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Rapid resolution liquid chromatography for monitoring the quality of stockpiled atropine preparations for injection

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We describe a rapid resolution liquid chromatography (RRLC) method for analyzing atropine sulfate, its degradation products (tropic acid, apoatropine, atropic acid) and other components (e.g. phenol, methylparaben) in injectable medicines that are used by the German armed forces in emergency situations. Chromatography is performed using an acetonitrile/phosphate buffer gradient (pH = 1.0) and an RP 18 column (50 x 4.6 mm, 1.8 μm) with the detection wavelength set at 220 nm. The concentration of the active ingredient (atropine sulfate) in the tested products ranges from about 1 mg·ml^{-1} to 10 mg·ml^{-1}. The concentrations of the detected degradation products range from 0.2% to 4.7% (tropic acid) in relation to the active pharmaceutical ingredient (API). Using shorter separation columns and smaller particle sizes of the stationary phase improved analysis time from 40 to 10 min and reduced the consumption of solvents by approximately 75%. Owing to the pressure conditions (< 200 bar), UHPLC (ultra high performance liquid chromatography) systems are not needed. Comparison of the atropine and tropic acid results obtained with the previously used HPLC (high performance liquid chromatography) method of the MAH (marketing authorization holder) show that there is no indication of a significant difference between the two methods. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: stockpiled atropine preparations; RRLC; tropic acid; apoatropine

Introduction

Military forces and civilian emergency services stockpile injectable atropine-containing medicines to be used as an antidote for the treatment of possible organophosphate poisoning.[1] These stockpiles include auto-injectors (e.g. combination auto-injectors for use in nerve agent poisoning, ATOX II ComboPen® auto-injectors, AtroPen® auto-injectors) as well as atropine-containing injection and infusion solutions, which can in Germany also be produced by pharmacies on the basis of standard marketing authorizations. These emergency medicines do not need to carry an expiry date. Since there is virtually no consumption of antidotes and many other medicines that are stockpiled for use in the event of an emergency, a disaster, or a pandemic, substances/products are stored for as long a period as possible not least for economic reasons. Since pharmaceutical companies usually grant a warranty period of 2 to 5 years on their products, the pharmaceutical quality of these products must be monitored throughout the entire storage period with a view to ensuring that the efficacy and safety of the products are not compromised.[2–4] Monitoring programmes require efficient methods that can identify and quantify atropine degradation products. The aim of this study was to investigate whether new column materials packed with sub 2 μm particles may help to characterize much more different products and batches also in relation to their individual storage facilities through the enhancement of established analytical performance. In addition this may also help to improve the evaluation of the safety of these products by means of representative sample sizes.

Although RRLC (rapid resolution liquid chromatography) and UHPLC (ultra high performance liquid chromatography) methods are already widely used by the pharmaceutical industry, their suitability for the aforementioned purposes must be assessed on a case-by-case basis. Unlike UHPLC, RRLC requires pressures that still allow conventional HPLC (high performance liquid chromatography) systems (< 400 bar) to be utilized. The economical use of UHPLC systems is currently limited, especially for official control laboratories, on account of the constantly changing spectrum of samples from market surveillance and the correspondingly different methods to be applied as authorized by the MAH (marketing authorization holder). These methods are often not adapted to new analytical developments for regulatory reasons.

Atropine sulfate is usually pharmaceutically analyzed and characterized on the basis of the HPLC method described in the European Pharmacopoeia (EP).[5] The EP recommends the use of octadecylsilyl silica gel with a particle size of 3 μm as the stationary phase and a column size of 100 x 4.6 mm. Sodium dodecyl sulfate in phosphate buffer (pH 3.3) and acetonitrile (approximately 30%) are used as the mobile phase in a gradient separation. The run time (without post-run time) is 20 min with detection at 220 nm. The EP tests for eight impurities (Imp), i.e. apoatropine (Imp A), noratropine (Imp B), tropic

