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Determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid in human plasma and urine by high-performance liquid chromatography with tandem mass spectrometric detection

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Abstract

A method is described for the determination of pilocarpine and its degradation products isopilocarpine, pilocarpic acid and isopilocarpic acid in human plasma and urine. The method is based on a simple sample preparation step – ultrafiltration for plasma and dilution for urine samples – followed by a reversed-phase liquid chromatographic separation of the analytes and detection by means of tandem mass spectrometry. Parameters affecting the performance of these steps are discussed. The high sensitivity and selectivity of the method allow low ng/ml concentrations to be determined for all compounds in plasma and undiluted urine, which enables the investigation of the metabolic fate and elimination of pilocarpine after oral administration to humans. © 1998 Elsevier Science B.V.

Keywords: Pilocarpine; Isopilocarpine; Pilocarpic acid; Isopilocarpic acid

1. Introduction

Pilocarpine is a parasympathicomimetic compound of natural origin, which has been used for decades in ophthalmic solutions to control intraocular pressure in patients suffering from glaucoma. As pilocarpine has been shown to stimulate salivation [1], it may also be orally administered for the treatment of patients with impaired secretion of the salivary glands (xerostomia), resulting from e.g., cancer radiation or drug therapy. Although many studies on the degradation of pilocarpine in aqueous or ophthalmic solutions have been reported [2], as yet little information is available on the metabolic fate and elimination pathways of pilocarpine following oral administration. In aqueous solution the drug is known to degrade by hydrolysis to pilocarpic acid or by epimerization to isopilocarpine, which in turn can be further hydrolysed to isopilocarpic acid (Fig. 1), all of which are pharmacologically inactive. These three degradation products are also potentially relevant in vivo.

Because of its widespread use, much attention has been devoted to the development of analytical methods for the determination of pilocarpine and its degradation products. Most methods employ high-performance liquid chromatography (HPLC) with e.g., an octadecyl [3,4], phenyl [5,6], cyano [7],

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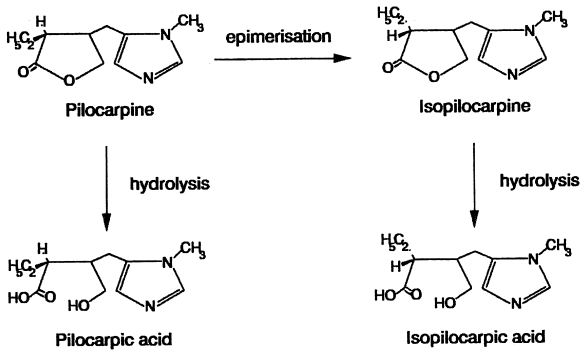

Fig. 1. Degradation pathways of pilocarpine.

unmodified silica [8] or β-cyclodextrin [9] phase in combination with an acidic (pH 2.5–4.0) eluent and UV absorbance detection. Typically reported chromatographic run times are in the order of 10–20 min and near or complete baseline separation can be achieved. However, the simultaneous determination of pilocarpine and its degradation products has so far been described exclusively in pharmaceutical preparations. For biofluids, no other assays than the determination of pilocarpine alone, in plasma [10,11] and aqueous humour [12] have been reported.

The bioanalysis of pilocarpine and its degradation products by LC–UV is complicated by two factors. First, the compounds lack chromophoric groups and are, thus, determined at low wavelengths (typically around 215 nm). This gives rise to substantial interference of endogenous components, which are difficult to remove because of the highly polar nature of the analytes. In plasma, a limit of quantitation of 10 ng/ml has been reported for pilocarpine [10] which was sufficient to determine concentrations after intravenous administration to anaesthetized dogs [13]. However, in order to accurately quantify pharmacokinetically useful levels after oral administration to humans, a low ng/ml limit of quantitation and, thus, a more sensitive and selective detection mode is required. Recently, a method was described employing pre-column derivatization of pilocarpine with 4-bromomethyl-7-methoxycoumarin and subsequent LC with fluorescence detection. This improved the detection limit to 1 ng/ml, but the derivatization needed 12–48 h for completion [11].

Second, the liquid–liquid extraction step, which has been described to be necessary for sample enrichment and clean-up in case of low-wavelength UV absorbance detection, does not extract the more polar pilocarpic and isopilocarpic acid, the determination of which is essential for the investigation of the metabolism of pilocarpine. Therefore, a method featuring a less discriminating sample preparation step is necessary. Further, in order to study the elimination pathways of the compound, a method for the determination of pilocarpine and its metabolites in urine would be useful.

