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## Note

# Simultaneous determination of atropine and its acidic and basic degradation products by mixed-column high-performance liquid chromatography

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The reversed-phase chromatographic separation of mixtures of acids and bases is often difficult as the chromatographic conditions that give a sufficient retention of the bases result in little or no retention of the acidic substances and *vice versa*, unless special precautions are taken.

The degradation products of atropine form such a mixture of acids and bases (Fig. 1)[1]. Numerous methods have been published for the determination of atropine, but only few include the determination of one or more of the degradation products[2–7]. A UV method[8] includes the three main degradation products (apoatropine, tropic acid and atropic acid) but the accuracy obtained is poor, even for atropine itself, the standard deviations ranging from 5 to 20%. The same compounds have been determined by a high-performance liquid chromatographic (HPLC) method[9], in which the retention problem is solved by using an ion-pair approach.

The aim of this study was to explore the chromatographic possibilities of using a combination of two medium-polarity column materials in an attempt to overcome the difficulties associated with the simultaneous determination of acids and bases by HPLC. A simple method has been developed for the determination of atropine and its main degradation products in a sample of atropine injection.

## EXPERIMENTAL

### *Chemicals*

Atropine sulphate complied with the tests given in the *Pharmacopoeia Nordica*[10]. Apoatropine hydrochloride was prepared from atropine sulphate as described by Hesse[11].

Tropic acid (purum; Fluka, Buchs, Switzerland) was recrystallized from benzene–diethyl ether. Atropic acid was prepared from tropic acid as described by Raper[12]. $\alpha$- and $\beta$-belladonnine were prepared from apoatropine as described by Küssner[13]. $\alpha$-Belladonnine crystallized from the reaction mixture and was recrystallized from ethyl acetate [m.p. 129° (corr.)]. The filtrate from the reaction mixture was evaporated, the residue was dissolved in diethyl ether and, on addition of hydrogen chloride in diethyl ether an amorphous solid consisting of a mixture of $\alpha$- and $\beta$-belladonnine hydrochloride was obtained.

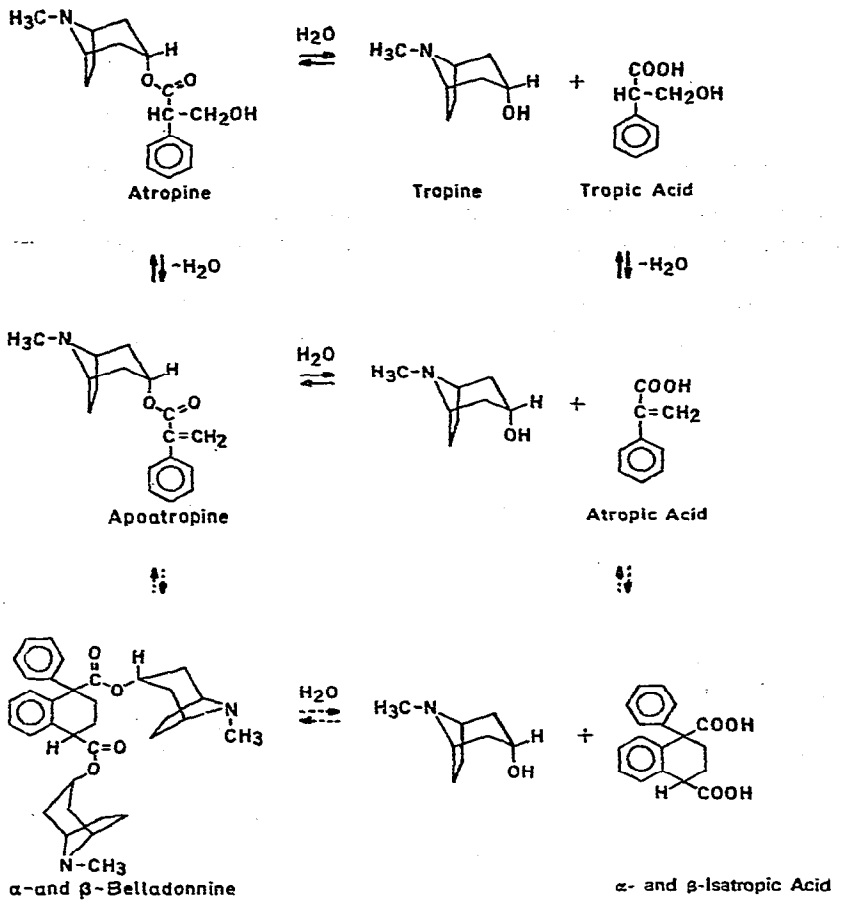


Fig. 1. Main degradation products of atropine[1].

