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Determination of atropine in pharmaceutical dosage forms containing vegetal preparations, by high-performance liquid chromatography with U.V. and electrochemical detection

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Abstract: A method to test the uniformity of Belladonna powder in multicomponent pharmaceutical dosage forms, by the determination of atropine, is described. The procedure involves a liquid–liquid extraction, followed by liquid chromatographic separation of atropine, apoatropine and scopolamine and quantification of atropine with either ultraviolet or electrochemical detection. The method is shown to be selective, sensitive and offers good reproducibility. A detailed study of the electrochemical properties of atropine also is undertaken.

Keywords: *Atropine; pharmaceutical preparations; HPLC; U.V. detection; electrochemical detection.*

Introduction

Tropane alkaloids contained in the vegetal preparations of plants such as Atropa belladonna and Datura stramonium are widely used as antispasmodic, anticholinergic and sedative agents. Because of their potency and toxicity their dosage levels are generally very low. Small quantities of synthetic alkaloids or raw materials are usually combined with large amounts of other substances in pharmaceutical dosage forms. This creates a significant analytical challenge for which numerous quantitative methods have been developed including; titrimetry [1], spectrophotometry [2], potentiometry [3], radioimmunoassay [4], thin-layer chromatography [5, 6], gas–liquid chromatography [7, 8] and high-performance liquid chromatography (HPLC) [9–19]. Thus far none of those methods are totally satisfactory.

A chromatographic system for the separation of atropine, scopolamine (a related alkaloid) and apoatropine (a degradation product of atropine) has been reported previously and used [14] to test the uniformity of Belladonna powder in multicomponent tablets. In this application, atropine, the main alkaloid contained in the cited vegetal preparation, was used as a marker to compare its concentration in the raw material and in the pharmaceutical dosage forms.

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Experimental

Chemicals and reagents

All chemicals and solvents used were of analytical reagent grade. Atropine, atropine sulphate, scopolamine hydrobromide and apoatropine hydrochloride were purchased from Fluka (Buchs, Switzerland). Belladonna powder and tincture were obtained from Cooper (Melun, France). These vegetal preparations meet the specifications of the *European Pharmacopoeia* [1]. The tested pharmaceutical dosage forms were commercially available tablets containing Belladonna powder (10%, m/m), with phenobarbital and caffeine added as active substances.

Voltammetric study of atropine

A polarographic analyser (Model 174 A; E.G. & G., Princeton Applied Research, Princeton, NJ, USA) was used in conjunction with a classical three-electrode stationary cell and an X–Y recorder (Model RE 0074, Omnigraphic; Houston Instruments, TX, USA) to obtain the current–potential curves. The working electrode was a rotating glassy carbon or platinum unit (Model EDI & Controvit; Tacussel, Villeurbanne, France) with a 3 mm diameter disk. The saturated calomel reference electrode (SCE) was placed in a compartment separated by a porous bridge from the measuring cell. The cell and the compartment were filled with the same solution. A platinum wire served as the auxiliary electrode. Recordings in direct current (d.c.), differential pulse (d.p.) and cyclic modes (c.v.) were made under various operating conditions as indicated in Fig. 1. Potentiostatic coulometry was realized at an applied potential of +1.2 V versus SCE with a large surface platinum electrode and stopped when the intensity of the electrolysis current reached 1% of the initial value. For all the voltammetric experiments, a solution of atropine base was used at a concentration of 5×10^{-4}M in an acetonitrile–water (99:1, v/v) mixture containing 0.1 M lithium perchlorate.

The postulated products of the electrochemical reaction (i.e. a secondary amino compound and formaldehyde) which occurs at a solid electrode, were identified by the following colorimetric methods. A 1 ml volume of the solution resulting from the coulometric experiment was evaporated to dryness under a gentle stream of nitrogen, the dried residue was added to 1 ml of an aqueous ammonia solution (20%, v/v), 1 ml of an aqueous 10^{-2}M cupric sulphate solution and 2 ml of a carbon disulphide solution in chloroform (5%, v/v). After vigorous shaking for 1 min, the colouration of the organic layer was observed. The other part of the electrolysed solution (about 50 ml) was steam-distilled and a portion (10 ml) of the distillate obtained (100 ml) was added to 10 ml of an aqueous solution of acetylacetone (2%, v/v) and ammonium acetate (20%, w/v). After heating at 60°C for 20 min, the expected colouration was observed.

