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# Determination of pilocarpine in aqueous humour by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

Kenji Matsuura*, Mituaki Kuwano and Hideo Takashina

*Central Research Laboratories, Santen Pharmaceutical Co., 9-19, Shimoshinjo 3-chome, Higashiyodogawa-ku, Osaka 533 (Japan)*

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ABSTRACT

A new method has been developed for rapid analysis and determination of pilocarpine in aqueous humour using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. The chromatography was carried out on a reversed-phase phenyl column with 0.1% acetic acid–acetonitrile (95:5, v/v). Pilocarpine and its analogues, isopilocarpine, pilocarpic acid and isopilocarpic acid, were separated. An aqueous humour sample was deproteinized with methanol. After evaporation, the residue was dissolved in the mobile phase. The method was applied to the analysis of the metabolite in aqueous humour after the topical application of 2% pilocarpine (w/v) eye-drops. The main metabolite, pilocarpic acid, was easily identified. The protonated molecular ion of pilocarpine was used for the determination. The calibration curve had a good linearity within the concentration range investigated (2 ng to 10 μg/ml). The limit of determination was estimated to be an aqueous humour concentration of *ca.* 2 ng/ml. The method was applied to the determination of unchanged pilocarpine after the topical application of 2% pilocarpine (w/v) eye-drops.

## INTRODUCTION

Pilocarpine hydrochloride, 3-ethyldihydro-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]-2(3*H*)-furanone monohydrochloride, has widely been used as a topical ocular agent for the reduction of the increased intra-ocular pressure associated with glaucoma. It is important to measure the concentration of pilocarpine in aqueous humour after topical application, because pilocarpine works on an iris-cilliary body that comes into contact with aqueous humour. Many assay methods have been developed to determine the concentration of pilocarpine in aqueous humour by high-performance liquid chromatography (HPLC) [1,2] and gas chromatography (GC) [3,4] with precolumn derivatization. In these methods, however, a long time is required for the derivatization of the N-methylimidazole ring of pilocarpine and the extraction with organic solvent.

In recent years, several liquid chromatographic–mass spectrometric (LC–MS) coupling systems and ionization methods have been described [5–7]. LC–MS has emerged as a powerful tool for the analysis of biological samples because it permits the separation and the ionization of non-volatile compounds without derivatization. Atmospheric pressure chemical ionization mass spectrometry (APCI-MS) has also found a variety of analytical applications in the biomedical field [8–11]. The direct analysis of pilocarpine in aqueous humour using LC–APCI-MS without derivatization might markedly shorten the time required for elaborate sample preparation or chemical derivatization.

* Corresponding author.

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This paper describes a method for the rapid analysis and determination of pilocarpine in aqueous humour using LC–APCI-MS.

## EXPERIMENTAL

### *Chemicals*

Pilocarpine hydrochloride and isopilocarpine nitrate were obtained from Aldrich (Milwaukee, WI, USA). Pilocarpic acid was prepared by hydrolysis of pilocarpine hydrochloride according to Bundgaard *et al.* [12]. Acetonitrile was of HPLC quality. The water used in the mobile phase was of Milli-Q grade (Millipore, Bedford, MA, USA), and the mobile phase was passed through a 0.45-$\mu$m filter. The other solvents and reagents were of analytical garde.

### *HPLC conditions*

The HPLC system consisted of a Hitachi Model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 100-$\mu$l loop, and a Hitachi Model 655A-52 column oven. The column was a Cosmosil 5Ph (250 × 4.6 mm I.D., 5 $\mu$m particle size; Nacalai Tesque, Kyoto, Japan). The column oven was maintained at 50°C. The mobile phase consisted of 0.1% acetic acid–acetonitrile (95:5, v/v). The flow-rate was set to 1.0 ml/min. In the case of the determination of pilocarpine in aqueous humour, a switching valve was placed at the rear of the column, and the eluate from the column was diverted to waste for 4 min after injection. Subsequently, the eluate was directed into the APCI interface for 4 min by switching the valve. During the wasting of the eluate, the same mobile phase was directed into the APCI interface by a Jasco Model 880-PU pump (Jasco, Tokyo, Japan).

