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Synthesis and identification of pilocarpic acid diesters, prodrugs of pilocarpine

TOMI JÄRVINEN,*† SEPPO AURIOLA,† PEKKA PEURA,† PEKKA SUHONEN,‡ ARTO URTTI‡ and JOUKO VEPSÄLÄINEN§

†*Department of Pharmaceutical Chemistry and* ‡*Department of Pharmaceutical Technology, University of Kuopio, P.O. Box 1627, SF-70211 Kuopio, Finland*
§*Huhtamäki Oy Leiras, Niittyhaankatu 20, P.O. Box 33, SF-33721 Tampere, Finland*

Abstract: A series of new pilocarpic acid diesters were synthesized to obtain prodrugs for pilocarpine with varying physico-chemical properties. Thermospray liquid chromatography–mass spectrometry (TSP-LC–MS), liquid chromatography with UV-detection (LC-UV) and NMR-spectroscopy were used for the identification of the synthetic products and for evaluation of their purity including typical impurities (pilocarpic acid monoester, pilocarpine). TSP-LC–MS-analysis was performed in the reversed-phase mode using acetonitrile (60%)–0.2 M ammonium acetate (40%) as mobile phase. In LC-UV-analysis chromatographic separation was carried out on a reversed-phase column and the mobile phase consisted of methanol (71%) and 0.02 M potassium dihydrogen phosphate, pH 4.5 (29%). Electron ionization-mass spectrometry (EI-MS) was used for elucidation of structures. Elemental compositions of the substances were verified with high resolution-mass spectrometry (HR-MS). The complete establishment of structures presented was based on ^{1}H-, and COSY-NMR-spectroscopy joined to TSP-LC–MS-analysis.

Keywords: *Prodrug of pilocarpine; pilocarpic acid diester; thermospray liquid-chromatography; NMR-spectroscopy; high resolution-mass spectrometry.*

Introduction

Pilocarpine is widely employed as a topical miotic for controlling the elevated intraocular pressure associated with glaucoma. However, the drug has significant delivery problems; only 1–3% or less of an instilled pilocarpine dose gains access to the internal ocular structures [1, 2]. Since the delivery problems of pilocarpine are dependent upon its physico-chemical properties (e.g. lipophilicity) the ocular absorption of pilocarpine can be improved by using prodrug derivatives. Bodor [3] has described quaternary ammonium salt prodrugs of pilocarpine and Bundgaard *et al.* [4] has reported various pilocarpic acid diesters as potentially useful pilocarpine prodrugs.

Thermospray LC–MS offers a possibility to analyse nonvolatile and thermally labile compounds, e.g. many drugs [5]. TSP ionization process is soft and the technique often enables verification of the molecular weight, because the TSP spectrum usually shows abundant protonated molecular ion or ammonium adduct ions and only few fragment ions [6, 7].

Electron ionization-mass spectrometry (EI-MS) usually produces fragment ions suitable for the structure elucidation. High resolution-mass spectrometry (HR-MS) offers a possibility to measure the accurate molecular weight and to verify the elemental composition of the compound.

Linking together data from chemical shifts, spin–spin couplings and intensity of each spectral line the NMR spectroscopy is a powerful method for identification and analysis of organic compounds.

In the work described here TSP-LC–MS, LC-UV, HR-MS and NMR have been applied to the analysis of new pilocarpic acid diesters. Pilocarpic acid diesters are thermally labile hence TSP-LC–MS was utilized to identify the synthetic products including the typical impurities (pilocarpine, pilocarpic acid monoester). TSP-LC–MS was also used to test the specificity of the LC-method used for analysis of physico-chemical properties of pilocarpic acid diesters by UV detection. Reversed-phase LC with UV detection (or other non-specific detectors) could not be applied as a sole method in identification (based on a comparison of retention times with standards) because the chemical standards were not available. Due to the absence of fragment ions in

* Author to whom correspondence should be addressed.

the thermospray mass spectra of pilocarpic acid diesters, more informative ionization techniques were needed for the structure elucidation of unknown compounds. Electron ionization mass spectrometry was applied to study mass spectral fragmentation of the pilocarpic acid diesters and the accurate molecular weights of the compounds were measured by HR-MS using electron impact ionization. The purity of the products of synthesis and the identification of the pilocarpic acid diesters and synthetic impurities were confirmed by NMR.