All other chemicals used were of reagent grade. The solvents were distilled before use.

*Apparatus*

The liquid chromatographic equipment consisted of a Waters M6000 pump, a Rheodyne Model 7120 injection valve, fitted with a 20-$\mu$l loop, an LDC Model 1203 UV detector operated at 254 nm and an Omniscribe Model 5111-5 recorder (Houston Instruments, Houston, Texas, U.S.A.).

Quantitations were performed by area measurement using an Autolab Minigrator (Spectra Physics, Darmstadt, G.F.R.). pH was measured on a PHM 26 pH meter (Radiometer, Copenhagen, Denmark). For the column switching experiments a Valco Model CV-6-UHPa-N60 valve was used. The tubing was 1/16-in. stainless steel (I.D. 0.25 mm) and connections were made with a Swagelok 1/16-in. tee.

*Columns and column packing*

The columns were of internally polished stainless steel (I.D. 4.65 mm). Fittings were modified Swagelok unions.

Each column was packed from a dilute slurry of the packing material in methanol by pumping 200 ml of methanol through the column at a pressure of 6400 p.s.i. generated by a Haskel Model 28646-4 pump. The packing materials were Nucleosil 5 CN and Nuceosil 5 $NH_2$ (Macherey, Nagel & Co., Düren, G.F.R.), both consisting of 5-$\mu$m spherical particles.

## RESULTS AND DISCUSSION

Atropine in aqueous solution is degraded by two main routes, namely hydrolysis and dehydration (Fig. 1)[1]. Dimerization of the dehydrated ester or of the acid resulting from the hydrolysis may occur, but under normal storage conditions the occurrence of these dimers is not expected in aqueous solutions of atropine[1]. Therefore, the following methods of analysis will be concerned with atropine and the three main degradation products (apoatropine, tropic acid and atropic acid). Tropine does not absorb UV light at 254 nm, and is not detected by the present analytical methods.

*Single-column experiments*

The two packing materials were initially studied separately in 10-cm columns. The mobile phases used were mixtures of methanol and sodium acetate buffers of varying pH and concentration. Figs. 2–4 show the influence of pH, buffer (acetic acid/acetate) concentration and methanol content on the retention of atropine, apoatropine, tropic acid and atropic acid. Only results for atropine and apoatropine

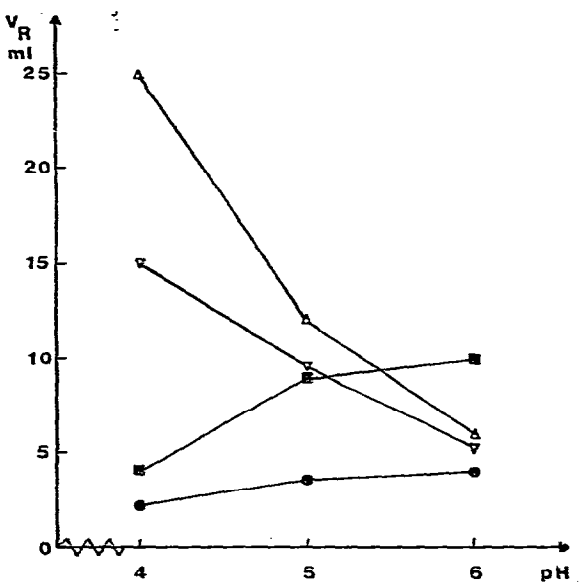


Fig. 2. Effect of pH on retention volumes. Symbols: (●) atropine and (■) apoatropine, chromatographed on a 10-cm Nucleosil 5 CN column; (▽) tropic acid and (△) atropic acid, chromatographed on a 10-cm Nucleosil 5 $NH_2$ column. Mobile phase: 0.05 *M* sodium acetate buffer–methanol (3:1, v/v).