### *APCI-MS conditions*

The APCI-MS system used was a Hitachi M-8093 APCI interface coupled to a Hitachi Model M-80B double-focusing mass spectrometer. The nebulizer and the vaporizer temperatures were maintained at 350°C and 390°C, respectively. The drift voltage was varied from 150 V to 190 V for the analysis of pilocarpine metabolite and set to 150 V for the determination of pilocarpine. The corona discharge current was 12 $\mu$A throughout the experiments. All mass spectra were gathered under a full-scan operation, scanning the range $m/z$ 1–600 in 4 s. The determination was carried out with selected-ion monitoring (SIM) by monitoring the protonated molecular ion, $m/z$ 209, for pilocarpine. Investigations of the optimal conditions were carried out with SIM by flow-injection without use of a column.

### *Calibration curve*

The determination of pilocarpine was based on the external standard method. Five-point calibration curves (triplicate injections) were created for the range from 0.3 ng to 1.5 $\mu$g (corresponding to aqueous humour concentrations from 2 ng/ml to 10 $\mu$g/ml) by plotting the peak area of pilocarpine *vs.* the amount of pilocarpine.

### *Aqueous humour samples*

Male albino rabbits each weighing *ca.* 2 kg were given a 20-$\mu$l or 50-$\mu$l dose of 2% pilocarpine(w/v) eye-drops topically in the eye. Rabbits were killed by marginal ear vein administration of pentobarbital sodium solution. Aqueous humour samples were obtained from the anterior chamber by entering the limbus with a 1-ml syringe.

### *Sample preparations*

A 0.2-ml portion of aqueous humour was deproteinized with 0.4 ml of methanol and centrifuged at 8000 *g* for 5 min. The supernatant was evaporated to dryness, and the residue was dissolved in 0.1 ml of the mobile phase. A 75-$\mu$l portion of the solution was subjected to LC–APCI-MS.

### *Precision and accuracy*

The intra-assay variation was determined by analysing sets of five aqueous humour samples containing pilocarpine at the concentrations of 10 and 100 ng/ml in aqueous humour. The inter-assay variation was measured by analysing samples at the concentration of 100 ng/ml over 5 days.

## RESULTS AND DISCUSSION

Fig. 1 shows the molecular structures of pilocarpine and its analogues, isopilocarpine, pilocarpic acid and isopilocarpic acid. Pilocarpine may epimerize to isopilocarpine under certain conditions, and pilocarpine is easily metabolized to pilocarpic acid in the cornea [13]. Therefore, the separation of these compounds during the analysis is important. The desired separation was achieved by Kennedy and McNamara [14] using a phenyl column and a mobile phase consisting of a 5% $KH_2PO_4$ solution. However, because the mobile phase consisting of phosphate buffer solution was not suitable for LC–APCI-MS system, we modified the mobile phase to 0.1% acetic acid–acetonitrile (95:5, v/v) after several investigations.

Figs. 2A and 2B show typical SIM chromatograms of these standard compounds (75 ng). These chromatograms were monitored at *m/z* 209, corresponding to the protonated molecular ion for pilocarpine (Fig. 3A) and the fragment ion for pilocarpic acid (Fig. 3B). Pilocarpic acid was prepared by the hydrolysis of pilocarpine [12], which contained isopilocarpic acid as a minor product. The retention times of pilocarpic acid, isopilocarpic acid, isopilocarpine and pilocarpine were 4.0, 4.3, 4.8 and 5.1 min, respectively. Baseline resolution of the corresponding