The unambiguous identification and evaluation of the purity of the pilocarpic acid diesters was accomplished by TSP-LC–MS, LC-UV, HR-MS and NMR.

Materials and Methods

Chemicals

Pilocarpine hydrochloride was kindly supplied by Huhtamäki Oy Leiras (Tampere, Finland). Acetyl chloride, propionyl chloride, valeryl chloride, octanoyl chloride, benzoyl chloride, 3-chloro-benzoyl chloride, cyclohexylmethyl bromide, fumaric acid and calcium sulphate were obtained from Aldrich (Steinheim, FRG). Ethyl acetate, 2-propanol, petroleum ether, chloroform, toluene and potassium carbonate were purchased from Merck (Darmstadt, FRG) and demethyl sulphoxide and benzyl chloride were from Merck (Munich, FRG). Diethyl ether, methanol (HPLC-grade), acetonitrile (HPLC-grade) were from Baker (Deventer, The Netherlands).

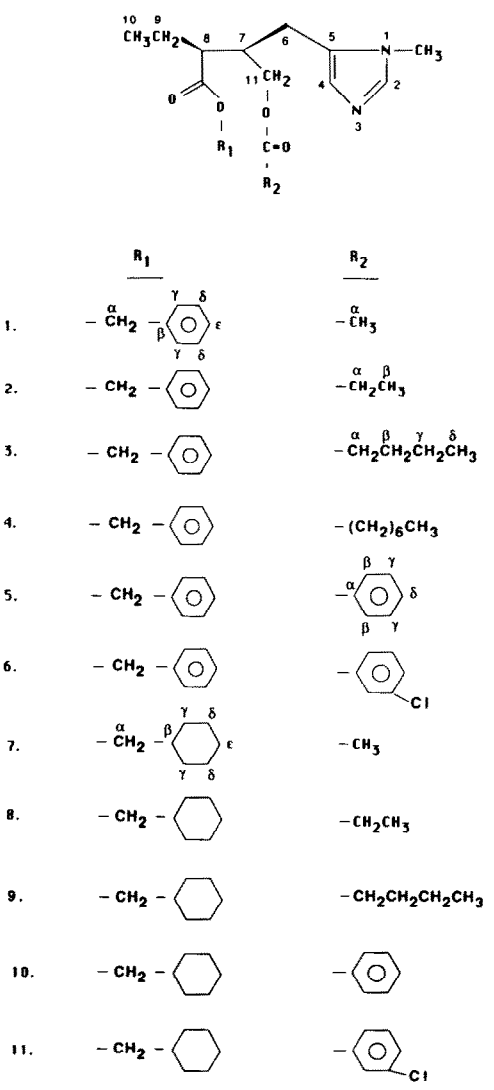

Figure 1
Structures of the pilocarpic acid diesters studied.

Synthesis of pilocarpic acid diesters

Various new alkyl and aralkyl pilocarpic acid diesters (Fig. 1) were prepared according to a previously described procedure [8]. Syntheses of starting materials; sodium pilocarpate and pilocarpic acid monoester, were modified from a previous report [4]. In the synthesis of pilocarpic acid monoester dimethylsulphoxide was used instead of dimethylformamide and the reaction time was extended to 3 days. All the compounds formed salts with 1.5 equivalents of fumaric acid.

Melting point and index of refraction

The melting points for pilocarpic acid diesters as fumarate salts were determined using a Reichert Thermovar apparatus (Wien, Austria) and were uncorrected. Refractive indices for pilocarpic acid diesters as a free base were measured with Atago Illuminator (Japan) at room temperature.

Liquid chromatography–mass spectrometry

The solvents were delivered by a Beckmann model 112 pump and samples were injected with a Rheodyne 7125 injector (loop volume 20 μl). The compounds were separated on a deactivated Supelcosil LC8-DB reversed-phase column (15 cm × 4.6 mm i.d., 5 μm). The isocratic solvent system was 0.2 M ammonium acetate–acetonitrile (40%–60%) and flow-rate was 1.0 ml min^{-1}.