In this paper, a method for the simultaneous determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid at the relevant levels in human plasma and urine is reported. It combines a straightforward sample preparation step (ultrafiltration for plasma and dilution for urine samples), a reversed-phase liquid chromatographic separation of the analytes on an octadecyl stationary phase and a highly selective detection by means of tandem mass spectrometry (MS–MS), using atmospheric pressure chemical ionization (APCI). The assay was applied to the analysis of plasma and urine samples from human volunteers after oral administration of a pilocarpine-containing dosage form.

2. Experimental

2.1. Chemicals

Pilocarpine hydrochloride and isopilocarpine nitrate were obtained from Sigma (St. Louis, MO, USA) and Aldrich (Bornem, Belgium), respectively. Ammonium acetate and disodium EDTA came from Merck (Darmstadt, Germany). Trifluoracetic acid was purchased from J.T. Baker (Deventer, Netherlands) and acetonitrile from BioSolve (Barneveld, Netherlands).

Water was purified using a Milli-Ro-15 or a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Equipment

A Waters (Milford, MA, USA) Model 717 plus autosampler was used to introduce 25-μl aliquots of the pretreated samples into the chromatographic system. Separation was performed by reversed-phase

HPLC using a 5 μm Inertsil ODS column (250×4.6 mm I.D.), obtained from Chrompack (Middelburg, Netherlands), which was conditioned at 25°C in a WO electronics (Langensdorf, Germany) Model BFO-04 column thermostat. A Perkin-Elmer (Beaconsfield, UK) Model 200 LC pump was used to deliver the eluent, a mixture of a 0.05 *M* ammonium acetate buffer (adjusted to pH 4.0 with trifluoroacetic acid)–acetonitrile (97:3, v/v), at a flow-rate of 1.0 ml/min. A Waters Model M510 fluid unit was used for the post-column addition of acetonitrile (1.0 ml/min).

Quantitation was performed by tandem mass spectrometric detection using a Perkin-Elmer Sciex API III plus mass spectrometer. Detection of the ions was performed in multiple reaction monitoring (MRM) mode, monitoring the decay of the *m/z* 209 parent ion to the *m/z* 95 daughter ion for all components in positive ion mode. APCI of the analyte species was performed using the heated nebulizer inlet probe at 500°C.

2.3. Standard solutions

A methanolic pilocarpine stock solution (1000 μg/ml as pilocarpine base) and a methanolic isopilocarpine stock solution (1000 μg/ml as isopilocarpine base) were used to prepare a working solution containing both pilocarpine and isopilocarpine at a concentration of 10.0 μg/ml in methanol.

Since no standards of pilocarpic acid and isopilocarpic acid are commercially available, these compounds were synthesized by chemical hydrolysis of pilocarpine and isopilocarpine, based on the method of Bundgaard and Hansen [14]. Under alkaline conditions, pilocarpine is converted to a mixture of pilocarpic and isopilocarpic acid (approximately 85:15) and isopilocarpine to virtually pure isopilocarpic acid.

To 10.84 mg of pilocarpine hydrochloride (equivalent to 10.00 mg of pilocarpic acid) 10.0 ml of 0.1 *M* sodium hydroxide was added and this solution was kept at room temperature for 2 h. Likewise, to 12.03 mg of isopilocarpine nitrate (equivalent to 10.00 mg of isopilocarpic acid) 10.0 ml of 0.1 *M* sodium hydroxide was added and this solution was kept at room temperature for 2 h. A stock solution of pilocarpic acid and isopilocarpic acid was obtained by mixing both solutions (1:1, v/v), and a working solution was prepared by diluting the stock solution 100-fold with water.

The concentrations of pilocarpic acid and isopilocarpic acid in the working solution were determined using the chromatographic system described above, but with UV absorbance detection at 214 nm. No pilocarpine or isopilocarpine were observed in the chromatograms nor any other peaks, indicating a complete conversion into the corresponding acids. Assuming both epimeres to have equal molar extinction coefficients, the concentrations of the compounds were found, based on their peak areas, to be 4.17 μg/ml and 5.83 μg/ml for pilocarpic acid and isopilocarpic acid, respectively. The described working solutions were used to prepare calibration and quality control samples by the addition of small volumes to blank human plasma or urine in such a way that all four compounds are present in the sample.