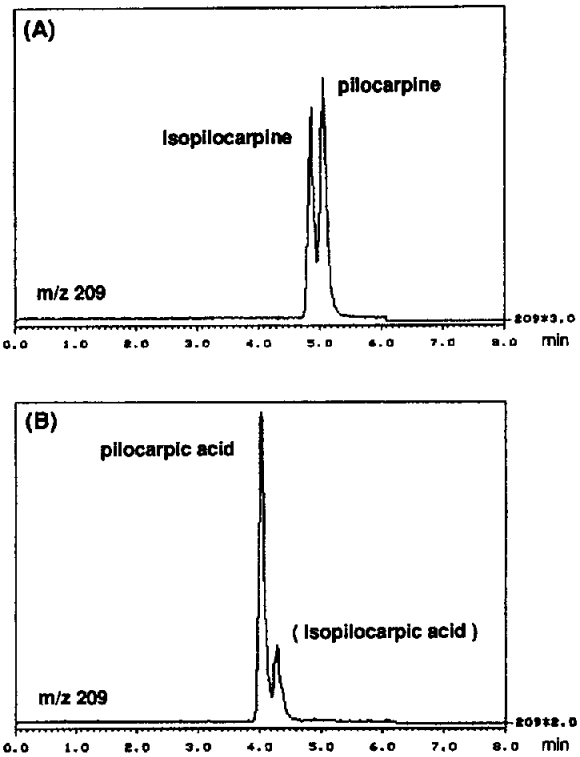
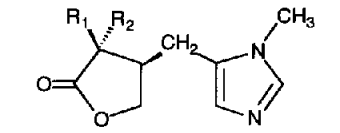
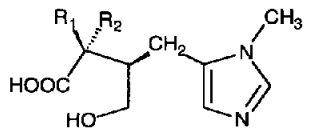



Fig. 2. SIM chromatograms of (A) a standard solution mixed with pilocarpine and isopilocarpine (each 1 μg/ml) and (B) pilocarpic acid obtained from hydrolysis of pilocarpine (1 μg/ml). Chromatographic conditions: mobile phase, 0.1% acetic acid–acetonitrile (95:5, v/v); column, Cosmosil 5Ph (250 mm × 4.6 mm I.D.); flow-rate, 1 ml/min.



$R_1 = C_2H_5$ $R_2 = H$ pilocarpine
$R_1 = H$ $R_2 = C_2H_5$ isopilocarpine



$R_1 = C_2H_5$ $R_2 = H$ pilocarpic acid
$R_1 = H$ $R_2 = C_2H_5$ isopilocarpic acid

Fig. 1. Molecular structures of pilocarpine and its analogues.

epimers was not achieved, but this did not affect the determination of pilocarpine and the analysis of its metabolite, because the epimerization rate of pilocarpine to isopilocarpine is low after topical application of eye-drops (Fig. 4).

Fig. 5 shows the total ion chromatogram (TIC) and mass chromatograms of *m/z* 209 and *m/z* 227, the protonated molecular ions for pilocarpine and pilocarpic acid, respectively, for an aqueous humour specimen taken 30 min after the topical application of 50 μl of 2% pilocarpine (w/v) eye-drops to a male albino rabbit. Figs. 3A and 3B show the mass spectra of pilocarpine and pilocarpic acid in aqueous humour. Pilocarpine and pilocarpic acid were identified with

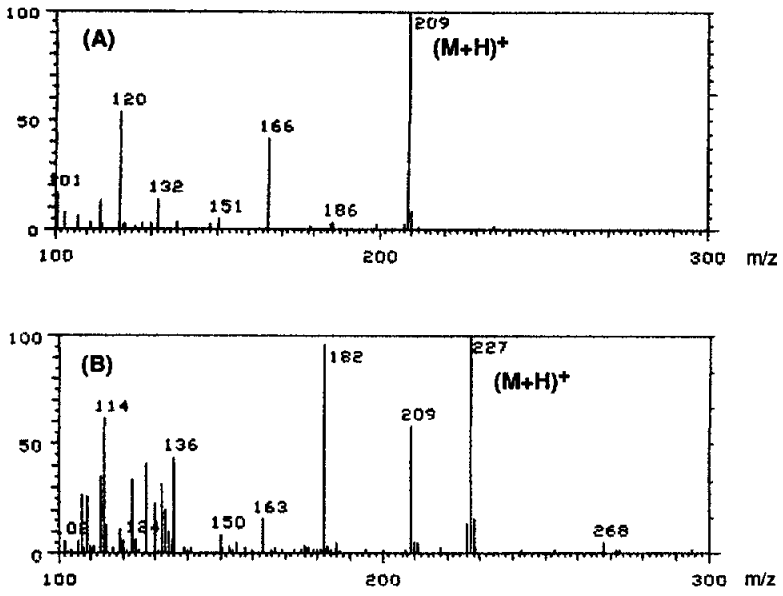



Fig. 3. Mass spectra of (A) pilocarpine and (B) pilocarpic acid present in aqueous humour.

these standard mass spectra and retention times on the mass chromatograms. Other metabolites were not observed in this analysis.