A VG thermospray–plasmaspray probe was coupled to a VG Trio-2 quadrupole mass spectrometer. The instrument was operated in

the thermospray ionization mode. The thermospray probe temperature was 165°C, the ion source temperature was 150°C and the repeller voltage was 170 V. The other ion source conditions were optimized daily.

Liquid chromatography

High-performance liquid chromatography was performed with a system consisting of a Beckman programmable solvent module 116, a Beckman variable UV-detector 166 (set at 215 nm), System Gold data module, Marathon autosampler equipped with column thermostat and a Rheodyne 7080-080 loop (20 μl) injector. A deactivated Supelcosil LC8-DB (15 cm × 4.6 mm i.d., 5 μm) reversed-phase column was used and the isocratic solvent system was 0.02 M KH_2PO_4, pH 4.5–methanol (29%–71%). The flow rate was 1.0 ml min^{-1}.

EI mass spectrometry

The EI mass spectra of the compounds were recorded on a VG 70-250SE magnetic sector mass spectrometer. The resolution of the instrument was adjusted to 10,000. The electron energy was 70 eV, ionization current 500 μA and the ion source temperature was 150°C. Samples were introduced to the mass spectrometer in a glass sample holder with a direct insertion probe. The probe temperature was raised from 30 to 500°C in 2–5 min.

The accurate mass measurement of the molecular ions was carried out automatically with the data system. Perfluorokerosene was used as the reference compound.

NMR spectrometry

^1H and COSY-NMR spectra were recorded on a Bruker AM-250 FT/ASPECT 3000 spectrometer using a 5 mm $^1H/^{13}C$-dual probe, operating at 250 MHz for the ^1H measurements. For ^1H measurements, samples (10–20 mg) were dissolved in 0.6 ml of CD_3OD with Me_4Si (0.1%) as an internal standard. The number of data points in the ^1H experiment was 32 kW, total relaxation time 16 s and pulse angle 45°. The COSY spectra (^1H–^1H-correlated) were acquired as 256*512 W matrices with zero filling to 512*512 W.

Results and Discussion

Synthesis and pilocarpic acid diesters

Although the need for pilocarpine prodrugs with desirable attributes has been generally recognized, the only previously described prodrug types were quaternary ammonium salts of pilocarpine [3] and diesters of pilocarpic acid [4]. Diesters of pilocarpic acid are potentially useful pilocarpine prodrugs due to their high solution stability and adequate rate of bioconversion to pilocarpine in the eye.

The hydrolysis and epimerization of pilocarpine is subject to specific base catalysis [9, 10]. During base hydrolysis pilocarpine undergoes epimerization to yield isopilocarpine, which rapidly hydrolyses to isopilocarpic acid [9]. In the synthesis of sodium pilocarpate the formation of sodium isopilocarpate varied from 10 to 18%. The obtained sodium pilocarpate was used without any further purification for the synthesis of pilocarpic acid monoesters.

Physico-chemical and analytical data for the diesters synthesized are given in Table 1. It should be noted that yields in the synthesis of pilocarpic acid monesters were very modest (30–50%). This was expected due to the

Table 1
Physico-chemical and analytical data of various pilocarpic acid diesters

Compound	Yield* (%) (fumarate)	η_d^{20} (free base)	m.p. (°C) (fumarate)	Formula (fumarate)
1	32	1.523	61–64	$C_{26}O_{10}N_2H_{32}$
2	54	1.482	60–63	$C_{27}O_{10}N_2H_{34}$
3	51	1.514	62–65	$C_{29}O_{10}N_2H_{38}$
4	66	1.510	69–72	$C_{32}O_{10}N_2H_{44}$
5	60	1.555	86–89	$C_{31}O_{10}N_2H_{34}$
6	84	1.543	80–81	$C_{31}O_{10}N_2H_{33}Cl$
7	68	1.498	84–87	$C_{26}O_{10}N_2H_{38}$
8	53	1.494	99–102	$C_{27}O_{10}N_2H_{40}$
9	48	1.494	73–75	$C_{29}O_{10}N_2H_{44}$
10	68	1.523	88–89	$C_{31}O_{10}N_2H_{40}$
11	71	1.531	89	$C_{31}O_{10}N_2H_{39}Cl$