2.4. Sample preparation

2.4.1. Blood

In order to prevent esterase-mediated breakdown of pilocarpine [15], blood samples (10 ml) of volunteers which were given an oral dose of pilocarpine, were collected over an excess of disodium EDTA (250 μl of a 5% solution) and immediately and thoroughly mixed. After centrifugation for 10 min at 1500 *g*, 4.0 ml of the plasma was transferred to a tube containing an additional 125 μl of 5% disodium EDTA. Blank plasma was handled identically. Plasma samples were stored at −20°C until analysis.

Sample preparation of the plasma was performed by ultrafiltration using Amicon (Capelle aan de IJssel, Netherlands) Centrifree cartridges with a molecular mass cut-off value of 30 000 Da. A 1.0-ml plasma sample was placed on top of the ultrafiltration membrane and centrifuged at 2000 *g* for 15 min. The ultrafiltrate was placed in an autosampler vial and a 25-μl aliquot was injected into the LC–MS–MS system. No internal standard was used.

2.4.2. Urine

Immediately after collection, the urine sample pH was adjusted with 0.1 *M* hydrochloric acid to 5, at

which value pilocarpine is chemically most stable [2]. Blank urine was handled identically. Urine samples were stored at −20°C until analysis.

Sample preparation of the urine was performed by diluting the urine sample 20-fold with a 0.20 *M* sodium acetate buffer (pH 3.7). The diluted urine sample was placed in an autosampler vial and a 25-μl aliquot was injected into the LC–MS–MS system. No internal standard was used.

2.5. Method performance

The specificity of the assay was checked by analysing blank human plasma or urine samples obtained from different subjects. The linearity of the method was established by plotting the peak heights found versus concentration of the analytes using eight calibration samples in triplicate. Accuracy and within-run and between-run precision were determined at four concentration levels by analysing samples in triplicate during five analytical runs. For plasma samples, the recovery was determined at three concentration levels by comparing the response of study samples with that of direct injections performed in the same run. Because of the nature of the sample preparation (mere dilution) no recovery was determined for urine samples. Stability of the compounds in plasma or urine after repeated ($n=5$) freezing and thawing cycles was assessed at two concentration levels and the stability of the compounds in the pretreated sample during storage in the autosampler was determined by repeated injection for a period of 15 h.

3. Results and discussion

3.1. Sample preparation

The need to recover four compounds of rather different polarity from plasma samples prompted the use of a simple deproteinization as the sample preparation step rather than liquid–liquid or solid-phase extraction, which are likely to discriminate between the compounds. Deproteinization traditionally is often achieved by precipitation of the plasma proteins with a strong acid or an organic solvent, followed by centrifugation. However, this approach turned out to be unsuitable for the present application. Acidification of the plasma samples by adding perchloric acid, sulphuric acid or hydrochloric acid resulted in highly irreproducible results, probably because of an increased rate of epimerization and/or conversion of the acids into pilocarpine, which is known to occur at low pH values [2]. On the other hand, the addition of methanol or acetonitrile to plasma in a 1:1 (v/v) ratio, which is minimally necessary for an efficient protein precipitation, gave rise to substantial band broadening on the LC system. This can be explained by the introduction of a relatively large amount of modifier upon the injection of the sample into a highly aqueous mobile phase.

By far the best results were obtained by ultrafiltration. This technique effects a very efficient removal of proteins under mild conditions; the only inherent disadvantage is a low recovery for highly protein bound compounds, which cannot pass through the membrane pores. The recoveries found for the present analytes were, however, essentially quantitative, which shows that they have a negligible protein binding and demonstrates the suitability of using ultrafiltration for sample preparation. As plasma ultrafiltrate contains relatively high salt concentrations, which might be detrimental for the mass spectrometer, the first 6 min of each chromatographic run the eluent was flushed to waste as a precaution.

3.2. Chromatography

Since pilocarpine and isopilocarpine are epimeres which only differ in their three-dimensional structure, it is not possible to distinguish them on the basis of their mass spectrometric characteristic: the ionization and fragmentation are similar for both compounds. Obviously, the same is true for the other set of epimeres, pilocarpic acid and isopilocarpic acid. Therefore, a chromatographic separation of the analytes had to be performed prior to their detection.