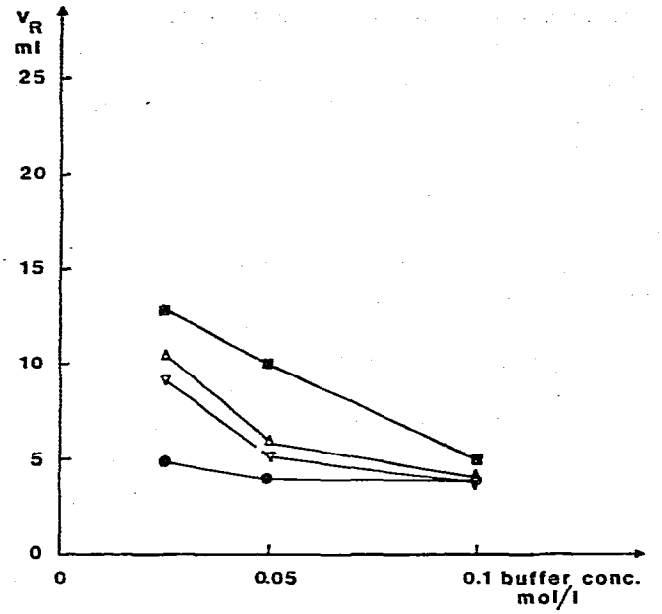


Fig. 3. Effect of buffer concentration on retention volumes. Symbols as in Fig. 2. Mobile phase: sodium acetate buffer (pH 6)–methanol (3:1, v/v).

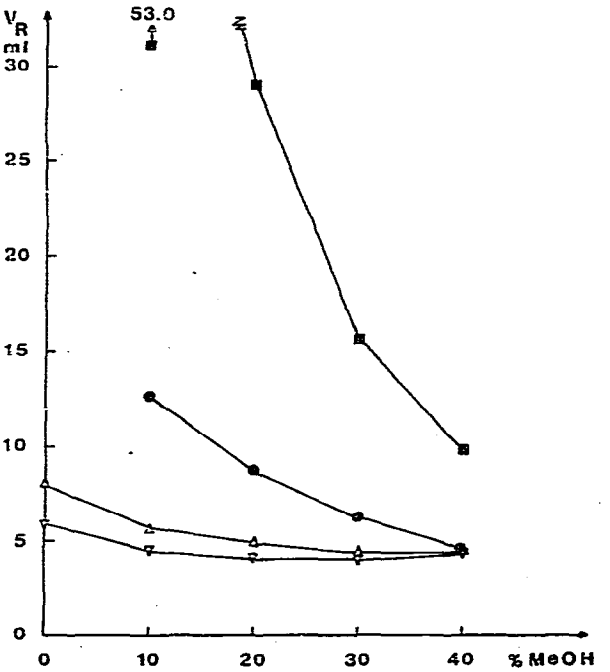


Fig. 4. Effect of methanol concentration on retention volumes. Symbols as in Fig. 2. Mobile phase: 0.1 *M* sodium acetate buffer (pH 6)–aqueous methanol (4:6, v/v).

on the Nucleosil 5 CN column and for tropic acid and atropic acid on the Nucleosil 5 $NH_2$ column are shown as in the other instances the substances eluted at or very close to the solvent front. Asuming the chromatographic system to be reversed phase on both columns as well as anion exchange on the Nucleosil 5 $NH_2$ column, the changes in retention with pH (Fig. 2) and buffer concentration (Fig. 3) are as one would expect. The underlying mechanism of the dependence of the retention volume on the methanol concentration in the mobile phase, however, seems to be complex (Fig. 4). A decrease in retention with increasing methanol concentration might be expected, but this is seen only at low methanol concentrations, and as the methanol content increases further even a small, but significant, increase in retention is seen.

