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Quantitative colorimetric and gas chromatographic determination of arecaidine propargyl ester[1]

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Abstract

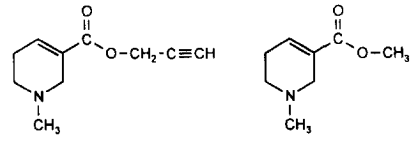
Arecaidine propargyl ester (APE) is a potent muscarinic agonist often used in pharmacological studies. To date, no sensitive quantitative analytical method for APE has been published. In this study, two methods for the quantitative determination of APE are compared: a colorimetric assay, based on the formation of the corresponding ferric(III)–hydroxamic acid complex, and a direct gas chromatographic method, using arecoline as the internal standard. The latter method was found to be more precise. The utility of the gas chromatographic assay was further demonstrated in a stability study of the drug in the biological fluid aqueous humor of rabbits.

Keywords: Arecaidine propargyl ester

1. Introduction

Cholinergic agonists have been used for many years in the treatment of glaucoma. The most commonly used cholinergic drugs are pilocarpine, aceclidine and carbachol. Many other cholinergic agents have been tested, but due to their poor corneal penetration or cholinergic side effects they have not been used clinically.

Arecaidine propargyl ester (APE) is a selective and highly potent muscarinic agonist that may offer a new perspective in the therapy of glaucoma. Although both are arecaidine esters, APE is considerably more potent than arecoline (Fig. 1) [1]. Although a lot of work has been done to characterize its



APE arecoline

Fig. 1. Chemical structures of the muscarinic agonists arecaidine propargyl ester (APE) and arecoline.

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[1] Dedicated to Prof. Dr. Karl Thoma (Munich, Germany) on the occasion of his 65th birthday.

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pharmacology [2–8], no sensitive quantitative analytical method has been published.

In this paper we describe a colorimetric and a gas chromatographic method for the quantitative determination of APE. In addition, the application of the gas chromatographic assay to stability studies of the drug in the aqueous humor of rabbits is reported. The determination of drug levels in this compartment of the eye is essential for an estimation of its corneal penetration and for the characterization of the pharmacokinetic behaviour of the compound in intraocular structures.

2. Experimental

2.1. Reagents and chemicals

Arecoline hydrobromide, hydroxylamine hydrochloride and hydrochloric acid were purchased from Fluka (Buchs, Switzerland). Dichloromethane, sodium hydroxide, ferric(III)–chloride hexahydrate and hydrochloric acid 0.1 *M* were obtained from Merck (Darmstadt, Germany). Sodium hydrogen carbonate was purchased from Roth (Karlsruhe, Germany). All reagents were of analytical grade and were used as received. The arecaidine propargyl ester hydrobromide (APE·HBr) was synthesized according to a method previously described [8]. All the solutions used for the assay were prepared with distilled water unless otherwise stated.

2.2. Instrumentation

For the colorimetric assay the absorption of the solutions was measured using a spectrophotometer type Beckman DU 7 (Beckman, Fullerton, CA, USA) at a wavelength of 514 nm.

The gas chromatographic system consisted of a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector system (FID) attached to a HP 3396 A integrator (Hewlett Packard, Bad Homburg, Germany). A 25 m×0.32 mm I.D. fused-silica capillary column with a stationary phase of film thickness 1.0 μm (FS-SE-54-CB-1.0, Macherey-Nagel, Düren, Germany), consisting of 5% phenylsilicone, 1% vinylsilicone and 94% methylsilicone, was used with helium carrier gas at a flow-rate of 1.1 ml min^{-1} at 200°C. The split–splitless injection port was used in the split mode with a split ratio of 6:1. A borosilicate liner HP 19251-60450 (Hewlett Packard), plugged with silanized glass wool, was installed. Both the injection port and the detector were held at a temperature of 250°C. The oven temperature was held isothermal for 2.0 min at 100°C, after which the temperature was raised from 100°C to 240°C at a rate of 10°C min^{-1} and then held constant at 240°C for a further 10 min.

2.3. Sample preparation

For the colorimetric assay an accurately measured volume of APE·HBr solution, containing between 0.8 and 2.5 mg of APE·HBr, was transferred to a 10.0 ml volumetric flask. Equal volumes (1.0 ml) of 10% aqueous solution of hydroxylamine hydrochloride and of 3.5 *M* sodium hydroxide solution were added. After mixing well, the solution was allowed to stand for 10 min at ambient temperature. Then 1.0 ml of 3.5 *M* hydrochloric acid was added slowly, followed by 1.0 ml of a 3.5 *M* solution of ferric(III)–chloride hexahydrate in 0.1 *M* hydrochloric acid. The resultant mixture was diluted with water to attain a final volume of 10.0 ml. Directly after sample preparation, the absorption was measured spectrophotometrically at 514.0 nm against distilled water as the blank.