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acid (Imp C), 6-hydroxyhyoscyamine (Imp D), 7-hydroxyhyoscyamine (Imp E), scopolamine (hyoscine, Imp F), littorine (Imp G), and an impurity of unknown structure (Imp H). This method[5] replaces the TLC (thin-layer chromatography) method described earlier[6] and the HPLC method that was described in a monograph on atropine [7] with a column size of 125 x 4 mm and a particle size of 4 μm (and a similar mobile phase). The run time and post-run time of a gradient separation is 30 min. Atropine sulfate solutions (preparations for subcutaneous, intramuscular or intravenous injection) that are produced on the basis of national standard marketing authorizations are analyzed using 125 x 3.9 mm columns packed with octadecylsilyl silica gel with a particle size of 5 μm. The detection wavelength is set at 254 nm. The components, however, are separated under isocratic conditions in acetate buffer with tetrabutylammonium hydrogen sulfate as an ion-pair reagent at a pH of 5.5.[8] Kirchhoff and Bitar[9] discuss RP 18 endcapped materials as stationary phase. They use a 125 x 4.6 mm column (5 μm) and a gradient separation with phosphate buffer (pH 2.5) and acetonitrile as mobile phase with detection at 215 nm. John *et al.*[10] developed a fast LC electrospray ionization tandem mass spectrometric (LC-ESI-MS/MS) procedure for the determination of atropine and tropane alkaloids in plasma using a 150 x 4.6 mm column, packed with RP 18, 5 μm particles. Boros *et al.*[11] used LC-ESI-MS for the determination of atropine and scopolamine in floral nectar. The chromatography was performed on novel fused core particles (2.7 μm; column 50 x 2.1 mm) with a gradient of aqueous formic acid and methanol. Russo *et al.*[12] described the analysis of scopolamine, 6-β-OH-hyoscyamine and littorine in plant extracts by UHPLC-UV – in comparison with UHPLC-ELSD (evaporative light scattering detection) – using RP 18 columns (1.7 μm, lengths: 50 and 100 mm) with acetonitrile/ trifluoracetic acid. In a review article, Aehle and Draeger[13] summarized the state-of-the-art of chromatographic and electrophoretic analysis of tropane alkaloids.

So to the best of our knowledge, studies on the analysis of stockpiled medicines containing atropine using sub-2 micron column particles or UHPLC are not published. A special problem in monitoring the pharmaceutical quality of atropine-containing medicines that are stored for a long period of time is the potential presence of other active substances and excipients (and their degradation products) and substances from primary packaging material in chromatography. Loch and Zimmermann[14] addressed the analysis and stability of atropine in two products (a combination auto-injector for use in nerve agent poisoning and the AtroPen® auto-injector) with glass/steel containers and investigated the formation of tropic acid as a function of storage time.

Experimental

Chemicals and reagents

Water (deionized) was prepared using a Milli-Q system by Millipore (Molsheim, France), consisting of Milli-Q Gradient, Q-Guard 2, Quantum Ex Ultrapure Organex Cartridge and Millipak Express 20. Acetonitrile was purchased from BDH Prolabo/VWR (Darmstadt, Germany) and o-phosphoric acid (analysis grade, ω (H_3PO_4) = 85%) from Merck KGaA (Darmstadt, Germany). Methyl 4-hydroxybenzoate, sodium salt (methylparaben) was purchased from Acros-Organics (Geel, Belgium), phenol (loose crystals, puriss., p. a., > 99.8%) from Fluka/Sigma-Aldrich (Seelze, Germany). Atropine Imp A (apoatropine, NMR assay about 90%) was obtained from Boehringer Ingelheim (Ingelheim, Germany), atropine Imp C (tropic acid, 99%, purum) from Fluka/Sigma-Aldrich (Seelze, Germany), atropine sulfate from Fagron (Barsbüttel, Germany) and the European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France). Atropine Imp B (noratropine) was also purchased from EDQM (Strasbourg, France). Atropine Imp F (scopolamine, > 99%) was purchased from Fluka/Sigma-Aldrich (Seelze, Germany). Solutions of atropine, its impurities, methylparaben and phenol were prepared in mobile phase A.