Sample preparation

A sample of finely powdered tablet (see Chemicals and Reagents) containing the equivalent of *ca* 600 μg (0.86 μmole) of atropine sulphate was accurately weighed into a 50 ml centrifuge tube. Liquid preparations (tinctures) were evaporated under reduced pressure in order to remove alcohol, before sampling. A 25 ml volume of 0.05 M sulphuric acid was added and the mixture vigorously shaken for 10 min and centrifuged (1350 g) for 5 min. A portion of the supernate (20 ml) was transferred to a separating funnel and extracted three times with diethyl ether (50 ml) for 10 min on each occasion. The organic layers were discarded, the aqueous phase was made alkaline (pH between 9

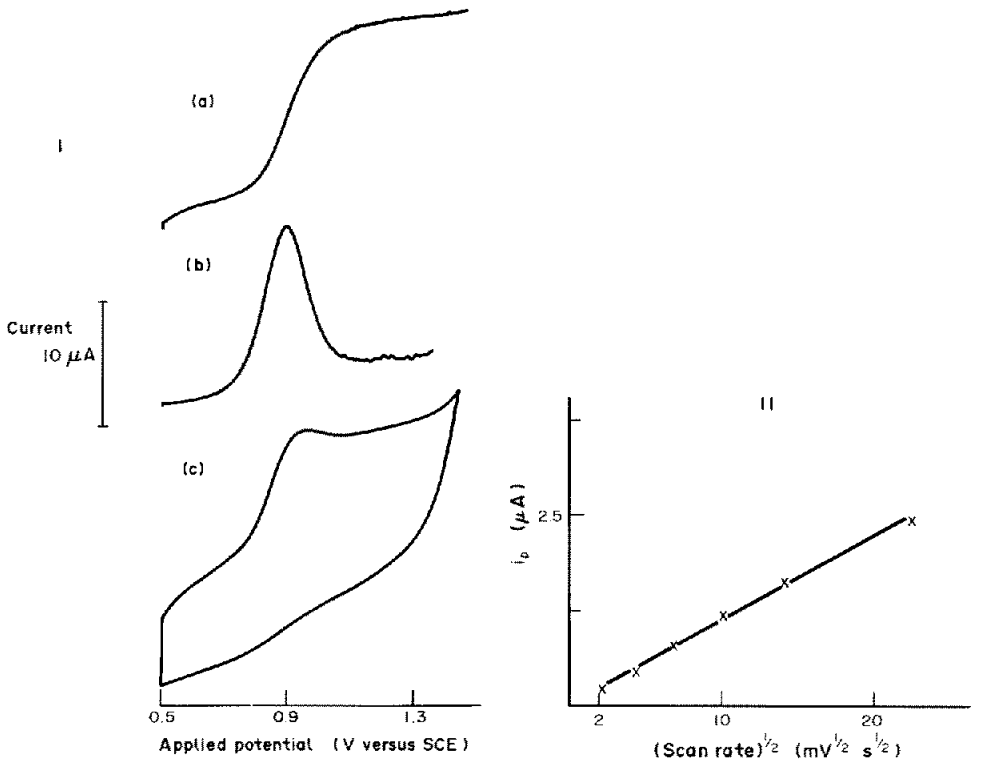


Figure 1
Electrochemical study of atropine at a glassy carbon electrode. (I) current–potential curves obtained in direct current (a), differential pulse (b) and cyclic (c) modes. Operating conditions are as follows: (a) rotating speed, 600 r.p.m.; scan rate, 2 mV s^{-1}; (b) rotating speed, 600 r.p.m.; scan rate, 5 mV s^{-1}; pulse height, 10 mV; pulse repetition, 0.5 s; (c) stationary electrode; scan rate, 200 mV s^{-1}. (II) peak current against scan rate behaviour for electrooxidation of atropine.

and 11) with the addition of 1 ml of an aqueous ammonia solution (20%, v/v) and extracted three times with dichloromethane (50 ml). The organic phases were collected, filtered through a phase-separator filter (Whatman 1-PS) and evaporated to dryness under reduced pressure, at a temperature which did not exceed 45°C. The dried residue was redissolved in acetonitrile (5 ml) and an aliquot (10 μl) was injected into the chromatograph. A sample of the vegetal preparation equivalent to the same amount of atropine sulphate was simultaneously treated as above. When electrochemical detection was used, sample size was reduced by a tenth.

Chromatographic conditions

The HPLC system used consisted of a ternary solvent delivery pump (Model SP 8700; Spectra-Physics, Santa Clara, CA, USA), an injection valve with a 10 μl sample loop (Model 7125; Rheodyne, Cotati, CA, USA), a U.V.–visible detector (Model LC 871; Pye Unicam, Cambridge, UK) and an integrator (Model 5020; Spectra-Physics). For electrochemical detection, a thin-layer electrolytic cell (Model LCC 231; Merck-Clevenot, Nogent-sur-Marne, France), fitted with glassy carbon working and auxiliary electrodes and a reference SCE, was used in connection with an electronic control unit

(Model E 230; Merck-Clevenot) and an integrator (Model ICR-1; Intersmat, Courtry, France).