Drug-metabolizing systems similar to those in the liver are present in various non-hepatic tissues. The distribution of drug-metabolizing enzymes in ocular tissues is also known [15]. Drug metabolism in ocular tissues is an important area to be fully explored in ocular pharmacokinetics. However, such studies have been limited because of the lack of the sensitivity required to detect metabolites. In this analysis, metabolites other than pilocarpic acid were not detected, but LC–APCI-MS should be very useful for investigation of the metabolites after topical application of drugs, because of its high sensitivity and the easy sample preparation.

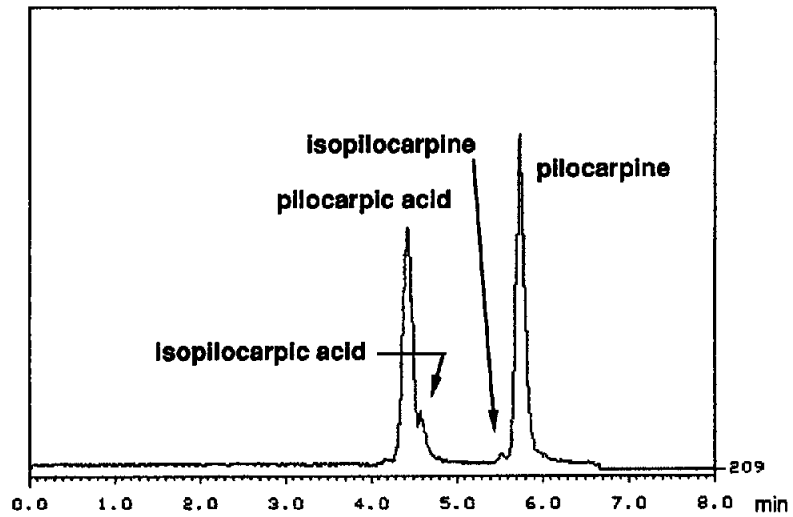



Fig. 4. SIM chromatogram obtained from aqueous humour taken 2 h after topical application of 2% pilocarpine eye-drops.

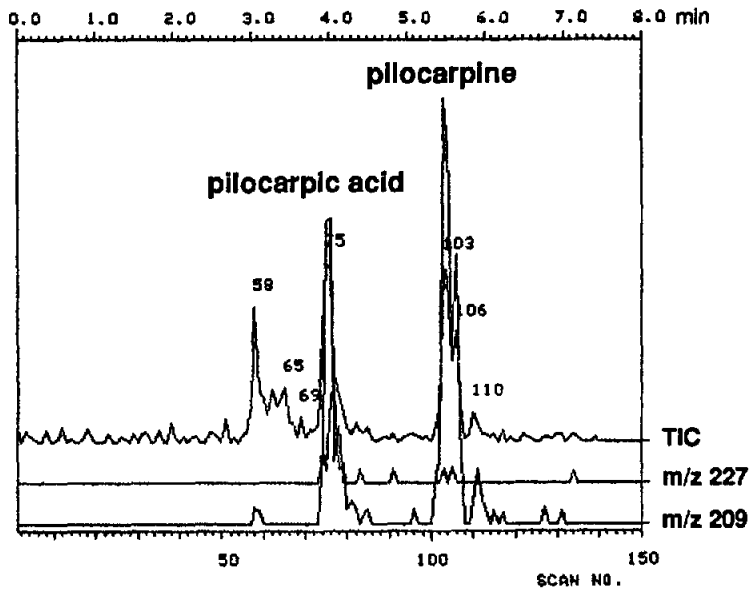



Fig. 5. Mass chromatograms of aqueous humour sample after topical application of 2% pilocarpine (w/v) eye-drops. A 75-$\mu$l sample solution was analysed by LC–APCI-MS.