HPLC system

The Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) was equipped with binary gradient pumps, auto sampler, auto injector, photo diode array detector (DAD SL, 80 Hz full-spectral data acquisition for ultra-fast LC; standard flow cell, 10 mm, 13 μl), vacuum degasser, column oven and solvent module; the system is controlled with ChemStation for LC 3D Systems (B 01.03) computer control software.

Liquid chromatographic conditions

Liquid chromatographic separation was achieved on a Zorbax® SB C18 RRHT (rapid resolution high throughput, Agilent Technologies), 50 x 4.6 mm, 1.8 μm, column with RRLC in-line filter (Agilent Technologies, 4.6 mm, 0.2 μm) using gradient separation (Table 1). As mobile phase A aqueous 3% o-phosphoric acid (*v/v*) was used and a mixture (*v/v/v*) of 3% H_3PO_4/ 7% water/ 90% acetonitrile as mobile phase B. The mobile phase was degassed and filtered through a PTFE (polytetrafluorethylene) membrane filter, pore size 1,2 μm (Sartorius, Göttingen, Germany). The flow rate of the mobile phase was 1.0 ml·min^{-1}. The column temperature was maintained at 50 °C and detection was set at a wavelength of 220 nm. The injection volume was 2 μl.

Test samples

For the validation of the method and for further investigations two different auto-injector products and one solution for injection had been investigated:

- AI 1(Kombinationsautoinjektor gegen Nervenkampfstoffvergiftung; MAH: Duphar (Amsterdam, the Netherlands); API: atropine sulphate (2 mg) and obidoxime chloride (220 mg) in 2 ml, single chamber).
- AI 2 (ATOX II-ComboPen®-Autoinjektor; MAH: Meridian Medical Technologies (MMT, Belfast, Northern Ireland); API chamber 1: atropine sulfate (2 mg·0.7 ml^{-1}), chamber 2: obidoxime chloride (220 mg·2 ml^{-1}); excipients: citric acid, sodium chloride, hydrochloric acid, sodium hydroxide, water).
- S1 (Atropinsulfat-Lösung, standard authorization; API: atropine sulphate (2 mg·ml^{-1} and 100 mg·10 ml^{-1}); labelled excipients: sodium chloride, hydrochloric acid, water).

Table 1. Stages of the RRLC gradient

Time (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0 - 0.2	84	16
0.2- 2.2	84→60	16→40
2.2 – 4	60	40
4 – 6	60→84	40→16
6 – 10	84	16
10 (restart)		

Precision

The intra-day precision of the method was determined by calculating the RSD of the peak area after analyzing six individual preparations of a pooled sample from the atropine chamber ($c_{API} \approx 0.57$ mg·ml^{-1}) of AI 1. The precision of the system was determined by calculating the RSD of the area after six-fold injection of one individual preparation. The inter-day precision of the method was evaluated using one analyst and one instrument in the same laboratory on six consecutive business days.

Specifity

Since detailed information on the formulations of the products AI 1 and AI 2 was not available, the specifity of the method was evaluated by analyzing an individual preparation containing a mix of the excipients of product S1 (water, sodium chloride [9 mg·ml^{-1}] and hydrochloric acid [10%], pH adjusted to 3.0–3.2, in accordance with the standard authorization) and an individual solution of citric acid (3.5 mg·ml^{-1}) as well as a blank of mobile phase A. To simulate the excipients of AI 2 in one preparation, equal parts of the solutions of simulating S1 and citric acid were mixed. Also some AI 2 products were stored for six months at ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) accelerated storage conditions (40 °C ± 2 °C/75% ± 5% relative humidity[15]).

Linearity

Individually prepared test solutions for the linearity of the method were prepared from atropine sulphate·H_20 at six concentration levels from 64 to 152% of the assay analyte concentration (0.31; 0.39; 0.48; 0.56; 0.65; 0.73 mg atropine·ml^{-1}). The peak areas versus concentration data were analyzed by least-squares linear regression.