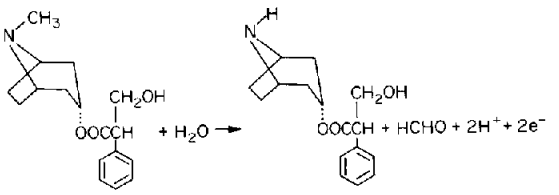
The spectrophotometric detector was set at 220 nm and the amperometric detector was operated at an applied potential of +1.2 V versus SCE.

The column (Hibar R.T., 250 × 4 mm; E. Merck, Darmstadt, FRG) was prepacked with LiChrosorb DIOL (7 μm). The mobile phase was an acetonitrile–0.0125 M sodium phosphate buffer pH 7.2 (20:80, v/v) mixture. This eluent was filtered through a 0.6 μm microfilter (type HVLP; Millipore, Bedford, MA, USA) and degassed before use. The flow-rate was 1 ml min^{-1}.

Results and Discussion

Electrochemical study

The polarographic behaviour of atropine [19] and its nitration product [2] has previously been described. Also the electrooxidation of atropine has been used as the basis of electrochemical detection in HPLC [14]. In the present work, the electrochemical reaction which occurs at positive potentials has been investigated by performing current–potential curves at rotating disc electrodes. Well-defined waves were only obtained by using acetonitrile containing lithium perchlorate (as supporting electrolyte) and low water content (1%, v/v). Similar half-wave potentials ($E_{1/2}$) were obtained with platinum and glassy carbon electrodes, which suggest that the same electrochemical process occurs in both cases. Cyclic voltammograms indicate an irreversible oxidative reaction (Fig. 1). Curves obtained in direct current and differential pulse modes permit the calculation of, respectively $E_{1/2}$ and E_p, both values close to +0.9 V. Otherwise, a linear relationship was obtained by plotting limiting current values versus the square root of the scan rates (from 5 to 500 mV s^{-1}), which demonstrates a diffusion-controlled electrochemical process. The functional group involved in this oxidation is probably the tertiary substituted nitrogen atom of the atropine molecule. An electrolytic N-dealkylation almost certainly occurs to produce formaldehyde and a secondary amino compound, as previously described for aliphatic tertiary amines [20]:



In order to confirm this mechanism, a potentiostatic coulometric experiment was undertaken. The postulated products were identified by colorimetric methods:
formaldehyde gave a yellow coloured lutidine adduct [21];
the secondary amino compound permitted the formation of a metal–dithiocarbamate complex giving a yellow–brown colour of the organic layer [22].

The solution of atropine before the controlled-potential electrolysis gives negative results for these two tests.

Addition of perchloric acid to a solution of atropine results in a disappearance of the electrooxidative wave on current–potential curves obtained at a rotating disc electrode.

This fact is not related to the degradation of atropine in acidic medium [23] but confirms the pH-dependent mechanism of tertiary amino compounds. The kinetic rate of the electrochemical process is enhanced at basic pH values [20]. Further studies concerning the influence of pH have been performed using the HPLC system in order to optimize the electrochemical detection.

Chromatographic conditions

Although the first reported LC analysis of atropine involved a normal-phase system [9], subsequent workers have used reversed-phase LC on bonded phases such as C_2 [16], C_8 [11, 18] and C_{18} [10, 12, 13, 15, 17]. Elution was usually effected in acidic media with the aid of an anionic [10, 15, 16] or a cationic [12, 13] pairing-ion. Use of the latter often appears necessary in order to reduce peak tailing resulting from the interaction of amino compounds with residual silanol groups. Oppositely charged pairing-ions have recently been combined in the mobile phase [16]. These systems permit separation of atropine from other related alkaloids, i.e. scopolamine [13, 14, 16, 17], from drugs used in combination in pharmaceutical preparations [10, 13, 18], or from degradation products (apoatropine [12, 14], tropic acid [12, 14, 15] and atropic acid [12]). Detection was generally by U.V. spectrophotometry at 254 nm. By lowering the wavelength to a value of 220 nm, a sensitivity of 20 ng per injection for atropine may be achieved [13]. Alternatively the detection limit (200 pg) may be reduced by use of a sophisticated post-column fluorimetric ion-pair technique [11]. In a previous report [14], electrochemical detection was tested for atropine, resulting in a sensitivity of 2 ng of atropine injected onto the column [13]. The current response measured during the electrooxidation of atropine at a glassy carbon electrode has been studied using amperometric detection as a function of the pH of the mobile phase and of the applied potential (Fig. 2). The response increased in intensity from pH 5.5 to 7.5. An eluent buffered at pH 7.2 was chosen in order to avoid any stationary phase degradation which may occur in alkaline

