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Sensitive high-performance liquid chromatographic assay for pilocarpine in biological fluids using fluorescence derivatisation

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Abstract

A sensitive assay for pilocarpine in biological fluids has been developed involving HPLC of a fluorescent derivative of 4-bromomethyl-7-methoxycoumarin. Pilosine as internal standard was added before the derivatisation step. The fluorescent derivatives were well resolved and separated from excess reagent and endogenous compounds on a cyanopropyl silica column. The detection limit of pilocarpine in biological fluids was 1.0 ng/ml and the assay was linear up to a concentration of 150 ng/ml. The assay was applied to a preliminary study of pilocarpine disposition in man after a single oral dose. This is the first report of pilocarpine excretion into saliva.

Keywords: Derivatization, LC; Pilocarpine

1. Introduction

Pilocarpine, a muscarinic agonist, has been widely used as a topical agent for the treatment of glaucoma [1]. Recently it has emerged as the drug of choice for the management of patients with xerostomia [2] either in association with Sjögren's syndrome [3] or following radiation therapy for head and neck cancer [4]. To date, no detailed information is available on the pharmacokinetics of pilocarpine in man due to the difficulty of analysing the low concentrations in vivo after a therapeutic dose. In order to rectify this situation we have developed a sensitive assay for pilocarpine which is capable of detecting the drug in biological fluids at therapeutic concentrations.

A number of assays for the determination of pilocarpine in aqueous humour have been developed. Mitra et al. [5] and Rendi et al. [6] derivatised pilocarpine using 4-nitrobenzyl bromide to increase the UV absorptivity for HPLC. The assay was linear in the concentration range 0.25–10 μg/ml with an approximate practical sensitivity limit of 0.05 μg/ml. Matsuura et al. [7] used HPLC–MS to determine pilocarpine but this method requires costly instrumentation. Bayne et al. [8] acylated pilocarpine with heptafluorobutyric anhydride and determined the derivative by GC with an electron capture detector. Although sensitive, this assay involved a complex series of extraction, derivatisation and clean-up steps.

The first assay developed specifically for plasma was by Weaver et al. [9] who used HPLC with UV detection at 214 nm to carry out a pharmacokinetic study in anaesthetised dogs [10]. Their assay had sufficient sensitivity to quantitate pilocarpine at

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concentrations as low as 10 ng/ml but it has not been applied to the determination of pilocarpine in man. In order to carry out a comprehensive study of the disposition, pharmacokinetics and pharmacodynamics of the drug in man, an assay with a lower quantitation limit is required.

A more sensitive strategy than derivatisation for UV detection is to produce a fluorescent derivative as suggested by Mitra et al. [5]. 4-Bromomethyl-7-methoxycoumarin (BrMmc) has been used as a fluorescence tag for carboxylic acids [11] but its application to the determination of amines has not been exploited. Yoshida et al. [12] applied BrMmc labelling to the trace analysis of pyrimidines and related compounds extracted from serum. Interference from excess reagent was removed by reacting it with 4-nitrobenzoic acid to give a non-fluorescent ester.

In the method reported here pilocarpine is extracted from biological fluids and analysed by HPLC of the fluorescent quaternary ammonium salt (QAS) on a cyanopropyl silica column. Pilosine is added as the internal standard before the derivatisation step. The assay has adequate sensitivity for the analysis of therapeutic concentrations of pilocarpine in biological fluids and has been applied to a preliminary study of pilocarpine disposition in man following an oral dose.