Accuracy

The accuracy of the method was evaluated through the determination of the recovery for atropine by standard addition. For this purpose, six-fold individually prepared solutions (solution 1, 2, 3) at three concentration levels were tested. Every solution was analysed twice by HPLC. The percentage recovery was calculated according to Kromidas [16] using the following formula:

$$\text{Recovery} = (S_1\text{-}S_2)/S_3 \bullet 100\ \% \qquad (1)$$

S_1: area of atropine signal in solution 1 (corresponding to 0.52 mg atropine·ml^{-1});
S_2: area of atropine signal in solution 2 (corresponding to 0.40 mg atropine·ml^{-1});
S_3: area of atropine signal in solution 3 (corresponding to 0.12 mg atropine·ml^{-1})

Solution 1: 0.5 ml test solution (sample: 0.48 mg atropine·ml^{-1}) + 0.1 ml calibration test solution (0.73 mg atropine·ml^{-1}, corresponding to the 152% calibration standard).;
Solution 2: 0.5 ml test solution (sample) + 0.1 ml solvent;
Solution 3: 0.5 ml solvent + 0.1 ml calibration test solution

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were determined according to DIN 32645 [17] by injecting seven different concentrations of different impurities/chemicals. The analytes in the stock solutions had a concentration of about 0.2% in relation to atropine. The absolute concentration of the analytes (except phenol: 1.08 μg·ml^{-1}) was 0.96 μg·ml^{-1}. The stock solution was diluted to concentrations of 0.025%, 0.05%, 0.075%, 0.10%, 0.125%, 0.15%, and 0.175%.

Qualification of instruments and quality management

The qualification of the HPLC equipment was performed according to the respective guideline of the EDQM[18] at level IV (in-use instrument checks) and level III (periodic and motivated instrument checks). This guideline supports the implementation of quality management aspects within the General European OMCL (official medicines control laboratory) Network (GEON). As the laboratory is accredited, all tests were performed in accordance with ISO/IEC 17025.

Results

Atropine hydrolyzes in aqueous solutions to Imp C and tropine, which is not detectable at 220 nm. Atropine can also dehydrate to Imp A. Apoatropine (Imp A) and tropic acid (Imp C) turn into atropic acid through hydrolysis or dehydration.[9] For this reason, investigations on the stability of atropine-containing solutions focus on the separation of atropine from Imp A, Imp C, and atropic acid (Figure 1). Although other impurities (B, D, E, F, G, H) are listed in the EP, we have not yet identified them in the investigated products. It is also necessary to separate these analytes from other components that are added as additional API (e.g. obidoxime chloride) or preservatives (e.g. phenol, methylparaben), or substances leached from packaging materials (e.g. rubbers, stoppers, chamber material, lubricants).

Figure 2 shows a typical chromatogram of an AI 1 sample. The signals of obidoxime (t_R 0.5) and phenol (t_R 2.6) dominate the atropine

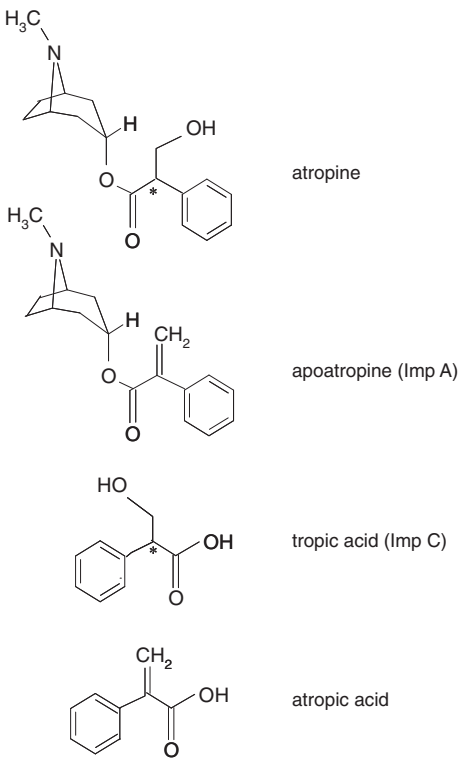

Figure 1. Atropine and its degradation products tropic acid, atropic acid and apoatropine in the investigated medicines for injection.