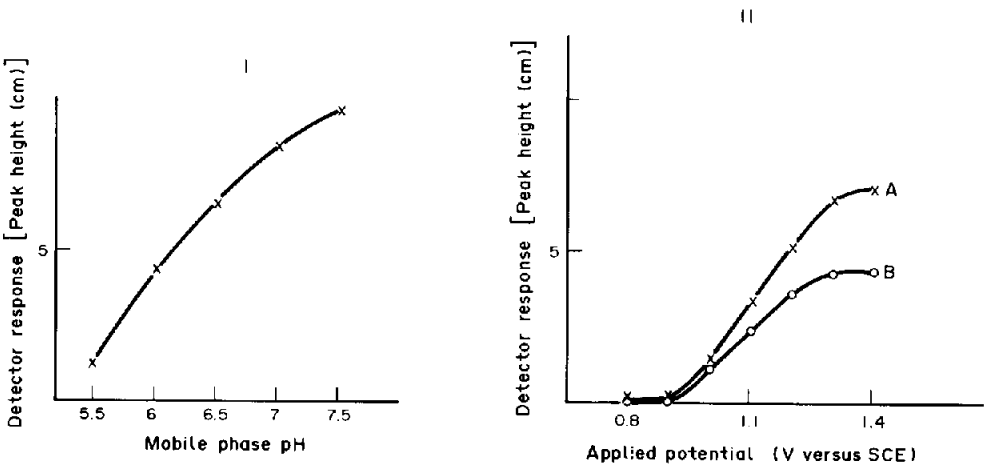


Figure 2
Electrochemical study of atropine using the HPLC system. (I) mobile phase pH dependence of current response for electrooxidation of atropine (applied potential: +1.2 V versus SCE). (II) hydrodynamic voltammogram of scopolamine (A) and atropine (B) obtained by repeated injection of 0.5 μg of each compound at various applied potentials.

medium, especially in the case of a modified silica. The hydrodynamic voltammogram obtained at pH 7.2 shows a current growth for applied potentials between +0.8 and 1.4 V versus SCE. Amperometric detection was operated at +1.2 V, which was considered to be the best compromise between a high response and a low residual baseline current.

The retention times of the test alkaloids on a reversed-phase column (C_8 or C_{18}) at pH 7.2 however were inconveniently long. Therefore, it was decided to use a hydrophilic stationary phase (LiChrosorb DIOL) which gave suitable retention times. The capacity factors k' were, respectively 1.4, 2.1 and 3.3 for scopolamine, atropine and apoatropine (Fig. 3). Resolution between adjacent peaks was calculated to be R_S (scopolamine/atropine) = 2.2 and R_S (atropine/apoatropine) = 1.9.

The polar column has the added advantage of offering good stability towards aqueous mobile phases thereby leading to reproducible solute capacity factors. Moreover, the chromatographic system was quickly equilibrated and resulting back pressure was low (less than 600 p.s.i.).

Analysis of raw materials and pharmaceutical dosage forms

The chromatographic system described above was applied to the identification of alkaloids contained in Belladonna preparations (tincture and powder). Peaks due to both scopolamine and atropine were observed on the chromatograms obtained from extracts of these vegetal products (Fig. 4). However, the scopolamine concentration was too low to be quantified with a coefficient of variation much less than 5%. Accordingly, atropine was chosen as a marker in order to test the conformity of pharmaceutical dosage forms containing Belladonna preparations. Peaks observed for atropine in the vegetal preparation alone and in the multicomponent preparation were compared for this