*Yield in synthesis of pilocarpic acid diester from pilocarpic acid monoester.

formation of the quaternary derivative of sodium pilocarpate during the alkylation. However, the quaternary derivative remained in the aqueous dimethylsulphoxide phase when the pilocarpic acid monoester was isolated from the reaction mixture. The reaction of the monoesters with the appropriate acid chlorides produced diesters as free bases in good yield, but they crystallized as fumarate salts incompletely reducing the yields of the synthesis. Nevertheless the crystallization solvent, ether–2-propanol–petroleum ether, was found to be the optimum solvent for diesters giving the best recovery of crystallization.

Identification by TSP-LC–MS

A satisfactory chromatographic separation for diester, monoester and pilocarpine was achieved with isocratic elution when 0.2 M ammonium acetate was used as the buffer in the mobile phase. Ammonium acetate offers the best sensitivity in acetonitrile–water and methanol–water solutions in thermospray analysis [11] and NH_4^+ decreases polar interactions by masking free silanol groups on the bonded stationary phase [12].

Figure 2A shows a typical thermospray mass chromatogram of a synthesis product (*O*-acetyl pilocarpic acid benzyl ester, compound 1). It can be seen that there are two extra peaks in the chromatogram, which were identified by the mass spectra obtained. The thermospray mass spectrum (Fig. 2B) of the first minor impurity (scan 23) had the protonated molecular peak $[M + H]^+$ at *m/z* 209. The retention time and the mass spectrum of the reference compound indicated the impurity to be pilocarpine. No important fragment ions were present in the mass spectrum.

Figure 2C shows corresponding mass spectrum from the second minor peak (scan 28). The mass spectrum from the scan 28 with retention time verified the second impurity to be pilocarpic acid benzyl monoester. The most intense fragments, which appeared at *m/z* 317 and *m/z* 209, had been assigned to the $[M + H]^+$ and [pilocarpine + H]$^+$, respectively. The ion *m/z* 209 may be formed by fragmentation of the benzyl substituent following the cyclization of the ion to pilocarpine or by thermal decomposition to pilocarpine in the heated thermospray capillary. A similar reaction occurs in the hydrolysis of pilocarpic acid monoesters to pilocarpine *in vitro* and *in vivo*.

The TSP-mass chromatograms of all the pilocarpic acid diesters were totally similar as shown in Fig. 2A. Typical impurities of diesters were pilocarpine and pilocarpic acid monoester as described in Fig. 2. However it should be noted that many synthetic products were very pure and no impurities were found.

The protonated molecular ion $[M + H]^+$ was a predominant peak in the positive ion TSP mass spectra of the all pilocarpic acid diesters during the LC–MS analysis using ammonium acetate as an electrolyte. A very typical thermospray mass spectrum of the pilocarpic acid diester is shown in Fig. 2D. The base peak at *m/z* 359 corresponded to the protonated molecular ion of *O*-acetyl pilocarpic acid benzyl ester (compound 1) (*m/z* = 358). The mass spectrum of the compound 1 showed a very weak peak at *m/z* 717 (below 1%), which may correspond to the attachment of two molecules of analyte plus hydrogen $[2M + H]^+$. Similar ions can also be found in some other spectra of the pilocarpic acid diesters. The ammonium adduct ion $[M + NH_4]^+$, which is a characteristic ion in the positive ion TSP mass spectra of many compounds, was not found in the mass spectra of pilocarpic acid diesters. Pilocarpic acid diesters are perceptibly more stable than pilocarpic acid monoesters in aqueous solutions. This character of the diester was also seen in the TSP mass spectrum, as the peaks at *m/z* 317 [pilocarpic acid monoester + H]$^+$ and at *m/z* 209 [pilocarpine + H]$^+$ are very weak (Fig. 2D).

During the development of any drug it is always necessary to identify the impurities formed during the synthesis, and the decomposition products formed as a result of e.g. stability testing. TSP-LC–MS method is suitable in identification of impurities at very low levels and hence this technique is very useful in evaluation of the purity of the synthetic products. As there was no data available on the thermospray spectra of pilocarpic acid diesters, a study was undertaken to characterize the behaviour of these compounds by positive TSP ionization. TSP mass spectra of pilocarpic acid diesters do not include important fragment ions and the structural information obtained is often insufficient for identification. However, TSP-LC–MS can provide molecular weight information for non-volatile, thermally or chemically labile compounds. Consequently this technique is of great interest in pharmaceutical research.