2. Experimental

2.1. Chemicals

Pilocarpine base obtained from BDH Chemicals (Poole, UK) was analysed by HPLC [13,14] and shown to contain 6.3% of pilocarpic acid. 4-Bromomethyl-7-methoxycoumarin (approx. 97% by HPLC) and pilocarpine hydrochloride for assay development were purchased from Sigma Chemical (St. Louis, MO, USA). Pilocarpine hydrochloride for human ingestion was from Sigma Pharmaceuticals (Melbourne, Australia). Isopilocarpine was obtained from Aldrich Chemical (Milwaukee, WI, USA) and pilosine from L. Light and Co. (Colnbrook, UK). HPLC-grade acetonitrile and methanol were from Mallinckrodt (Paris, KY, USA). Ethylenediaminetetraacetic acid (EDTA) dipotassium, HPLC-grade chloroform, AnalaR-grade diethylamine and orthophosphoric acid were from BDH. Analar-grade acetone obtained from Rhone Poulenc (Melbourne, Australia) was dried over molecular sieves (4 Å) and distilled. Diethyl ether was dried over sodium. Water for HPLC was distilled and passed through a reverse osmosis Milli-Q Reagent Water System. The mobile phase was filtered through a 0.45-μm filter and degassed for 30 s with sonicator before use. Plasma for assay development was obtained from the Blood Bank, Dunedin Hospital. Blood samples for the disposition study were collected in silicone coated Vacutainer tubes (10 ml). Samples of all biological fluids were preserved by addition of an aqueous solution of EDTA dipotassium (14%).

2.2. Apparatus

Melting points were determined on an electrothermal capillary type apparatus; temperatures were uncorrected. Infrared spectra were obtained on a Bio-Rad Model FTS-7 infrared spectrophotometer with a Data station 3200. NMR spectra were obtained on a 200-Hz Gemini NMR spectrometer using deuterated methanol as solvent and the methanol peaks as internal references. Mass spectra were recorded on a Kratos MS80RFA spectrometer using fast-atom bombardment, with xenon as accelerator gas and nitrobenzyl alcohol as support matrix at a scan rate of 3 s/dec and a resolution of 1000. A Savant Speed Vac concentrator Model SVC-200H equipped with refrigerated vapour traps RVT 4104 and VP 100 and a two-stage Savant pump was used for solvent evaporation. All glassware was silanized by treating with dimethyldichlorosilane vapour for 12 h followed by deactivation by methanol vapour.

The HPLC system consisted of a Shimadzu AS10 isocratic pump with a Model RF530 fluorescence detector. The excitation and emission wavelengths were set at 324 and 400 nm, respectively. Injection was via a 100-μl fixed-loop Rheodyne injector. Separation was achieved at 37°C using a cyanopropyl silica column (220×4.6 mm) and guard column (20×4.6 mm) both from Brownlee Labs. The mobile phase was a mixture of 3 m*M* diethylamine adjusted to pH 3.5 with 1 *M* phosphoric acid and acetonitrile (70:30) at a flow-rate of 1 ml/min. The

Peak Simple chromatography data system from SRI Instruments (Torrance, CA, USA) was used for data acquisition and analysis. Area under the curves (AUC) for concentration–time profiles were calculated using Minim version 3.0 [15].

2.3. Preparation of derivatives

2.3.1. Pilocarpine

A solution of BrMmc (160 mg, 0.6 mmole) in 150 ml of dried acetone was added to pilocarpine (50 mg, 0.24 mmole) in a round bottom flask. The flask was stoppered, protected from light, and stirred for 2 days. A white precipitate separated from solution. This was filtered off, washed with acetone (10 ml) and diethyl ether (3×10 ml), and then dried under vacuum overnight at room temperature. The filtrate was evaporated under vacuum to a volume of ca. 20 ml before diethyl ether (30 ml) was added. A white precipitate formed which was separated, washed and dried as before. The two crops of the product were examined by TLC on silica plates developed in 1.0% glacial acetic acid in methanol. Both crops gave a single identical spot (R_F=0.15) of 4-(3′-pilocarpinyl)methyl-7-methoxycoumarin bromide (75 mg; 65% yield), m.p. 223–226°C; IR $\nu_{C=O}$ 1766, 1736, ν_{C-O} 1177, 1147 cm^{-1}; ^1H NMR (methanol-d_4) 1.12 (3H, t, CH_3), 1.50–1.95 (2H, m, CH_2), 2.55–3.00 (3H, m, C_4–H, C_8–H), 3.86 (3H, s, NCH_3), 3.94 (3H, s, OCH_3), 4.0–4.4 (3H, m, C_3–H, C_5–H), 4.83 (2H, s, NCH_2), 5.91 (1H, s, δ-lactone), 7.01 (2H, m, aromatic), 7.67 (2H, m, aromatic, heterocyclic) ppm; mass (FAB) *m/z* 875, 873 (quaternary cation plus quaternary ammonium bromide), 477, 475 (quaternary ammonium bromide-1), and 397 (quaternary cation); UV (methanol) λ_{max} 210, 218 and 324 nm, molar absorptivities $2.3 \cdot 10^4$, $2.2 \cdot 10^4$ and $1.6 \cdot 10^4$, respectively; fluorescence (methanol) λ_{ex} 324, λ_{em} 400 nm; Found: C, 55.48; H, 5.34; N, 5.79; Br, 16.57. $C_{22}H_{25}BrN_2O_5$ requires C, 55.36; H, 5.28; N, 5.87; Br 16.74%.