A rather large number of different stationary phases have been described to effect a separation between pilocarpine and its degradation products, but in our hands the choice of the column type proved to be very critical. Although the best performance in terms of separation efficiency and speed of analysis

(baseline separation in 10 min) has been reported for a chiral, β-cyclodextrin-based phase [9], in order to reduce peak tailing a high concentration of ammonium sulphate has to be added to the eluent, which makes the method incompatible with mass spectrometric detection. Various types of other phases, less efficient but with the possibility of being coupled to MS, (phenyl, cyano, octadecyl and polymer phases) were tried, but the only column yielding an acceptably efficient and reproducible separation of the four analytes within 20 min was an Inertsil octadecyl type. Typical chromatograms are shown in Figs. 3 and 4.

3.3. Detection

Full scan mass spectra revealed the almost exclusive formation of m/z 209 ions for all four analytes (data not shown). These correspond to the $[M+H]^+$ ions for pilocarpine and isopilocarpine (molecular mass 208); pilocarpic acid and isopilocarpic acid (molecular mass 226) might lose water and form $[M-H_2O+H]^+$ ions in the ion source. Under MS–MS conditions applying a collision energy of 27.5 eV, the m/z 209 parent ions of all analytes were found to fragment mainly to m/z 95 and 96 daughter ions, which is illustrated in Fig. 2 for pilocarpine and pilocarpic acid. The m/z 95 daughter ion most probably corresponds to the positively charged (1-methyl-imidazole)methylene moiety which all compounds have in common. The, somewhat less abundant, m/z 96 daughter ion probably represents a protonated form of the moiety mentioned above.

Because of the high water content of the LC eluent, the post-column addition of acetonitrile in a 1:1 ratio was found to be profitable in order to obtain a good nebulization of the liquid phase and enhance response stability. In fact, the detector stability thus obtained allowed sample analysis without the use of an internal standard. Obviously, this was also possible because the sample preparation techniques used gave rise to the quantitative recoveries for all analytes.

Interestingly, using the described detection conditions, a substantial peak tailing was observed for pilocarpic acid and isopilocarpic acid, whereas this phenomenon was found to a much lesser extent for pilocarpine or isopilocarpine (see e.g., Fig. 4C). However, when UV detection was used instead, no peak tailing was found for these two analytes either. Apparently, this behaviour is not due to the chromatographic separation but originates from the detection part of the system. Furthermore, essentially no peak tailing occurred for these analytes when the, much less abundant, m/z 227 ($[M+H]^+$) parent ion was selected instead of the m/z 209 ($[M-H_2O+H]^+$) parent ion. This indicates that the observed peak tailing is related to the loss of water, which can take place in the heated nebulizer probe or the desolvation chamber. It is to be expected that the peak tailing is the result of an adsorption/desorption process rather than a gas phase process and it is most likely that the acids, contrary to pilocarpine and isopilocarpine, are adsorbed onto the inner surface of the heated nebulizer probe where a thermal degradation is induced, followed by desorption.

3.4. System performance – plasma

The described mass spectrometric conditions appeared to be highly selective. Despite the non-specific sample preparation, virtually no interferences were found in the chromatograms, as is illustrated for a blank plasma sample in Fig. 3a. Detection limits were rather determined by chemical noise than by interference of endogenous plasma compounds; they were estimated to be 0.5 ng/ml for pilocarpine and isopilocarpine and 1.0 ng/ml for pilocarpic acid and isopilocarpic acid, using 25 μl of ultrafiltrate. For practical reasons, the lowest points of the calibration curves were taken as the lower limits of quantitation (LLQ). These levels were 2.00 ng/ml for pilocarpine and isopilocarpine, 4.17 ng/ml for pilocarpic acid and 5.83 ng/ml for isopilocarpic acid. A chromatogram of a plasma sample spiked at the LLQ levels is presented in Fig. 3B.

Assessment of the within-run precision with four plasma pools containing relevant concentrations of all analytes resulted in coefficients of variation (C.V.s) below 9.2% (Table 1). The between-run C.V.s were higher (up to 19.1% for the LLQ level), which indicates a relatively high day-to-day variation for the present instrument settings. The accuracy of the method as expressed in the bias was between +3.1% and +14.8% for all compounds.