To determine the concentration of pilocarpine in aqueous humour with high sensitivity, the APCI interface parameters were optimized. Fig. 6 shows the effect of drift voltage and nebulizer temperature on the ion intensity of the protonated molecular ion for pilocarpine (*m/z* 209). At each nebulizer temperature of 330°C, 350°C and 370°C, the drift voltage was varied from 110 to 210 V, and the vaporizer temperature was maintained at 390°C throughout the investigation. A 100-ng amount of pilocarpine was injected, and the ion intensity was measured in the SIM mode. The drift voltage and nebulizer temperature giving the highest abundance of the ion were 150 V and 350°C, respectively, using the mobile phase of 0.1% acetic acid–acetonitrile (95:5, v/v).

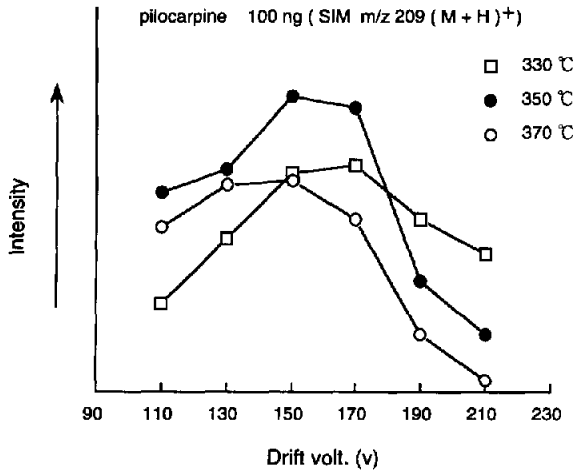



Fig. 6. Effect of drift voltage and nebulizer temperature on the intensity of the $(M + H)^+$ ion for pilocarpine.

Under these conditions, when the corresponding peak area was plotted against the amount of pilocarpine, a linear relationship was observed in the range from 0.3 ng to 1.5 $\mu$g (corresponding with 2 ng/ml to 10 $\mu$g/ml in the concentration of aqueous humour). The correlation coefficient for this plot was found to be 0.9999.

Table I shows the precision and the accuracy for the whole sample preparation procedure and LC–MS analysis by analysing known amounts (10 and 100 ng/ml in aqueous humour) of pilocarpine. Fig. 7A and B shows typical SIM chromatograms obtained from blank aqueous humour and blank aqueous humour spiked with 10 ng/ml of pilocarpine. The determination of pilocarpine was performed by the external standard

TABLE I

INTRA-DAY AND INTER-DAY PRECISION SIM OF ASSAY

| Concentration added (ng/ml) | Found concentration, mean ± S.D. (ng/ml) | C.V. (%) |
|---|---|---|
| *Intra-day precision (n = 5)* | | |
| 10 | 9.94 ± 0.23 | 2.3 |
| 100 | 96.45 ± 2.91 | 3.0 |
| *Inter-day precision (n = 5)* | | |
| 100 | 96.86 ± 4.58 | 4.7 |

method, because there were no analogues that showed similar chromatographic behaviour to pilocarpine and it was not affected by interfering compounds in the aqueous humour. However, pilocarpine has an N-methylimidazole ring, which gives high proton affinity, the intensity of the protonated molecular ion at $m/z$ 209 was very strong, and the ion was stable throughout the analysis of pilocarpine. The result at each level of spiking was satisfactory with respect to the reproducibility, and there was no interference peak in the blank aqueous humour sample. The limit of determination was estimated to be an aqueous humour concentration of *ca.* 2 ng/ml, at a signal-to-noise ratio of 3.