*Column combinations*

On the basis of the results obtained on single columns, a mobile phase of 25% (v/v) methanol in 0.025 *M* sodium acetate buffer (pH 5) was chosen for the following experiments. The data collected on single columns were used to calculate the column combination that would most efficiently give the desired separation of atropine, apoatropine, tropic acid and atropic acid. The separation conditions could thus be adjusted to suit situations in which special emphasis was to be laid on the analysis of one or more of the compounds.

For technical reasons columns shorter than approximately 5 cm cannot be made. The fastest separation possible with a combination of columns would then be obtained using two 5-cm columns of Nucleosil 5 $NH_2$ and Nucleosil 5 CN, respectively, connected in series as shown in Fig. 5a. As can be seen, the separation of the acids was satisfactory; hence, it should be possible to increase the speed of separation by packing a 5-cm column with a mixed bed of Nucleosil 5 CN and Nucleosil 5 $NH_2$. A separation on such a column, consisting of Nucleosil 5 CN and Nucleosil 5 $NH_2$ in the proportions 2:1 is shown in Fig. 5b. The use of this column reduced the analysis time by a factor of 2–3. Quantitation of atropine itself in these fast separations was not possible as it was eluted close to the solvent front.

Quantitation of atropine can be effected by using a longer column of Nucleosil 5 CN to increase the relative retention of atropine, as shown in Fig. 5c. This resulted in a correspondingly increased retention of apoatropine and thus in a lower detection limit for apoatropine and for the two acids.

This situation could be improved by the use of a medium-sized Nucleosil 5 CN column and column switching (Fig. 5d). This markedly improved the detection limit for apoatropine but, as the tubes and connections had not been modified to minimize peak broadening, no improvement was seen for the acids.

*Analysis of atropine injection*

The applicability of the chromatographic system to the analysis of atropine injection was demonstrated. A combination of a 5-cm Nucleosil 5 CN and a 5-cm Nucleosil 5 $NH_2$ column was used. The retention volumes of the degradation products of atropine, including the compounds that are formed only under severe conditions, and for atropine, scopolamine and homatropine are listed in Table I, which also includes the detection limits (three times the detector noise) of the three degradation products normally found (apoatropine, tropic acid and atropic acid). Standard graphs