2.3.2. Pilosine

Pilosine (24.1 mg, 0.084 mmole) was mixed with BrMmc (35 mg, 0.13 mmole) in acetone (75 ml) in a round-bottom flask. The flask was stoppered, protected from light and kept at room temperature for two days. The solution was evaporated under vacuum to about 20 ml before diethyl ether (30 ml) was added. A white precipitate was formed which was filtered, washed with acetone and diethyl ether and dried under vacuum to yield a QAS (23.8 mg, 51%), m.p. 175–178°C; IR ν_{OH} 3261, $\nu_{C=O}$ 1762, 1718, ν_{C-O} 1155, 1137 cm^{-1}; mass (FAB) *m/z* 475 (quaternary cation), 369 (quaternary cation minus C_6H_5CHO); UV (methanol) λ_{max} 210, 218sh, and 324 nm, molar absorptivities $3.4 \cdot 10^4$, $1.96 \cdot 10^4$ and $3.2 \cdot 10^4$, respectively; fluorescence (methanol) λ_{ex} 324, λ_{em} 400 nm; found: C, 57.12; H, 4.84; N, 4.55; Br, 15.58. $C_{27}H_{27}BrN_2O_6$ requires C, 58.39; H, 4.90; N, 5.04; Br, 14.3%.

2.4. Assay procedure

Stock solutions (0.01%) of pilocarpine hydrochloride in water and pilosine in methanol were prepared and kept at 4°C. The solutions were stable for at least 1 month. A solution containing pilosine (50 μl, 0.50 μg/ml) was added to 3-ml samples of plasma which were extracted with chloroform (2×3 ml). The combined chloroform extracts were evaporated to dryness. A solution of BrMmc in acetone (200 μl, 0.08%) was added to each residue and the tubes tightly closed and allowed to stand at 37°C for 48 h. The mixture was then evaporated to dryness and the residue dissolved in mobile phase (1.0 ml). After centrifuging at 15 000 rpm (20 000 *g*) for 5 min the supernatant was injected into the HPLC apparatus. Aliquots of urine (50 μl) or saliva (1.5 ml) were treated with sodium bicarbonate solution (10%, 5 μl and 90 μl respectively) then analysed in a similar way to plasma.

2.5. Investigation of derivatisation conditions

To investigate the optimum conditions for derivatisation, solutions containing both pilosine (100 ng) and pilocarpine free base (100 ng) in methanol were evaporated to dryness and BrMmc solution (200μl, 0.08%) added to the residues. Mixtures were incubated at 30, 40 and 50°C for 12, 24 and 48 hours. Each mixture was evaporated, dried and analysed as described above. The effect of reagent concentration was studied using chloroform extracts of blood bank plasma (3 ml). After evaporating to dryness, solutions containing pilosine (100 ng) and

pilocarpine (100 ng) in methanol were added to the residues. Each mixture was evaporated to dryness and a solution of BrMmc (200 μl of 0.02, 0.04, 0.06, or 0.08%) added. The reaction mixtures were incubated at 37°C for 48 h and the resulting solutions treated as above.