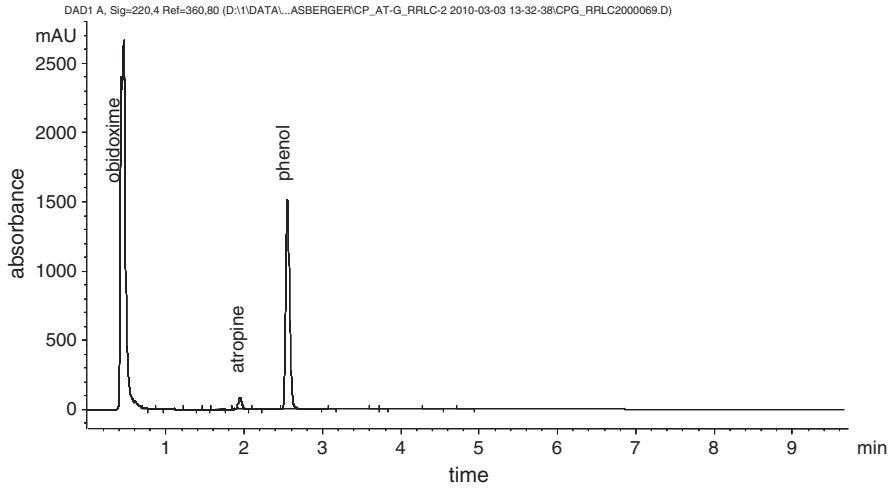

Figure 2. Separation of obidoxime (≈ 27 mg·ml^{-1}), atropine (≈ 0.2 mg atropine·ml^{-1}) and phenol (≈ 1.0 mg·ml^{-1}) in AI 1 on Zorbax® SB C18 RRHT, 50 x 4.6 mm, 1.8 µm, aqueous phosphoric acid acetonitrile gradient (Table 1), flow rate 1.0 ml·min^{-1}, oven temperature 50 °C, UV detection at 220 nm, injection volume 2 µl.

peak (t_R 1.8) in the chromatogram. Figure 3 demonstrates the separation of the API in the atropine containing chamber from the AI 2. These solutions are unpreserved; nevertheless phenol may be found, caused by leaching from the primary packaging materials.

During the validation of the method the linearity calibration plot ($y = 1300.2x + 5.7587$; $R = 0.9993$; $s_y = 5.592$; $s_{x0} = 0.0043$) over the recommended range (0.31–0.73 mg atropine·ml^{-1}) demonstrated a good correlation between the peak area and the concentration of the analyte. The precision of the method ranges between 0.57% (system precision) and 0.80% (inter-day precision). The accuracy studies (studied range: 0.12/0.40/0.52 mg atropine·ml^{-1}) demonstrate a recovery for atropine of 98%. The calculated peak symmetry for atropine (t_R 1.8 min) is 0.5.

Table 2 summarizes the calculated LODs and LOQs for the tested impurities/analytes as well as the data for the retention. Figure 4 illustrates the separation for the individual analytes (scopolamine, noratropine, tropic acid, phenol, methylparaben, apoatropine, atropic acid) at the 0.5 µg·ml^{-1} level.

For evaluating specifity, test solutions with the excipients of the different products to be analyzed were prepared based on the available product information. As an example, Figure 5 demonstrates that the signals of a preparation simulating the excipients of AI 2 showed no interference with the API, impurities or the other components of interest; comparable results were achieved with a blank (mobile phase A), a citric acid solution and for obidoxime.

For a first evaluation of the applicability of the method for a new stockpiled product (AI 2) samples of this product were tested after six months storage at ICH accelerated storage conditions.[15] Although new signals did appear in the chromatogram (Figure 6), the respective peaks do not interfere with the analytes of interest. Three additional signals in the time window from 0.6 to 1.0 min are significant. It is worth mentioning that the signals for tropic acid (t_R 2.1), phenol (t_R 2.6) and methylparaben (t_R 3.3) increased in comparison to the non-high-temperature-exposed sample (Figure 3).