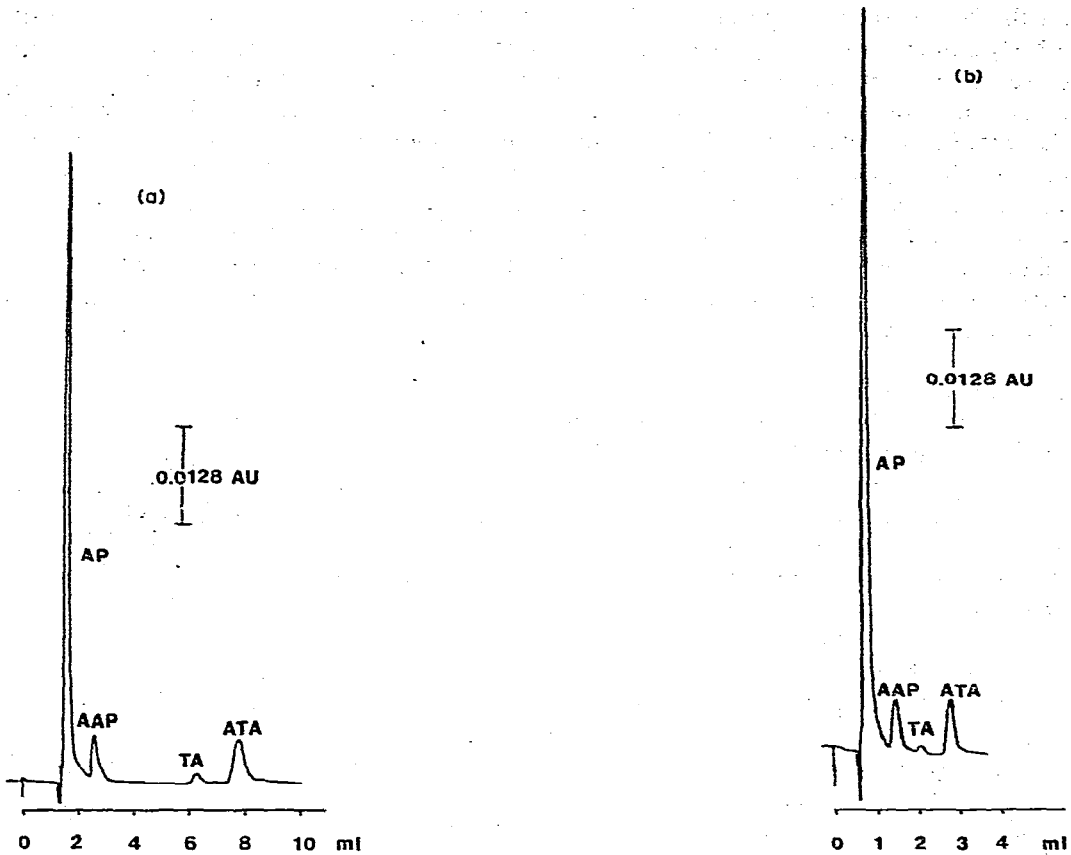


Fig. 5.

TABLE I

RETENTION DATA AND DETECTION LIMITS FOR ATROPINE AND ITS DEGRADATION PRODUCTS

Columns: 5-cm Nucleosil 5 CN and 5-cm Nucleosil 5 $NH_2$ connected in series. Mobile phase: 0.05 *M* sodium acetate buffer (pH 5)–methanol (3:1, v/v), ($V_0$ = 1.2 ml).

| *Sample* | *k'* | *Minimum detectable amount (ng)* |
|---|---|---|
| Atropine sulphate | 0.57 | — |
| Apoatropine hydrochloride | 2.0 | 5 |
| Tropic acid | 2.4 | 40 |
| Atropic acid | 3.0 | 2 |
| α-Belladonnine | 3.5 | — |
| β-Belladonnine | 5.9 | — |
| β-Isatropic acid | 3.0 | — |
| Scopolamine | 0.36 | — |
| Homatropine | 0.44 | — |