Linear regression analysis yielded correlation co-

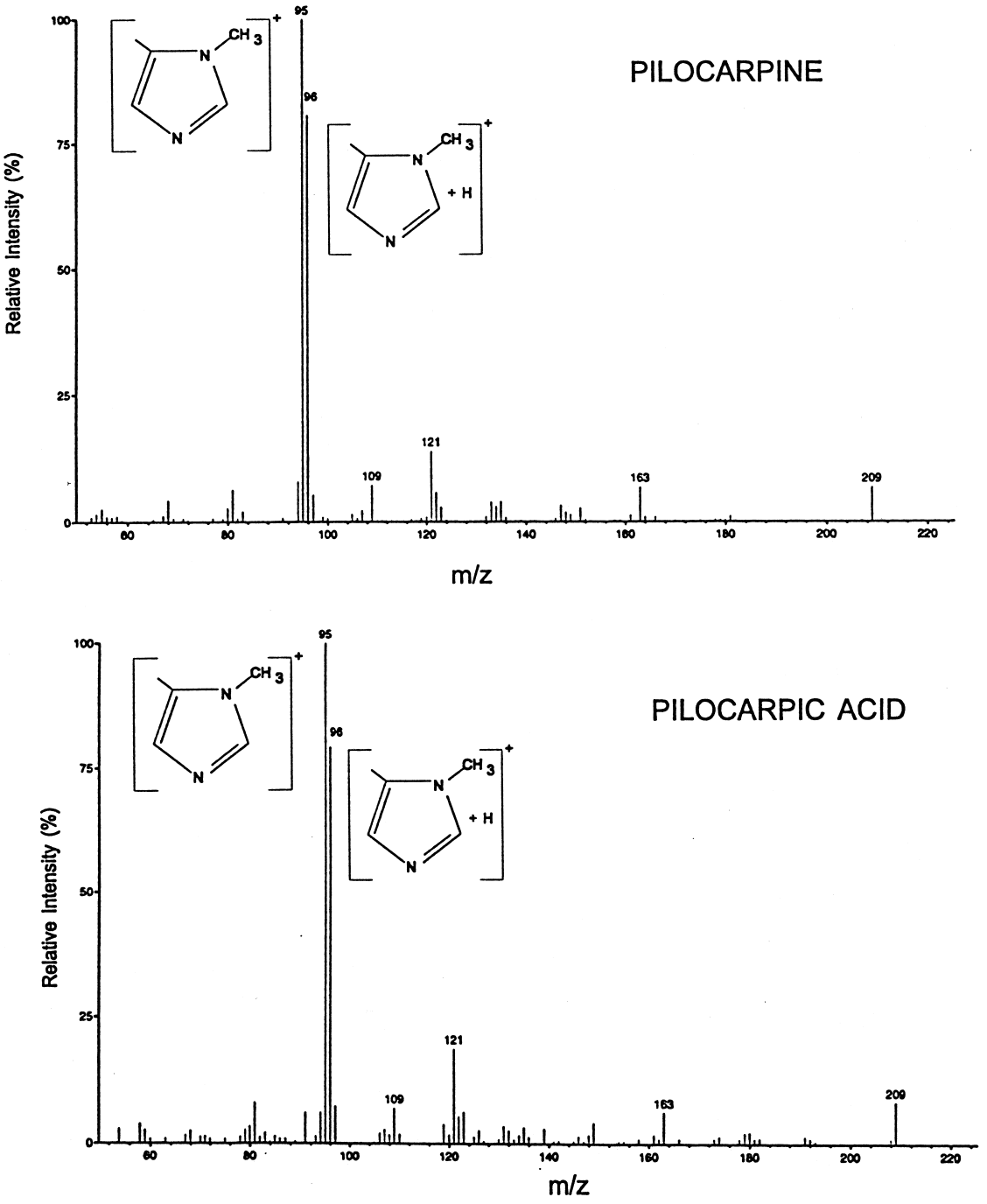

Fig. 2. MS–MS spectra of pilocarpine and pilocarpic acid showing the fragmentation from *m*/*z* 209 parent ions.

