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Short communication

Determination of neostigmine in human plasma and cerebrospinal fluid by high-performance liquid chromatography with ultraviolet detection

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Abstract

A high-performance liquid chromatographic assay coupled with ultraviolet detection has been developed for the determination of neostigmine in human plasma and cerebrospinal fluid. A novel solid-phase extraction procedure was first used for this analyte and allowed good recovery (89±4.5%) together with ease and speed of execution. The method was sensitive, reproducible (C.V.<4.5%) and accurate (100±6.6%) over the range 2.6–167.0 ng/ml neostigmine concentrations in plasma or cerebrospinal fluid, and was applied successfully to study the pharmacokinetics of neostigmine in patients suffering from chronic postoperative abdominal pain.

Keywords: Neostigmine

1. Introduction

Neostigmine, 3-[[(dimethylamino)carbonyl]oxy]-*N*,*N*,*N*-trimethylbenzenaminium methylsulfate (Fig. 1), a reversible cholinesterase inhibitor, has been extensively used in the treatment of myasthenia gravis and during reversal of non-depolarizing neuromuscular blockade [1]. Recently, it has been investigated in patients for the relief of post-surgical pain after abdominal [2] or gynaecological surgery [3] after intrathecal and epidural injections. Quantitative determination of neostigmine concentration in plasma and cerebrospinal fluid (CSF) is a prerequisite for further pharmacokinetic–pharmacodynamic studies in this promising area.

Previous methods for the determination of neostigmine in plasma were based on liquid–liquid ion-pair extraction which was a tedious and time-consuming procedure [4–9]. The limit of sensitivity of these assays varied from 2–5 ng/ml plasma using GC with a nitrogen-sensitive detector [5,7] to 0.1–0.2 μg/ml plasma utilizing HPLC with UV [4,8] and a fluorescence detector [6]. In view of the recent use of

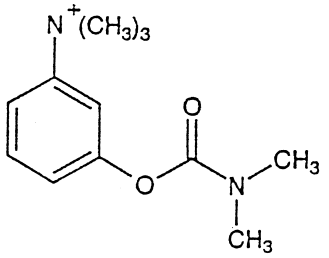

Fig. 1. Chemical structure of neostigmine.

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neostigmine as an adjunct in anaesthesia, a method previously described [10] for muscle relaxants was successfully adapted and proved to be specific and more sensitive for the determination of neostigmine in human plasma and CSF.

2. Experimental

2.1. Chemicals and reagents

Neostigmine was obtained from Aldrich (Milwaukee, WI, USA) and *N*-methyl-laudanosine, the internal standard, was kindly provided by Glaxo Wellcome (NC, USA). Solvents of HPLC grade were purchased from Anachemia (Montreal, Canada) and all others chemicals were from Fisher Scientific (Montreal, Canada).

2.2. Instrumentation

The HPLC system consisted of a Constametric 4100 pump (TSP, Riviera Beach, FL, USA), a model Spectra System AS 3000 injector (Spectra-Physics Analytical, Freemont, CA, USA) and a model SM 4000 UV detector (Milton Roy, Riviera Beach, FL, USA) set at 200 nm. The column (15 cm×4.6 mm I.D.) was packed with 5-μm Spherisorb SCX (Phenomenex, Torrance, CA, USA). Mobile phase, consisting of a mixture (56:44) of 24 m*M* sodium sulfate in 0.43 m*M* sulfuric acid and acetonitrile, was prepared daily. The solvent flow-rate was 2.0 ml/min and the chromatographic system was operated at room temperature.

2.3. Standard solutions

A 10 mg/ml stock solution of neostigmine methylsulfate was prepared in HPLC water and further diluted to 5 μg/ml to obtain the working solution. Concentrations are expressed as neostigmine base in the text. A 0.1 mg/ml stock solution of *N*-methyl-laudanosine was prepared in a 0.5 m*M* sodium sulfate solution and further diluted to 1 μg/ml.

2.4. Calibration curves

A pool of drug-free plasma spiked with 167.0 ng/ml neostigmine was serially diluted with drug-free plasma to yield standards at various concentrations down to 2.6 ng/ml. Calibration curves were generated by weighted regression ($1/X^2$) of the analyte/internal standard peak–height ratio vs. standard concentration of the analyte.