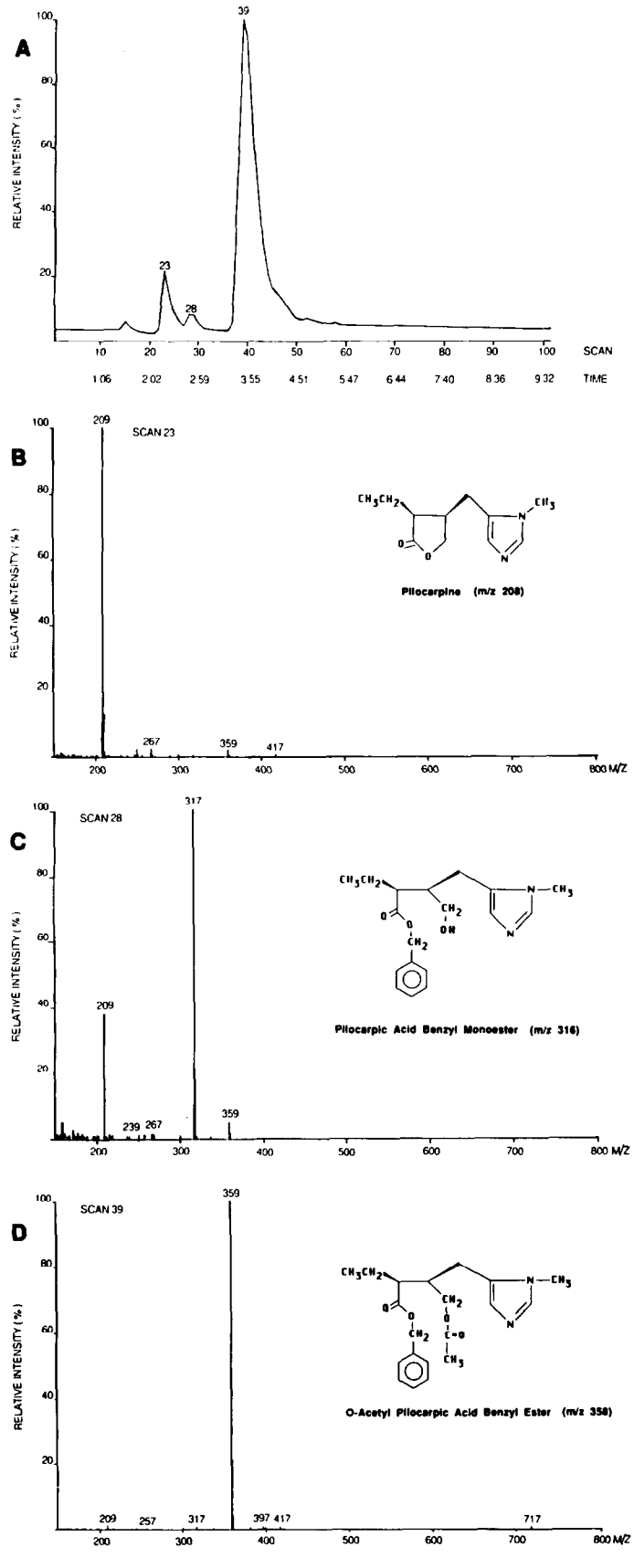

Figure 2
Thermospray mass chromatogram of *O*-acetyl pilocarpic acid benzyl ester (compound 1) (A), thermospray mass spectrum from scan 23 (B), thermospray mass spectrum from scan 28 (C) and thermospray mass spectrum from scan 39 (D).