2.6. Assay validation

A series of standard solutions (50 μl) containing pilosine (0.50 μg/ml) and pilocarpine hydrochloride (equivalent to 3.0, 2.0, 1.0, 0.5, and 0.1 μg/ml of free base) were used to spike plasma samples (3 ml) and prepare standard curves. Regression analysis was carried out and standard curves were shown to be linear over this range. The intra-day (n=4) and inter-day (n=4) variabilities at a concentration of 5 ng/ml were 4.81 and 6.9%, respectively and at 150 ng/ml were 2.85 and 3.18%, respectively. The detection limit of pilocarpine in plasma was 1.0 ng/ml (signal-to-noise ratio ≥4).

The efficiency of the extraction from plasma was evaluated by comparing plasma spiked with pilocarpine and pilosine with evaporated plasma extracts spiked with the same amounts of pilocarpine and pilosine. The peak height ratios (mean±S.D., n=3) of pilocarpine to pilosine obtained from the plasma samples spiked with 1 ng and 80 ng were 101.04±1.07% and 99.45±0.54% of those determined by spiking evaporated plasma extracts.

2.7. Human study

A healthy, fasted male subject (49 years, 85 kg) was given a single dose of pilocarpine hydrochloride (equivalent to 10 mg pilocarpine free base), dissolved in 5 ml of water followed by 10 ml of water. Venous blood samples (10 ml) from the resting arm were collected at various times over a period of 5 h. The blood was collected in silicone coated Vacutainer tubes containing 1.0 ml of 14% EDTA dipotassium. Plasma was obtained by centrifuging at 2000 g for 10 min. Saliva samples were collected over 2 h and urine over 7.5 h into preweighed containers. The biological fluids were stored on ice and analysed within 24 h.

3. Results and discussion

Fluorescent QAS of pilocarpine and pilosine were prepared from the respective bases and BrMmc and characterised by IR, NMR, mass spectrometry and elemental analysis. The structures of the derivatives are shown in Fig. 1 and the compounds are analogous to derivatives prepared by Mitra et al. using 4-nitrobenzyl bromide [5]. The fluorescence spectra of the derivatives in methanol are identical so that derivatisation with BrMmc appears eminently suitable for the sensitive detection of pilocarpine by HPLC using pilosine as an internal standard. These solutions and solutions in the mobile phase were stable for at least one week at 4°C.

3.1. Chromatography

Typical chromatograms of blank plasma and a plasma sample containing pilocarpine to which pilosine was added as internal standard are shown in Fig. 2. The retention times of the derivatives of pilocarpine and pilosine were 13.86 and 12.36 minutes respectively and the resolution factor (R_s) was 1.36. No interfering peaks were observed at these retention times for blank plasma (Fig. 2a). Similar chromatograms were obtained from urine and saliva samples. The fluorescent QAS were retained on the column by increasing the pH and decreasing the concentration of diethylamine in the mobile phase, while the retention of the unreacted reagent was not significantly affected by these factors. Acetonitrile was more effective than methanol in eluting the unreacted reagent. The derivative

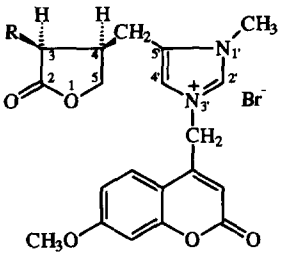

Fig. 1. Structures of 4-methyl-7-methoxycoumarin quaternary ammonium bromides of pilocarpine, R=CH_3CH_2, and pilosine, R=C_6H_5CHOH.