This method was applied to the determination

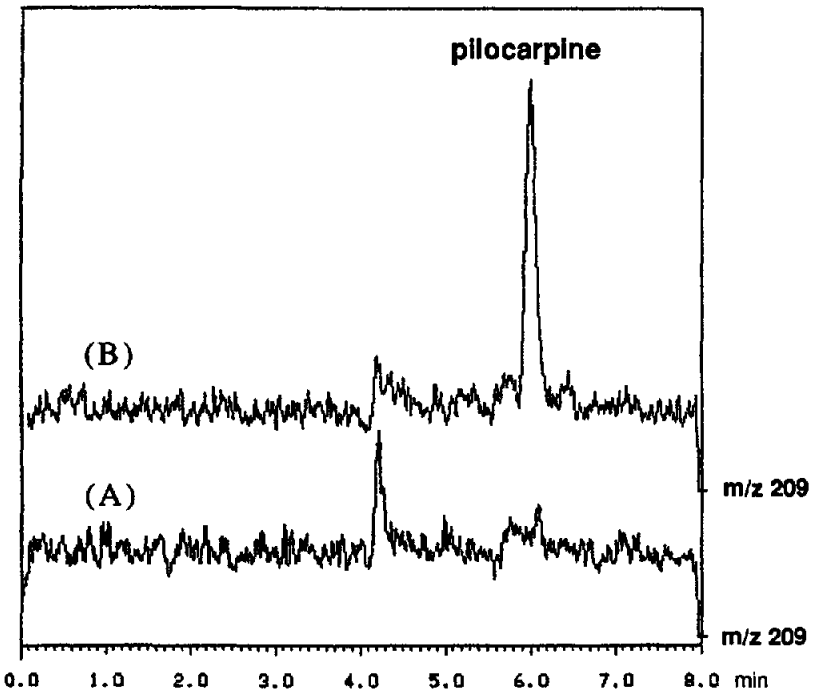



Fig. 7. SIM chromatograms obtained from (A) blank aqueous humour and (B) blank aqueous humour spiked with 10 ng/ml of pilocarpine.

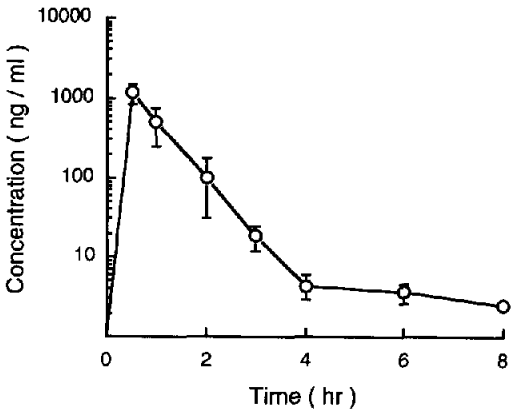



Fig. 8. Concentration–time curve of pilocarpine in aqueous humour after topical application of 2% pilocarpine (w/v) eye-drops. Values are mean ± S.D.

of the aqueous humour level of pilocarpine in the male albino rabbit ($n$ = 4, for each point) after topical application of 20 $\mu$l of 2% pilocarpine (w/v) eye-drops. Fig. 8 shows the time-course of the pilocarpine concentration in aqueous humour, and Fig. 4 shows the SIM chromatogram for aqueous humour taken 2 h after the topical application of eye-drops.

The aqueous humour is a transparent liquid that fills the space between the cornea and lens, and supplies oxygen and nutrition to the cornea and lens. It also regulates the intra-ocular pressure precisely. It is sealed from extracellular fluids, and contains scarcely any protein [16]. Even so, in this study the aqueous humour samples were deproteinized with methanol, to maintain the quality of the separation and to prevent the accumulation of protein during the determination of pilocarpine in hundreds of samples. However, the direct injection of up to 50 aqueous humour samples was possible, using the valve-switching technique. Thus, aqueous humour is more suitable than blood or urine for LC–MS analysis without elaborate sample preparation.

Although pilocarpine is a polar compound and has an apparent chromophore in the imidazole system, it cannot be determined in aqueous humour at low concentrations. It must be converted into a derivative that can be analysed by GC or HPLC with high sensitivity. The base-catalysed epimerization of pilocarpine to isopilocarpine, and the base-catalysed ring-opening to pilocarpic acid were reported previously [17]. Therefore, we developed a rapid analysis and determination method for pilocarpine in aqueous humour using LC–APCI-MS. The sensitivity, the selectivity and the rapidity were better than those of the HPLC [1,2] and GC methods [3,4]. These findings suggest that LC–APCI-MS is the most suitable method for the analysis of the drug in aqueous humour.

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