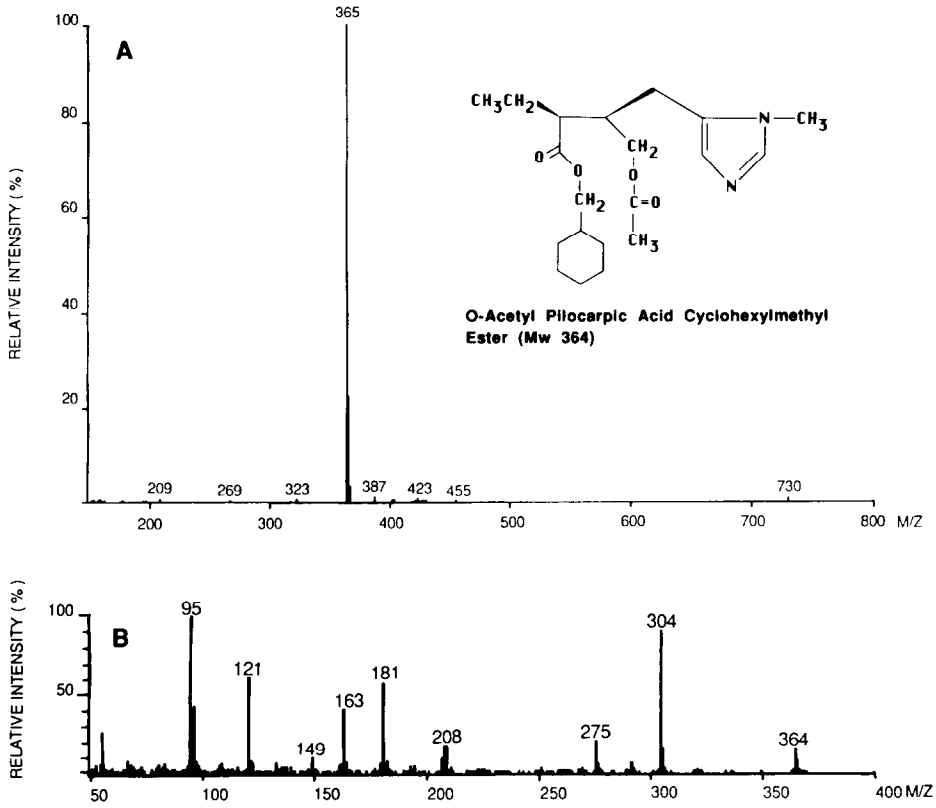

Figure 3
Thermospray mass spectrum (A) and EI mass spectrum (B) of *O*-acetyl pilocarpic acid cyclohexylmethyl ester (compound 7).

EI mass spectrometry

All the pilocarpic acid diesters were also identified by recording the EI mass spectra and the accurate molecular weights of the compounds were determined using high resolution MS.

The EI mass spectrum of the *O*-acetyl pilocarpic acid cyclohexylmethyl ester (compound 7) exhibits at low intensity molecular ion M^+ at *m/z* 364 (Fig. 3B). Compared to the TSP spectrum (Fig. 3A) the EI spectrum shows more intensive fragmentation. The ion at *m/z* 304 may be formed by loss of R_2COO- and the ion at *m/z* 208 by fragmentation of the R_1-group following the cyclization of the ion to pilocarpine. Fragmentation of pilocarpine involved the cleavage of the imidazole ring to give a base peak at *m/z* 95. All the other pilocarpic acid diesters had completely similar EI mass spectra as described in Fig. 3B.

The elemental composition of the molecular ions were determined by measuring the accurate mass with HR-MS. The error between observed and calculated mass was below 2.1 mmu for all the compounds studied (Table 2). This gives reliable verification of the elemental composition of the synthesized pilocarpic acid diesters.

NMR-spectroscopy

Pertinent NMR data are currently required among other documents for identification and documentation of drug substances. The proton NMR data of the pilocarpic acid diesters 1–11 have been collected in Table 3. Our assignments of the diester structures were based on ^1H–^1H-correlated COSY experiment from two representative compounds, 1 and 7. The chemical shifts of measured compounds were in good agreement with pilocarpus alkaloids determined by Link and Bernauer [13].