2.5. Sample preparation

Bond Elut phenyl solid-phase extraction cartridges were used. The cartridges were pre-conditioned with 1 ml of acetonitrile, followed by 1 ml of 5 m*M* sulfuric acid. CSF was diluted 1:1 with drug-free plasma before the extraction. Plasma standards or patient samples (1 ml) and the internal standard solution (100 μl) were combined in the reservoir and then aspirated through the sorbent. Cartridges were washed with 1 ml of 5 m*M* sulfuric acid, followed by a 50:50 methanol–water mixture (v/v). Analytes were eluted twice with 300 μl of a 60:40 mixture of acetonitrile–sodium sulfate solution (80 m*M* in 5 m*M* sulfuric acid). The eluate obtained was then reduced to approximately 250 μl in a Speed-Vac Plus concentrator (Model SC 210A, Savant Instruments, Farmingdale, NY, USA). A volume of 150 μl was injected onto the analytical column.

2.6. Recovery

The recovery of neostigmine from plasma was determined in quadruplicate at concentrations of 167.0, 41.8 and 10.4 ng/ml. Plasma samples spiked with known amounts of analyte and internal standard were extracted as usual. Blank plasma samples containing the internal standard only were extracted and subsequently spiked with the same amount of analyte to give the 100% reference prior to evaporation. Recovery was assessed by comparing the analyte/internal standard peak–height ratios in the two corresponding sets of extracts.

2.7. Precision and accuracy

Intra-assay precision was assessed from quadruplicate spiked plasma samples at concentrations of

Table 1
Recovery and within-assay reproducibility of neostigmine assay

Concentration added (ng/ml)	Recovery (%)[a]	Coefficient of variation (%)
167.0	85±1	2
41.8	82±6	7
10.4	100±7	7

[a] Results are expressed as mean±SD, $n=4$.

167.0, 41.8, 10.4 ng/ml on the same day. Inter-assay precision was calculated from calibration curves obtained on five separate occasions.

Drug-free plasma was spiked with neostigmine to yield ten concentrations between 2.6 and 167.0 ng/ml and assayed blindly. The accuracy was evaluated by comparing the estimated concentration of the analyte to the nominal value.

2.8. Clinical study

This method was used to determine neostigmine plasma and CSF concentrations in patients suffering from non-cancer chronic abdominal pain. Plasma (5 ml) and CSF samples (at least 0.5 ml) were withdrawn immediately before and 0.33, 0.66, 1, 2, 3, 4, 6, 8, 10 and 12 h after the administration of a 1.5 mg dose of neostigmine via the epidural route. Samples were immediately put on ice and subsequently frozen at −70°C until HPLC analysis. The CSF concentration over time profile of neostigmine for one patient is presented.

3. Results and discussion

The mean recovery of the analyte in human plasma averaged 89% with an overall coefficient of variation of 4.6% for the three concentrations tested (Table 1). A potential biological matrix effect was excluded by comparing the results obtained when neostigmine was spiked directly in plasma or in 1:1 plasma–CSF mixture. Dilution with plasma was preferred to direct CSF injection to avoid the necessity of duplicating calibration curves and QC samples.

In preliminary experiments, calibration curves ranging from 2 ng/ml to 1000 ng/ml showed a good linearity ($r^2=0.9920$) with an acceptable degree of precision (2.3% at 2 ng/ml) . However, the in vivo CSF concentrations of neostigmine were generally found to be less than 100 ng/ml and the upper range of the calibration curve was subsequently adjusted to 167.0 ng/ml (Table 2).

The intra-assay (Table 1) and inter-assay (Table 2) reproducibilities gave mean coefficients of variation of 5.3% and 3.7%, respectively. The mean accuracy proved to be 93.14% and individual values ranged from 82% to 102%.

Representative chromatograms of plasma extracts are presented in Fig. 2. Although not interfering with the analyte, an endogenous peak was usually observed at 2.5 min in all drug-free plasma used for calibration curves (Fig. 2A). In one patient, chromatograms of plasma extracts obtained before (Fig. 2B) and 2 h after (Fig. 2C) the administration of a 1.5 mg epidural dose of neostigmine show a retention time of 3.8 min for the internal standard

Table 2
Calibration curves of neostigmine in plasma and inter-assay reproducibility

Concentration (ng/ml)	*n*	Extrapolated concentration (ng/ml)[a]	Coefficient of variation (%)
2.6	5	2.7±0.1	2.3
5.2	5	5.3±0.2	4.1
10.4	7	10.3±0.8	7.7
20.9	7	19.5±0.9	4.8
41.8	8	40.2±0.5	1.3
83.5	8	85.0±2.0	2.4
167.0	8	179.4±6.1	3.4

[a] Results are expressed as mean±SD.