In order to prepare the samples for the gas chromatographic determination, 100.0 μl of an APE solution, containing between 0.10 μg and 8.5 μg APE·HBr, were transferred to a conical mini vial (Mini Vial 3.0 ml, Alltech, Unterhaching, Germany). 100.0 μl of a 0.004% solution of arecoline hydrobromide, 600.0 μl of a 3% solution of sodium hydrogen carbonate and 1.0 ml of dichloromethane were added. The biphasic mixture was shaken for about 2 min. After separation of the phases, the organic compound was transferred to a second vial and evaporated under a gentle stream of nitrogen to a volume of about 30 μl. A 2 μl volume of this sample was injected into the GC system by the technique of hot needle injection.

2.4. Calibration curves

A calibration curve for the photometric assay was prepared by using 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 ml of an aqueous stock solution containing 824 mg l^{-1}

APE·HBr. After addition of the reagents to the volumes of stock solution as described above, the samples were diluted with water to a final volume of 10.0 ml. The absorbance measured was correlated to the concentration (mg l^{-1}) of APE·HBr present.

For the gas chromatographic assay, a calibration curve was prepared by using 2.5, 5.0, 10.0, 25.0, 75.0 and 100.0 μl of an aqueous stock solution containing 42.8 mg l^{-1} APE·HBr. Additionally, 75.0 and 100.0 μl volumes of a second stock solution containing 85.6 mg l^{-1} APE·HBr were employed. The solutions were diluted with distilled water to a final volume of 100 μl. The internal standard, arecoline hydrobromide, was added to the solution at a concentration of 38.6 mg l^{-1}. These solutions were than extracted and analyzed by GC as described under sample preparation. For the calibration curve the concentration (mg l^{-1}) of APE·HBr in the aqueous drug solution used for sample preparation was correlated to the ratio between the areas of the APE and arecoline peaks.

2.5. Recovery

The recovery of APE·HBr using the proposed photometric and GC methods was determined by comparing actual sample concentrations with concentrations calculated using the calibration curve. In the case of the photometric method, the samples were prepared by reacting 0.5 ml, 1.0 ml and 2.5 ml (=concentration levels: low, medium, high) of an aqueous stock solution containing 1600 mg l^{-1} APE·HBr according to the method of sample preparation described above. In the case of the GC method, 25.0 μl, 50.0 μl and 100.0 μl (=concentration levels: low, medium, high) of aqueous solutions containing APE·HBr in a concentration of 80 mg l^{-1} were analyzed according to the method of sample preparation described above.

2.6. Stability of the color

Samples of APE·HBr, prepared according to the method described for the photometric assay, were stored at 25°C over a period of 40 min. Within this period, the absorbance of the ferric(III)–hydroxamic complex was measured at intervals of 10 min.

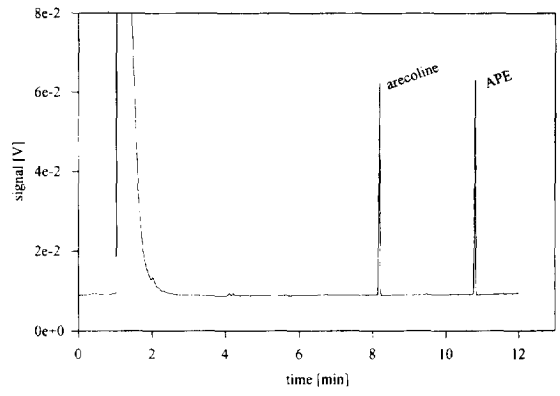

Fig. 2. Representative chromatogram of the gas chromatographic assay: the retention times for arecoline (internal standard) and APE were 8.2 min and 10.8 min, respectively. 100.0 μl of an 30 mg l^{-1} APE·HBr solution and 100.0 μl of a 40 mg l^{-1} solution of arecoline hydrobromide were used for the sample preparation.

2.7. Identification of constituents

A typical chromatogram is shown in Fig. 2. The total retention times for APE and arecoline, used as the internal standard, were 10.8 min and 8.2 min, respectively.

2.8. Stability of APE in aqueous humor

Six New Zealand White Albino Rabbits (Thomae, Biberach a.d. Riss, Germany) were killed by rapid injection of 1 ml T61® (Hoechst, Frankfurt, Germany) into the marginal ear vein. The anterior chambers of both eyes were punctured and 200 μl of aqueous humor were aspirated with a syringe. The samples were immediately pooled in a vial and placed in an ice bath until incubation with APE.