The limits of detection of impurities and solvent residues was lower than 0.5 mole %. Impurities were identified and quantitated using signals from methyl triplets (CH_3CH_2-) in ethyl side chains and from aromatic signals. The amounts of diester and different by-products were also determined by comparing

Table 2
Measured and calculated accurate mass of the pilocarpic acid diesters

Compound	Observed mass	Calculated mass	Error (mmu)	Elemental composition (free base)
1	358.1895600	358.1892600	−0.3	$C_{20}O_4N_2H_{26}$
2	372.2052310	372.2049310	−0.3	$C_{21}O_4N_2H_{28}$
3	400.2346040	400.2362040	1.6	$C_{23}O_4N_2H_{32}$
4	442.2818810	442.2831810	1.3	$C_{26}O_4N_2H_{38}$
5	420.2054215	420.2049215	−0.5	$C_{25}O_4N_2H_{28}$
6	454.1678070	454.1659070	−1.9	$C_{25}O_4N_2H_{27}Cl$
7	364.2362820	364.2362079	−0.1	$C_{20}O_4N_2H_{32}$
8	378.2497410	378.2518579	2.1	$C_{21}O_4N_2H_{34}$
9	406.2838444	406.2831581	−0.7	$C_{23}O_4N_2H_{38}$
10	426.2504270	426.2518579	1.4	$C_{25}O_4N_2H_{34}$
11	460.2127690	460.2128856	0.1	$C_{25}O_4N_2H_{33}Cl$

Table 3
^{1}H chemical shifts for pilocarpic acid diesters (all the compounds measured as 1.5 equivalents salts with fumaric acid)

	Compounds										
	1	2	3	4	5	6	7	8	9	10	11
Proton											
2	8.40	8.37	8.37	8.35	8.34	8.33	8.41	8.39	8.38	8.36	8.35
4	7.16	7.14	7.14	7.12	7.16	7.16	7.18	7.17	7.17	7.20	7.20
N-Me	3.65	3.65	3.65	3.65	3.67	3.67	3.77	3.76	3.76	3.77	3.78
6	2.68	2.67	2.66	2.66	2.80	2.79	2.75	2.75	2.75	2.87	2.87
7	2.34	2.34	2.33	2.34	2.53	2.52	2.36	2.36	2.34	2.54	2.55
8	2.54	2.54	2.54	2.54	2.64	2.64	2.51	2.51	2.50	2.61	2.61
9	1.69	1.69	1.69	1.69	1.76	1.76	1.67	1.67	1.67	1.75	1.75
10	0.87	0.87	0.87	0.88	0.90	0.91	0.91	0.91	0.91	0.94	0.95
11	4.05	4.06	4.06	4.06	4.33	4.33	3.89	3.89	3.89	3.82	3.83
R_1^α	5.14	5.13	5.14	5.13	5.10	5.10	4.08	4.09	4.08	4.34	4.35
R_1^β	—	—	—	—	—	—	1.77*	1.77*	1.77*	1.70*	1.70*
R_1^γ	7.35	7.35	7.35	7.35	7.32	7.32	1.72	1.72	1.72	1.67	1.67
R_1^δ	7.35	7.35	7.35	7.35	7.32	7.32	1.24	1.24	1.25	1.19	1.20
R_1^ϵ	7.35	7.35	7.35	7.35	7.32	7.32	1.03	1.03	1.03	0.96	0.96
R_2^α	1.97	2.28	2.29	2.28	—	—	2.02	2.33	2.32	—	—
R_2^β	—	1.07	1.54	1.56	7.96	†	—	1.10	1.58	7.97	‡
R_2^γ	—	—	1.32	1.28§	7.47	†	—	—	1.35	7.49	‡
R_2^δ	—	—	0.90	0.87	7.60	†	—	—	0.92	7.62	‡

* Assignment of chemical shifts for cyclohexyl ring is not perfect because of broad and complicated structure of spectral lines.
† Shift of 3-chlorobenzoyl ring: 7.89, 7.86, 7.63 and 7.47 ppm.
‡ Shift of 3-chlorobenzoyl ring: 7.92, 7.89, 7.64 and 7.49 ppm.
§ 8 H broad multiplet.

the integrals of α-protons of ester side chains. No significant degradation procedures were observed during NMR measurement.

Conclusions

In summary, the methods developed in the present paper allow rapid and unambiguous identification and estimation of the purity of the pilocarpic acid diesters. TSP-LC–MS is an especially useful technique in identification of impurities, particularly if the supposed impurities or decomposition products are polar or thermolabile compounds and no reference compounds are available.

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