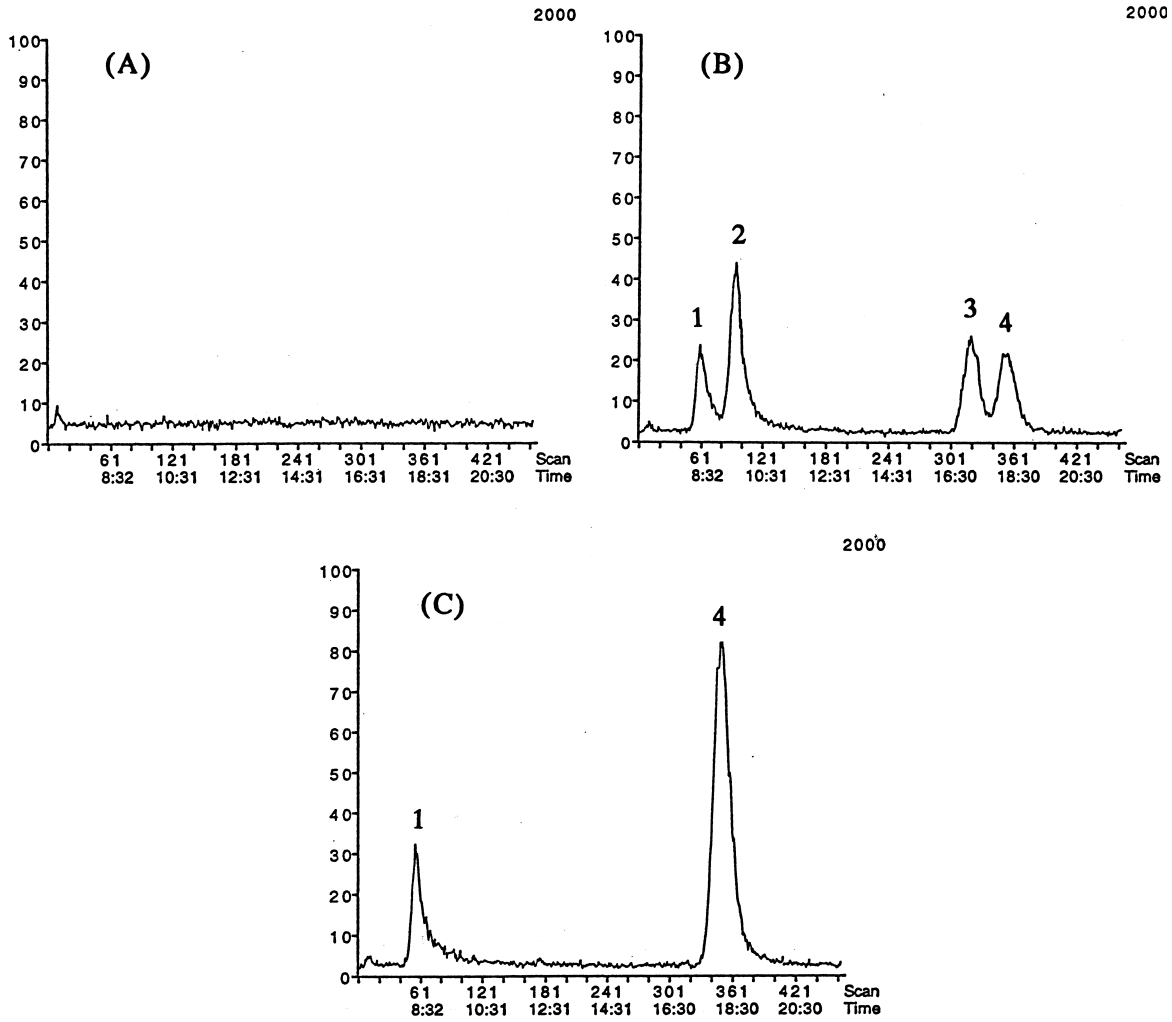

Fig. 3. LC–MS–MS chromatograms of human plasma samples. (A) Blank plasma; (B) blank plasma spiked with the four analytes at their respective LLQ levels; (C) plasma obtained from a subject administered 10 mg of pilocarpine hydrochloride, 3 h after intake. 1=Pilocarpic acid, 2=isopilocarpic acid, 3=isopilocarpine and 4=pilocarpine.

efficients of the calibration curves above 0.9959 for pilocarpine, above 0.9985 for isopilocarpine, above 0.9938 for pilocarpic acid and above 0.9966 for isopilocarpic acid.

As can also be concluded from Table 1, the absolute analytical recoveries for pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid are quantitative and consistent over the concentration ranges studied.

No deterioration was observed for any of the analytes after repeated freezing and thawing of plasma samples (five cycles), nor was there a consistent change in their concentrations during storage for 15 h at 4°C.