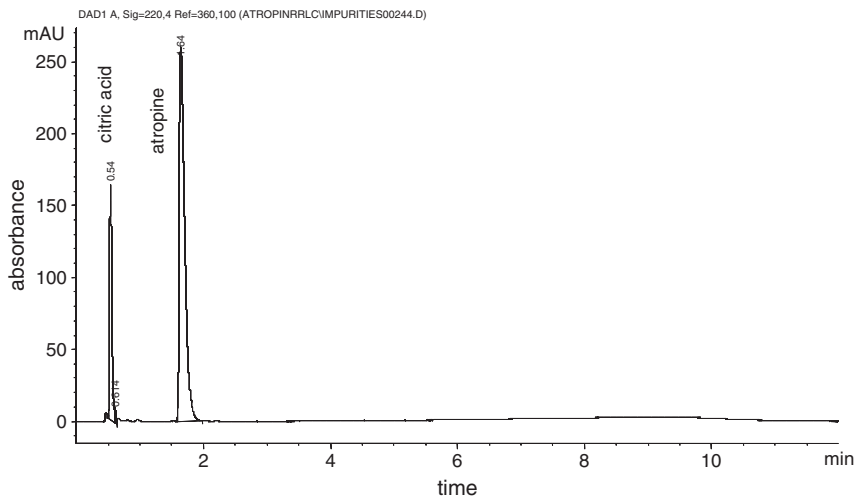

Figure 3. Atropine (≈ 0.5 mg atropine·ml^{-1}) analysis in AI 2 after product procurement on Zorbax® SB C18 RRHT, 50 x 4.6 mm, 1.8 µm, aqueous phosphoric acid acetonitrile gradient (Table 1), flow rate 1.0 ml·min^{-1}, oven temperature 50 °C, UV detection at 220 nm, injection volume 2 µl.

Table 2. Retention times and estimated LOD and LOQ (for standard solutions)

	Retention Time (t_R) (min)	Relative retention (r_G)	LOD (μg·ml^{-})	LOQ (μg·ml^{-})
Scopolamine	1.1	0.6	0.14	0.43
Noratropine (Imp B)	1.6	0.9	0.37	1.56
Atropine	1.8	1	not determined	not determined
Tropic acid (Imp C)	2.1	1.2	0.03	0.09
Phenol	2.6	1.4	0.02	0.06
Methylparabene	3.3	1.8	0.01	0.03
Apoatropine (Imp A)	3.4	1.9	0.06	0.20
Atropic acid	3.9	2.2	0.23	0.76

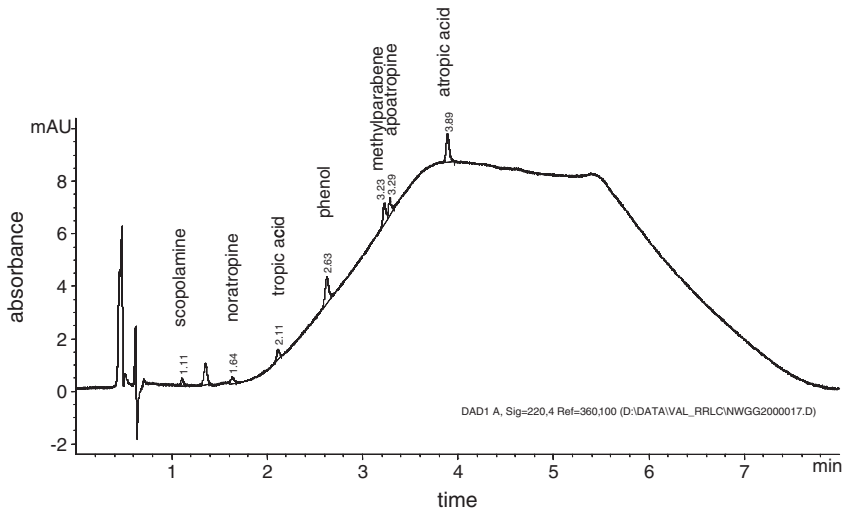

Figure 4. Determination of LOD and LOQ; chromatographic separation with a mix of analytes (concentration level 0.5 μg·ml^{-1}) on Zorbax® SB C18 RRHT, 50 x 4.6 mm, 1.8 μm, aqueous phosphoric acid acetonitrile gradient (Table 1), flow rate 1.0 ml·min^{-1}, oven temperature 50 C, UV detection at 220 nm, injection volume 2 μl. Remark: the signal with t_R 1.35 is under investigation.