Volumes of 0 μl, 100 μl and 500 μl aqueous humor were added to 450.0 μl volumes of an APE solution in isotonic phosphate buffer pH 7.4 containing 84.4 mg l^{-1} APE·HBr. The mixtures were diluted with phosphate buffer to 1000.0 μl and stirred in an Eppendorf Thermomixer 5436 (Hinz, Hamburg, Germany) at 37°C and 500 min^{-1} over a period of 300 min. At predetermined times, 100.0 μl aliquots of the mixtures were withdrawn and 100.0 μl of a 0.004% solution of arecoline hydrobromide was added. The samples were then heated to 100°C (30 s) in order to inactivate the enzymes of the aqueous humor.

The concentration of APE·HBr in the aqueous

humor samples was determined as a function of time using the gas chromatographic assay.

3. Results and discussion

The molecular structure of the muscarinic agonist arecaidine proparyl ester (APE) contains no functional groups conducive to a simple colorimetric assay (Fig. 1). The formation of hydroxamic acid and the subsequent colorimetric assay of its iron complex is a useful approach to the quantitative determination of drugs (e.g., esters) for which no direct photometric assay exists [9,10]. In addition to its application to ester structures, this method has been used extensively for the assay of various lactones, anhydrides, amides and nitriles. For the proposed photometric method, the formation of the corresponding ferric(III)–hydroxamic acid complex of APE was adapted from a method previously described for pilocarpine by Brochmann-Hanssen et al. [11]. Absorption spectra of the solutions obtained exhibited a maximum at 514 nm.

Plotting the measured absorbance against the APE concentrations led to a calibration curve for the colorimetric assay of APE·HBr that was linear over a concentration range between 41.2 and 412.0 mg l^{-1} ($y=0.00341x+0.03525$, S.D. slope 0.0001, S.D. intercept 0.02465, standard error 0.03167, $r=0.9983$, $n=6$). The limit of quantitative detection in the solution used for the colorimetric assay is 41.2 mg l^{-1} APE·HBr. Since the latter concentration results after addition of the derivatisation reagents, the detection limit in the biological samples is 68.7 mg l^{-1}. The recovery (Table 1) and its associated confidence intervals was similar at the three concentrations tested, and close to 100%.

As pointed out previously [11], the formation of the ferric(III)–hydroxamic acid complex yielded unstable colors. Therefore, the stability of the color was determined over a period of 40 min by measuring the change in absorption with time. To avoid interference from the formation of gas bubbles on the walls of the cell, the cell was freshly filled with the colored solution prior to each measurement. Solutions at concentration 164.8 mg l^{-1} showed an initial absorption of 0.6462, which decreased over 40 min to 0.6195, corresponding to a reduction in absorbance of 4.1%. For this reason, measurement immediately either after filling, or at a defined time after sample preparation, is crucial to accuracy and reproducibility of the results. Storage of the samples after conversion to the colored iron complex should be avoided.

The sensitivity of the proposed colorimetric method was insufficient for quantitative determinations of APE in the biological fluid aqueous humor. Drug concentrations typically achieved in ocular tissues after topical application of eye drops are in the range of 10^{-5}–10^{-4} *M*. Based on the molecular weight of 260.1 g mol^{-1}, the minimum aqueous humor concentrations of APE·HBr is expected to be about 3.0 mg l^{-1}. For this reason, and to enhance selectivity for APE, a gas chromatographic assay was de-

Table 1
Recovery data for the colorimetric and gas chromatographic assays at different concentration levels

	Colorimetric assay			Gas chromatographic assay		
	Low (80 mg l^{-1})	Medium (160 mg l^{-1})	High (400 mg l^{-1})	Low (20 mg l^{-1})	Medium (40 mg l^{-1})	High (80 mg l^{-1})
Recovery (%)	96.74	99.43	98.18	101.64	99.71	98.04
S.D. (%)	3.17	3.76	1.02	1.93	1.37	1.25
Confidence interval (P=95%)						
Upper limit (%)	100.68	102.12	99.45	104.04	100.97	99.59
Lower limit (%)	92.80	96.74	96.91	99.24	98.44	96.49
Maximum (%)	101.48	104.37	99.16	104.48	101.29	99.07
Minimum (%)	94.83	94.65	96.74	99.76	97.63	96.36
Number of data	5	10	5	5	7	5

The gas chromatographic assay was found to be more accurate than the spectrophotometric assay with respect to recovery.

veloped. Arecoline hydrobromide was used as the internal standard (Fig. 1).