3.5. System performance – urine

As with plasma, no matrix interference was observed in blank urine samples (Fig. 4A). The detection limits for the present method, which includes a 20-fold dilution, were estimated to be 10 ng/ml for pilocarpine and isopilocarpine and 20 ng/ml for

Table 1
Summary of accuracy, precision and recovery of the analytes in plasma samples

Analyte	Nominal concentration (ng/ml)	Bias (%)	Within-run precision (%)	Between-run precision (%)	Recovery (%)
Pilocarpine	2.00	5.6	6.9	15.1	N.D.
	5.00	13.7	4.2	1.9	106.3
	20.0	5.6	3.0	5.1	101.8
	40.0	7.3	2.6	4.4	106.5
Isopilocarpine	2.00	9.5	6.0	6.0	N.D.
	5.00	4.3	3.4	5.7	98.8
	20.0	5.9	1.9	6.6	100.9
	40.0	7.1	1.8	6.0	107.3
Pilocarpic acid	4.17	12.7	9.2	19.1	N.D.
	8.34	11.0	6.5	12.2	106.0
	20.9	6.0	3.6	9.0	100.5
	33.4	3.1	8.1	12.9	98.1
Isopilocarpic acid	5.83	14.8	4.8	10.7	N.D.
	11.7	11.4	5.1	12.3	101.4
	29.2	8.2	3.0	16.7	97.0
	46.6	4.4	3.4	15.8	96.9

N.D.=Not determined.

pilocarpic acid and isopilocarpic acid. It is to be expected that for undiluted urine detection limits will be 20-times lower, which makes the method equally sensitive as the one for plasma. The LLQ values for the analytes were 40.0 ng/ml (pilocarpine and isopilocarpine) 83.4 ng/ml (pilocarpic acid) and 117 ng/ml (isopilocarpic acid). Fig. 4B shows a chromatogram of urine spiked at these levels.

Accuracy and precision data (Table 2) were comparable to those found for plasma: within-run C.V. below 11.2% and between-run C.V. below 19.5% for all analytes. The observed bias was between −5.5% and +10.5%.

Method linearity was established over the ranges 40.0–10 000 ng/ml for pilocarpine and isopilocarpine, 83.4–8340 ng/ml for pilocarpic acid and 117–11 700 for isopilocarpic acid. Correlation coefficients were above 0.9991, above 0.9979, above 0.9948 and above 0.9979, respectively.

Also here, no deterioration was found for any of the analytes after five freeze–thaw cycles or storage at 4°C for at 15 h.

3.6. Application to study samples

Chromatograms of a plasma sample obtained 3 h after drug administration and of a urine sample obtained approximately 8 h after drug administration are shown in Fig. 3C and Fig. 4C, respectively. For all analytical runs in which the study samples were analysed, calibration curves were recorded with coefficients of correlation above 0.995 for all analytes in both plasma and urine. Per analytical run, quality control samples were analysed in duplicate at three levels, which are similar to the three highest validation levels. For all analytes, the average accuracy (expressed as the percentage bias from the nominal value) and the average precision (expressed as the C.V.) found at these levels were better than 8.6% and better than 10.6%, respectively, for plasma and better than 10.7% and better than 8.7%, respectively, for urine, which is consistent with the validation data presented in Table 1. The reproducibility (expressed as the mean relative difference between the results of duplicate assays of randomly selected