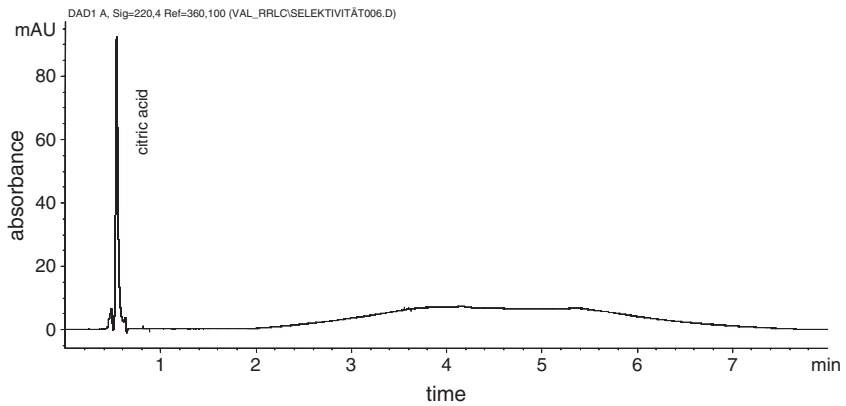

Figure 5. Chromatographic separation of a solution simulating the excipients of AI 2 on Zorbax® SB C18 RRHT, 50 x 4.6 mm, 1.8 μm, aqueous phosphoric acid acetonitrile gradient (Table 1), flow rate 1.0 ml·min^{-1}, oven temperature 50 °C, UV detection at 220 nm, injection volume 2 μl.

Figure 7 demonstrates that the method will also be suitable to test AtroPen® auto-injectors. This product contains phenol (t_R 2.6 min) as a preservative.

The comparison of the atropine and tropic acid results (HPLC method of the MAH and RRLC) in 10 batches of AI 1demonstrate that there is no statistically significant difference between input groups (t-test; atropine: P = 0.423; tropic acid: P = 0.440).

With the developed RRLC method, 104 batches of AI 1 and 9 batches of S1 were examined (Table 3). The API-solution of auto-injectors (production 1987–1989) contained tropic acid up to 4.7% (% of API) whereas apoatropine was only found in the S1 samples; atropic acid was not detectable in these samples. The pH of atropine-containing product solution is added for information purposes as the stability of atropine in aqueous solutions depends on this parameter.[19]

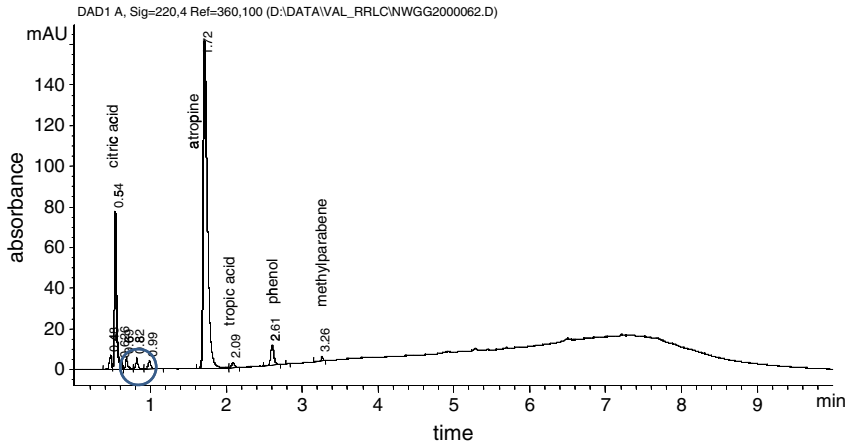

Figure 6. Atropine analysis in AI 2 (stored for six months at 40 C ± 2 C/75% ± 5% relative humidity) on Zorbax® SB C18 RRHT, 50 x 4.6 mm, 1.8 µm, aqueous phosphoric acid acetonitrile gradient (Table 1), flow rate 1.0 ml·min^{-1}, oven temperature 50 °C, UV detection at 220 nm, injection volume 2 µl.