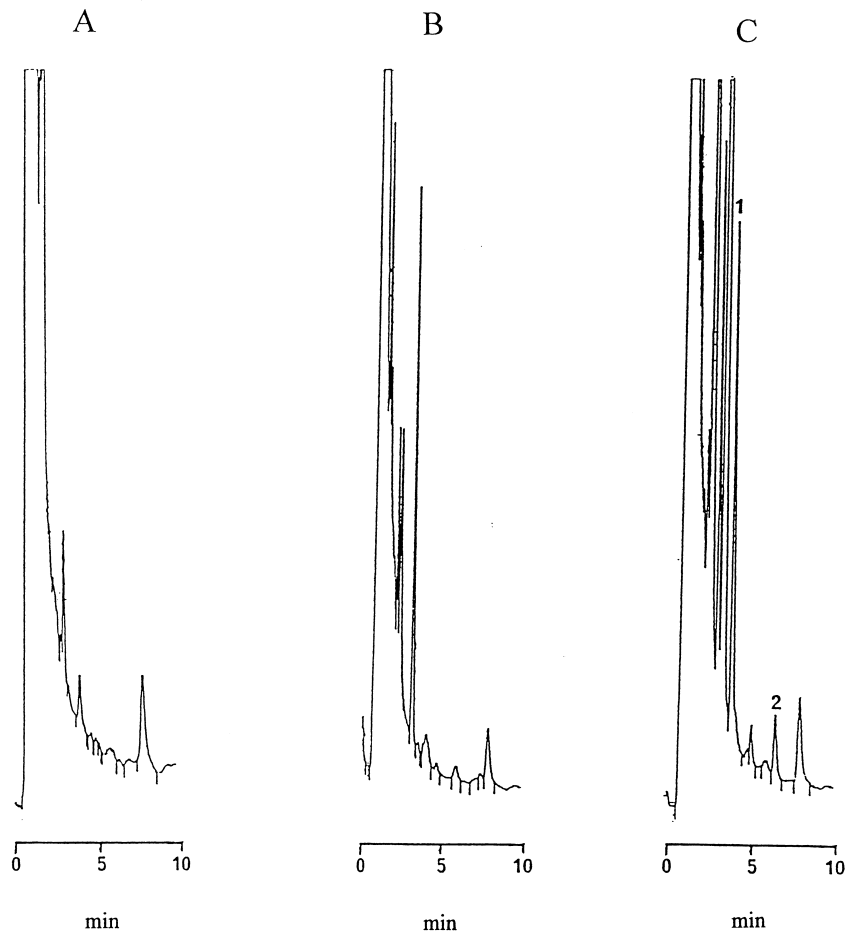

Fig. 2. Chromatographic profiles of extracts from drug-free plasma (A) and plasma obtained from one patient before (B) and 2 h after (C) a 1.5 mg epidural dose of neostigmine methylsulfate. Peaks: 1 = internal standard, 2 = neostigmine; Abs at 200 nm.

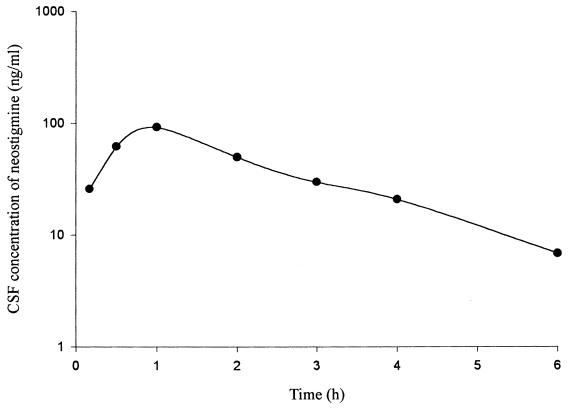

Fig. 3. Concentration–time curve for neostigmine in human cerebrospinal fluid following the epidural injection of a 1.5 mg dose of neostigmine sulfate.

N-methyl-laudanosine and 6.5 min for neostigmine. The temporal profile of neostigmine CSF concentrations for this patient is shown in Fig. 3. Peak concentrations of 93 ng/ml were reached at 1 h and declined gradually to 6.9 ng/ml after 6 h. In plasma, peak concentrations of 27 ng/ml were reached at 2 h and are therefore not represented.

4. Conclusion

The method presented herein for the determination of neostigmine in human plasma or CSF is sensitive, reproducible and readily applicable to pharmacokinetic studies in humans. One major advantage

of this assay is the ease of sample preparation and the reproducibility of solid-phase extraction.

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