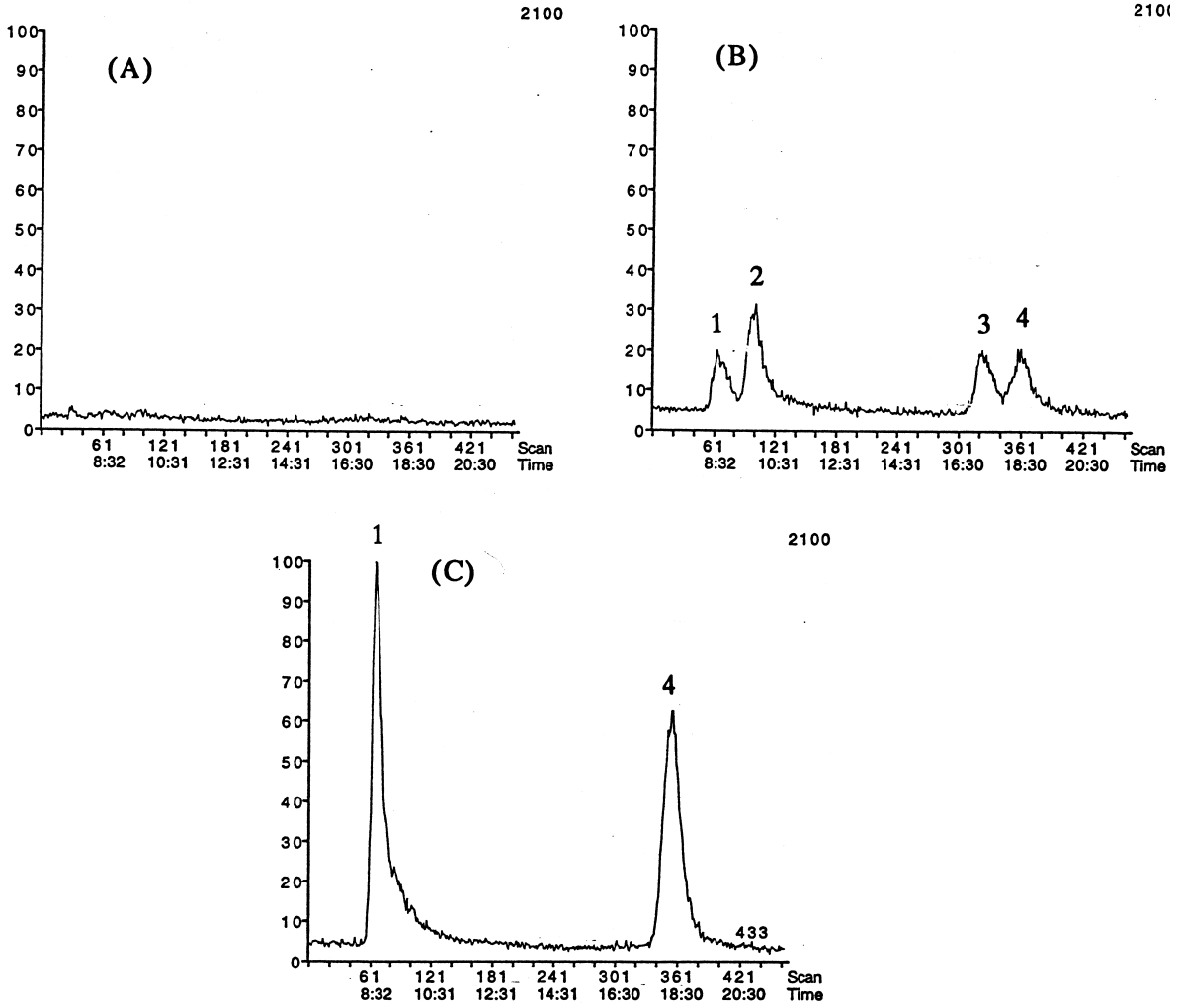

Fig. 4. LC–MS–MS chromatograms of human urine samples. (A) Blank urine; (B) blank urine spiked with the four analytes at their respective LLQ levels; (C) urine obtained from a subject administered 10 mg of pilocarpine hydrochloride, approximately 8 h after intake. 1=Pilocarpic acid, 2=isopilocarpic acid, 3=isopilocarpine and 4=pilocarpine.

samples and the original results) was better than 7.9% for plasma and better than 8.9% for urine, respectively.

4. Conclusions

A straightforward and highly selective LC–MS–MS method has been developed for the simultaneous determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid in plasma and urine samples. Owing to the simple sample preparation procedures involved, the qualitative recovery and because of the high sensitivity and selectivity, this method is very suitable for routine analysis in clinical trials with pilocarpine formulations. Since no isopilocarpine and isopilocarpic acid appear to be formed after oral administration which, obviously,

Table 2
Summary of accuracy and precision of the analytes in urine samples

Analyte	Nominal concentration (ng/ml)	Bias (%)	Within-run precision (%)	Between-run precision (%)
Pilocarpine	40.0	−5.5	7.3	19.5
	250	4.8	3.4	6.2
	2800	7.7	2.7	2.7
	7500	5.6	2.7	5.0
Isopilocarpine	40.0	5.7	7.5	17.2
	250	5.2	4.9	3.5
	2000	8.2	3.4	2.8
	7500	6.7	2.4	3.9
Pilocarpic acid	83.4	10.5	15.8	15.8
	209	4.1	8.5	5.6
	1668	3.1	6.3	5.1
	6255	3.0	5.6	6.6
Isopilocarpic acid	117	10.5	11.2	4.0
	292	4.1	6.8	9.2
	2332	3.1	6.2	5.8
	8745	3.0	5.4	4.7

was not known when the method development was started, for future use the chromatographic conditions could be adapted by using a stronger mobile phase to decrease the chromatographic run time and increase sample throughput.

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