 nm, respectively.

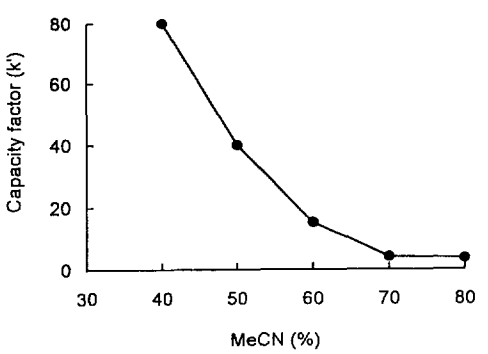

Fig. 3. Influence of acetonitrile content in the mobile phase on the capacity factor (k') of atropine–1-AN.

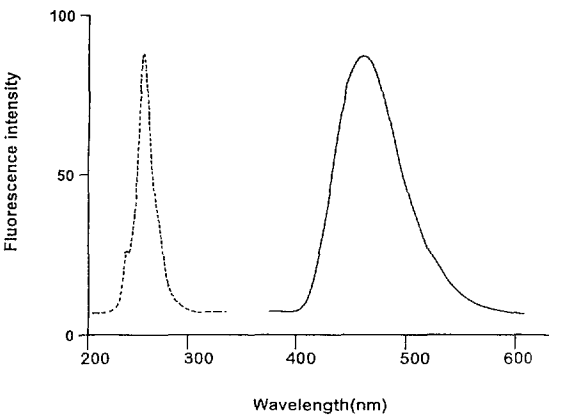

Fig. 4. Fluorescence excitation (dashed line) and emission (solid line) spectra of atropine–1-AN.

Fig. 5 shows the chromatograms of (A) a derivatized standard solution, (B) a reagent blank and (C) a derivatized commercial tablet sample. The atropine ester peak was clearly separated from impurities from the derivatization reaction.

The calibration graph of the atropine peak-area was found to be linear over the concentration range 0.05–1.0 μg/ml of seven sample solutions. The linear regression coefficient was 0.999 ($n=3$), the slope was 176.5 and the intercept on the ordinate was −4.8. The repeatability of this procedure was also adequate; the relative standard deviation (RSD) for 0.5 μg/ml of atropine was 2% ($n=5$). The detection limit of atropine under the selected conditions was about 0.01 μg/ml, with a 10 μl injection volume (signal-to-noise ratio, $S/N=3$).

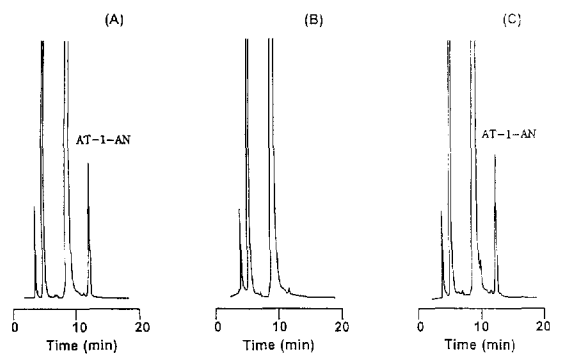

Fig. 5. Chromatograms of (A) a derivatized standard solution, (B) a reagent blank and (C) a derivatized commercial tablet sample. Peak: AT-1-AN=atropine–1-AN.

Table 1
Determination of atropine in pharmaceutical preparations

Preparations	Declared	Atropine	
		Calculated amount[a]	Amount found[b]
A. (Fine granule)	Scopolia extract, 11 mg/g	0.10–0.12 mg/g	0.10 mg/g
B. (Tablet)	Scopolia extract, 4.25 mg/tablet	0.04–0.05 mg/tablet	0.04 mg/tablet
C. (Powder)	Scopolia extract, 10 mg/g	0.09–0.11 mg/g	0.10 mg/g
D. (Granule)	Scopolia extract, 10 mg/g	0.09–0.11 mg/g	0.11 mg/g
E. (Capsule)	Belladonna extract, 6.5 mg/capsule	0.06–0.08 mg/capsule	0.06 mg/capsule
F. (Tablet)	Atropine sulfate, 0.5 mg/tablet	0.43 mg/tablet	0.43 mg/tablet
G. (Injection)	Atropine sulfate, 0.5 mg/ml	0.43 mg/ml	0.43 mg/ml

[a] Japan Pharmacopoeia 12th scopolia extract is listed as containing 0.90–1.09% atropine and belladonna extract is listed as containing 0.95–1.15% atropine.
[b] Each value is the mean of four determinations.

The proposed procedure was applied to the determination of atropine in pharmaceutical preparations. The results obtained from the analysis of pharmaceutical preparations of atropine are shown in Table 1 and a chromatogram of the 1-AN derivative of atropine from a commercial tablet sample is shown in Fig. 5C. Both the derivatization and chromatographic separation were not influenced by other compounds; for example, water-soluble vitamins (B_1 and B_2), caffeine, chlorpheniramine maleate, albumin tannate, lysozyme hydrochloride, crude drugs (geranium herb, fennel, amomum seed, clove, glycyrrhiza, cinnamon bark and swertia herb). These results indicate that, in each instance, the values are within acceptable limits. The minimum recovery was 94%. The relative standard deviation (RSD) for the repeatability was 4% ($n=5$). The chromatograms of all other samples also showed a sharp peak for atropine ester with no interference from other substances.

In conclusion, HPLC using 1-AN as a derivatization reagent was very useful in the determination of atropine, and offers a significant improvement in sensitivity over previously reported HPLC methods with UV absorbance detection. We believe that the availability of this new method, with its increased sensitivity and selectivity, will be very useful for the determination of atropine in pharmaceutical preparations.

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