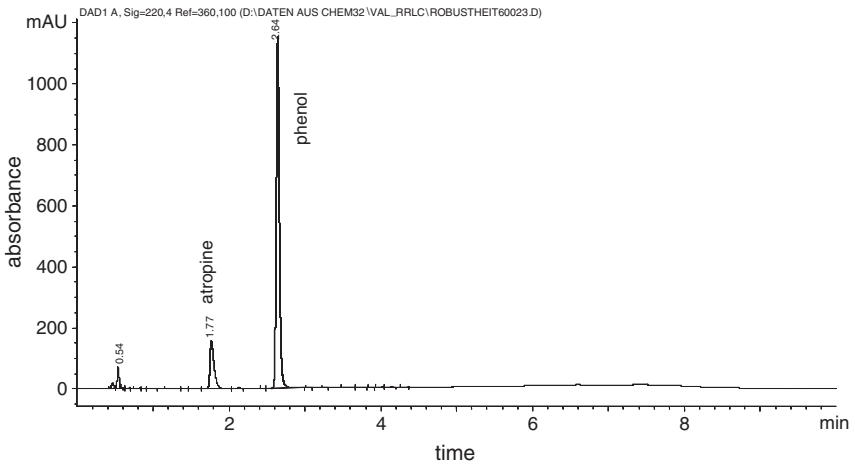

Figure 7. Chromatographic separation of atropine (≈ 0.5 mg atropine·ml^{-1}) and phenol (≈ 0.8 mg ·ml^{-1}) in an AtroPen® auto-injector on Zorbax® SB C18 RRHT, 50 x 4.6 mm, 1.8 µm, aqueous phosphoric acid acetonitrile gradient (Table 1), flow rate 1.0 ml·min^{-1}, oven temperature 50 °C, UV detection at 220 nm, injection volume 2 µl

Table 3. Tropic acid, atropic acid, and apoatropine in products.

	Production, batches (year, number of investigated batches)		pH
AI 1	1987 (n = 30)		2.4 - 2.5
	1988 (n = 41)		2.3 - 2.6
	1989 (n = 33)		2.3 - 2.6
S1 (2 mg·ml^{-1})	2000, 2001(n = 8)		3.4 - 3.5
S1 (100 mg·10 ml^{-1})	2007 (n = 1)		3.2
	atropic acid (% of API)	apoatropine (% of API)	tropic acid (% of API)
AI 1 (1987)	n. d.	n. d.	1.9 - 3.9
AI 1 (1988)	n. d.	n. d.	2.3 - 4.7
AI 1 (1989)	n. d.	n. d.	2.3 - 3.6
S1 (2 mg·ml^{-1})	n. d.	0.2 – 0.4	0.6 - 0.8
S1 (100 mg·10 ml^{-1})	n. d.	0.4	0.4

n. d.: not detected

Conclusion and discussion

RRLC substantially improves the speed of analysis of atropine-containing preparations. A time and solvent reduction of at least 75% was achieved. Depending on the actual objective, this improvement may be used to include more samples (e.g. from different locations or batches) into quality monitoring programmes in order to increase the safety of all stockpiled products. Another

important point with regard to authorized products is that methods must prove – regardless of their validation – that they will generate results comparable to approved methods.

Preliminary investigations on the application of the RRLC method to other products (atropine API and AtroPen® auto-injectors) indicated the separation of atropine and its known impurities.

Further investigations must also be conducted on the identification of degradation products and components leached from packaging materials during long-term stockpiling.

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