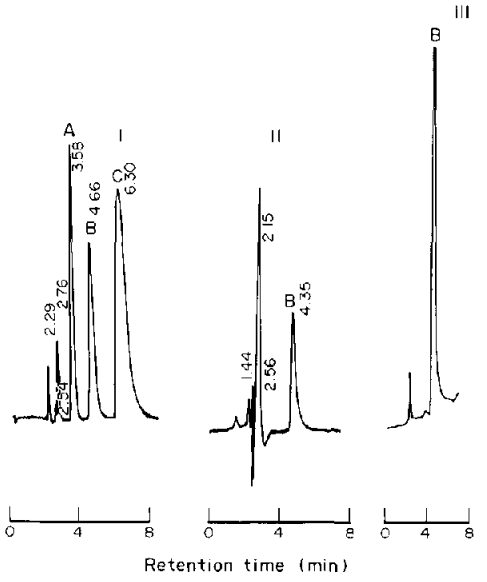


Figure 3
Chromatograms of scopolamine (A), atropine (B) and apoatropine (C), eluted using a LiChrosorb DIOL (7 μm) column (250 × 4 mm i.d.) with acetonitrile–sodium phosphate buffer 0.0125 M (pH 7.2) (20:80, v/v) at a flow rate of 1 ml min^{-1}. Detection by spectrophotometry (λ = 220 nm; 0.04 a.u.f.s.) for I and II, or by amperometry (E = +1.2 V versus SCE; 1 μA. f.s.) for III. *ca* 1 μg of each compound was injected.

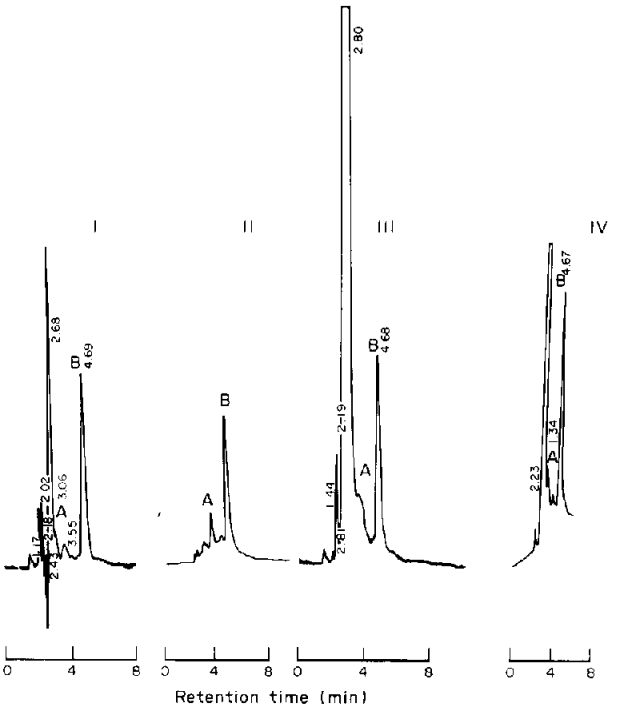


Figure 4
Chromatograms obtained from extracts of a Belladonna powder (I), tincture (II) and a multicomponent tablet (III, IV). Peaks: scopolamine (A) and atropine (B). Detection by U.V. spectrophotometry (I, III) or by amperometry (II, IV). For other experimental conditions, see Fig. 3 and Experimental section.

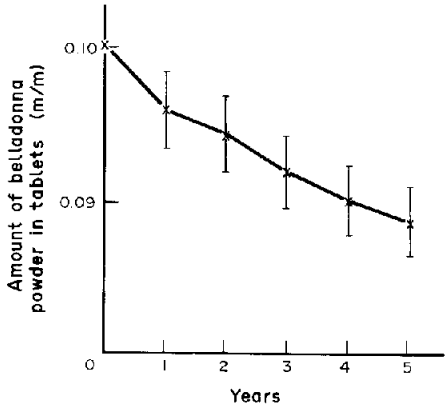


Figure 5
Stability study of multicomponent tablets containing Belladonna powder over a five year storage period, using HPLC with U.V. detection.

purpose. A symmetry factor of nearly 1.0 for the atropine peak permits a choice between height and area measurement as recommended for liquid chromatographic assays in the *European Pharmacopoeia*. No interferences with the atropine assay have been observed with the pharmaceutical preparations studied. The co-extracted drugs (phenobarbital, caffeine), which are not completely removed from acidic medium by ether extraction are weakly retained and are eluted well before the analytes. The choice between electrochemical and spectrophotometric detection for a specific application may be based on the following criteria. Amperometric detection offers an increase in sensitivity of at least ten-fold and a wider linear response for atropine (between 1×10^{-6} and 5×10^{-3}M) under the present experimental conditions. However, the RSD obtained for ten replicate assays (including extractive and chromatographic steps) was better with U.V. spectrophotometry (2.6%) than with amperometry (4.2%). The assay values obtained for the pharmaceutical preparations studied were included in a ±10% range of the theoretical amount (0.1% m/m). The mean experimental value was 0.0948 ± 0.0025% m/m ($n = 10$), with U.V. spectrophotometry.

The method also was used to study the stability of tablets over a five-year storage period. A statistic decrease in the amount of atropine was observed of *ca* 10% over five years (Fig. 5).

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