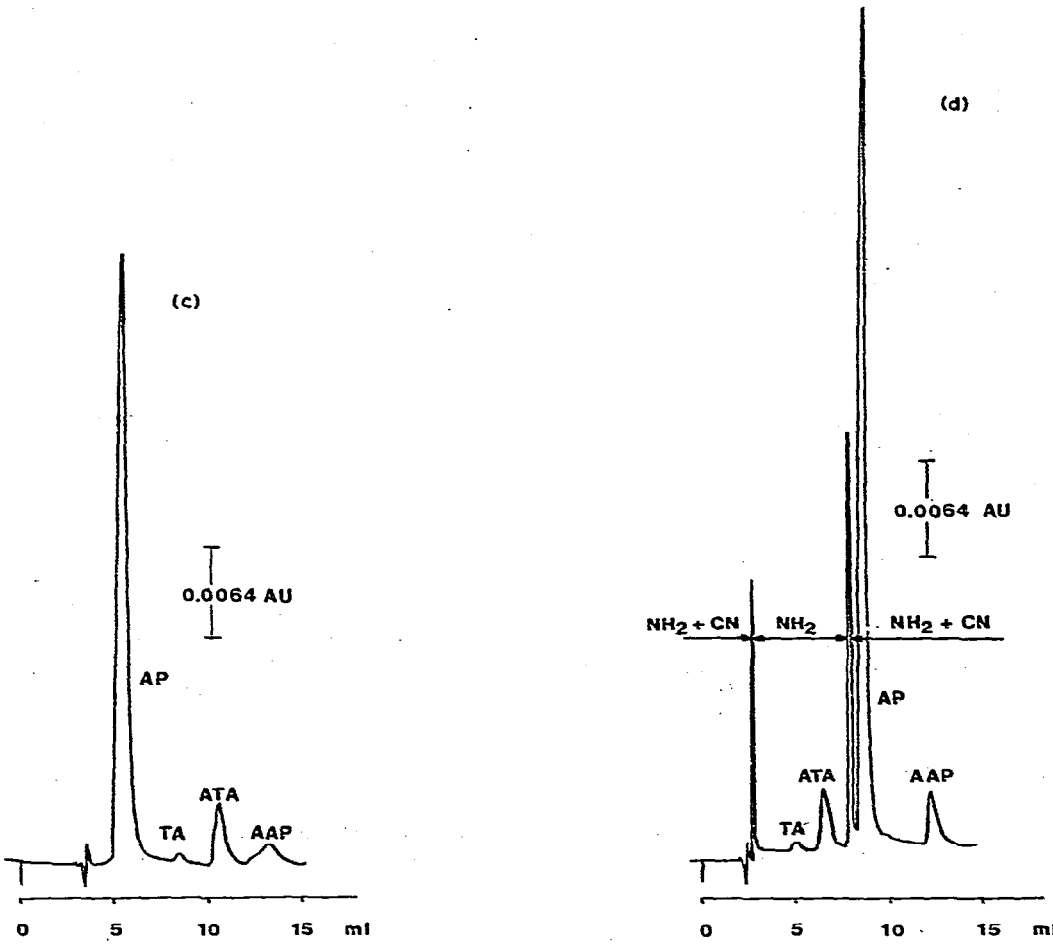


Fig. 5. Separation of atropine and degradation products. Substances: atropine sulphate 36.8 mg/ml (AP), apoatropine hydrochloride 0.12 mg/ml (AAP), tropic acid 1.96 mg/ml (TA) and atropic acid 0.13 mg/ml (ATA). Sample volume: 2 $\mu$l. Mobile phase: 0.05 *M* sodium acetate buffer (pH 5)–methanol (3:1, v/v). Columns: (a) 5 cm Nucleosil 5 CN and 5 cm Nucleosil 5 $NH_2$ connected in series. (b) 4.4 cm containing a mixture of Nucleosil 5 CN and Nucleosil 5 $NH_2$ (2:1, w/w). (c) 15 cm Nucleosil 5 CN and 5 cm Nucleosil 5 $NH_2$ connected in series. (d) 5 cm Nucleosil 5 $NH_2$ and 10 cm Nucleosil 5 CN with column switching.

were constructed for apoatropine hydrochloride in the range from 2 to 20 $\mu$g/ml, for tropic acid from 10 to 100 $\mu$g/ml and for atropic acid from 1 to 10 $\mu$g/ml. The three graphs passed through the origin and were linear in the concentration ranges examined.

An example of a chromatogram from a 15-year-old sample of atropine injection (2 mg/ml) is shown in Fig. 6. The sample contained an amount of apoatropine corresponding to 0.52% degradation of the original amount of atropine sulphate, tropic acid corresponding to 4.3% degradation, but no detectable amounts of atropic acid.

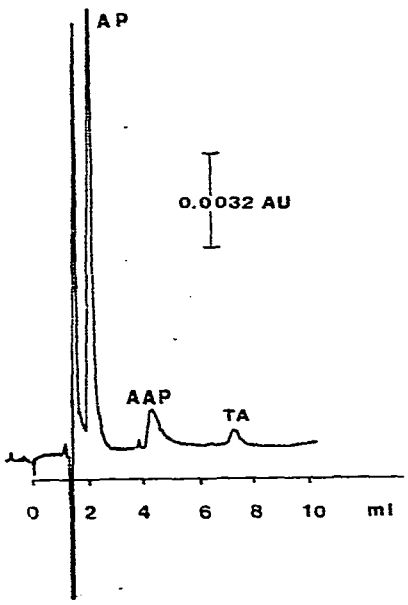


Fig. 6. Analysis of a sample of atropine injection. Substances: atropine (AP), apoatropine (AAP) and tropic acid (TA). Sample volume: 5 $\mu$l. Columns: 5 cm Nucleosil 5 CN and 5 cm Nucleosil 5 $NH_2$ connected in series. Mobile phase: 0.05 *M* sodium acetate buffer (pH 5)–methanol (3:1, v/v).

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