The calibration curve for the gas chromatographic assay determined by plotting the APE concentration against the ratio area of the APE peak to area of the arecoline peak was linear over a concentration range between 1.07 mg l^{-1} and 85.6 mg l^{-1} (y= 0.03212x+0.01373, S.D. slope 0.00036, S.D. intercept 0.01455, standard error 0.03022, r=0.99957, n=9). The recovery at three concentration levels and the confidence interval of the mean are shown in Table 1. The gas chromatographic assay was found to be more accurate than the spectrophotometric assay with respect to recovery. The enhanced sensitivity of the gas chromatographic method enables the quantification of concentrations 70 times lower than under photometric conditions and, therefore, determination of the drug levels expected in aqueous humor samples. The limit for a qualitative recording of the drug was determined to be 100 μg l^{-1}.

The dichloromethane extracts of APE·HBr and the internal standard were stored at a temperature of −20°C over a period of several days in order to prevent any solvent evaporation. Within this time the samples remained stable with regard to the ratios of the APE peak areas to the areas of the arecoline peaks. Furthermore, no impurity peaks were detected after storage.

To evaluate the utility of the proposed gas chromatographic method for quantification of APE in aqueous humor samples of rabbits, the stability of the drug in presence of different amounts of the biological fluid was evaluated. Previously it had been shown that eye compartments such as the cornea, iris–ciliar body and aqueous humor contain esterases capable of hydrolysing model compounds like α-naphthyl acetate [12,13]. A blank aqueous humor sample prepared according to the gas chromatographic sample preparation procedure showed no peaks and was not different from the baseline chromatogram. This demonstrates that the extraction procedure using dichloromethane was selective for the drug and internal standard over products of esterase cleavage such as arecaidine and propargylalcohol. This selectivity was expected, since the acidic compound arecaidine is not extracted into dichloromethane under the given sample preparation procedure and propargylalcohol has the same retention as dichloromethane under the chromatographic conditions employed.

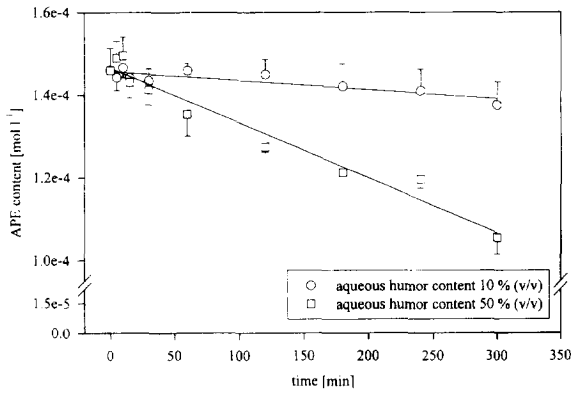

Fig. 3. Time dependent reduction of the APE content in the aqueous humor of rabbits. APE was used at a concentration of $1.46 \cdot 10^{-4}$ M. This was in the range of drug concentrations typically achieved in ocular tissues.

A time dependent reduction in the APE content was observed in the presence of the aqueous humor (Fig. 3). Over the same period of 300 min, a slight reduction in the content of the APE control solutions from $1.460 \cdot 10^{-4}$ M to $1.428 \cdot 10^{-4}$ M was also observed. This reduction was not significant (P= 0.05; n=4; Students t-test). In order to determine the esterase activity for the hydrolysis of APE, the slopes of the decomposition curves, representing the velocity of hydrolysis, were calculated by linear regression analysis. By extrapolation to the decomposition velocity of the drug in pure aqueous humor, the esterase activity was calculated to be $2.68 \cdot 10^{-7}$ mol l^{-1} min^{-1}. Taking into account the protein concentration in the aqueous humor of rabbits (554 mg l^{-1}) [14] the esterase activity was calculated to be $4.83 \cdot 10^{-4}$ U mg^{-1} (Unit=μmoles of substrate hydrolyzed per min). The result suggests that significant metabolization of APE must be expected after topical application to the eye and subsequent penetration into intraocular structures.

4. Conclusions

Two different methods for the quantitative determination of arecaidine propargyl ester (APE) were

compared. The colorimetric assay was developed as a fast and facile method, with the restriction that the lower limit of quantification is about 68.7 mg l^{-1}. In contrast, the gas chromatographic assay has a minimum quantifiable concentration in the range of 1.0 mg l^{-1}. With respect to the recovery, the GC method was found to be more accurate. The gas chromatographic method described was shown to be suitable for determining concentrations of APE in aqueous humor, a necessary step in the characterization of the pharmacokinetics of the drug after topical application to the eye. The stability of the drug in the aqueous humor of rabbits was evaluated. A time-dependent degradation of APE occurred, indicating that the drug is subject to attack by esterases present.

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