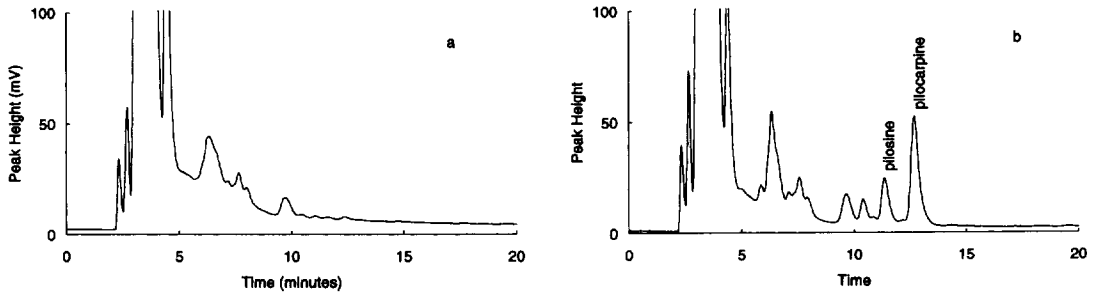

Fig. 2. Chromatograms obtained from (a) blank plasma and (b) plasma from a human volunteer 10 min after oral administration of pilocarpine. In the latter chromatogram pilosine (25 ng) was added to plasma (3 ml) before extraction and derivatisation.

of isopilocarpine was not resolved from that of pilocarpine under these conditions.

3.2. Derivatisation

To optimise the conversion of pilocarpine to its fluorescent derivative, a range of reaction conditions was investigated. For sensitive analysis at low concentrations of pilocarpine, incubation with BrMmc at 37°C for 48 h was deemed optimum. At 30°C the reaction was incomplete after 12 h whereas at 50°C additional HPLC peaks were observed suggesting the reagent or the derivative was undergoing some decomposition. BrMmc appeared to react with pilocarpine a little more slowly than with pilosine but the ratio of the peak heights of the two derivatives did not vary significantly with time. The yield of derivatives did not increase greatly with incubation times exceeding 12 h so that a reaction time of 12 h could probably be used with little loss of sensitivity.

We also investigated the reagent concentration required to maximise derivatisation in the presence of endogenous amines extracted from plasma. Although the ratio of the peak heights of pilocarpine to pilosine did not change with increasing the reagent concentration, the actual peak heights did increase. However, since the solubility of BrMmc in acetone at 37°C was only slightly greater than 0.08%, higher concentrations of reagent were not used.

3.3. Human study

Pilocarpine was first detected in plasma 5 min after ingestion of a single 10-mg oral dose. The plasma concentration–time profile is shown in Fig. 3a. The maximum plasma concentration, C_{max}, was 53.8 ng/ml observed after 30 min (T_{max}). The AUC was 87.4 h ng ml^{-1} and the elimination rate constant was 0.014 min^{-1}. The amount of pilocarpine excreted unchanged in the urine was 16% of the oral dose (Fig. 3b) with a first order rate constant for excretion of 0.014 min^{-1}. Pilocarpine was also detected in saliva and we are currently comparing the concentration of pilocarpine secreted in the saliva with the concentration detected in plasma.

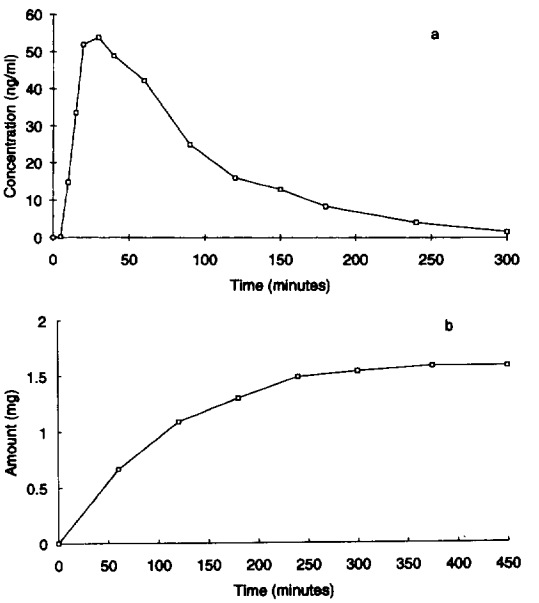

Fig. 3. (a) Concentration of pilocarpine in plasma and (b) cumulative amount of pilocarpine excreted in urine from a human volunteer at various times after oral administration of 10 mg pilocarpine.

We have also detected a significant amount of an unkown metabolite in plasma and urine which is not present in saliva. The metabolite is neither pilocarpic acid, which is not extracted from the plasma, nor isopilocarpine, whose fluorescent derivative is not resolved from that of pilocarpine. Further investigations into the identification of this metabolite are underway.

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