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Concentrations of quinolinic acid in cerebrospinal fluid measured by gas chromatography and electron-impact ionisation mass spectrometry
Age-related changes in a paediatric reference population

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Abstract

A simple method for the determination of the excitotoxin, quinolinic acid (QUIN) in cerebrospinal fluid (CSF) is described. QUIN, in lyophilized samples, was silylated by *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide in a single-step reaction at 65 to 70°C to form a di-*tert.*-butyldimethylsilyl ester. Neither pre-purification of QUIN from CSF nor post-derivatisation sample clean-up was required. The derivatives were analysed by gas chromatography–electron impact mass spectrometry resulting in a prominent and characteristic $[M-57]^+$ fragment ion which was used for quantitation. 2,6-Pyridine dicarboxylic acid, a structural analog of QUIN, was used as the internal standard. The detection limits for injected standards are in the femtomole range. CSF QUIN was found to be age-related and three preliminary reference ranges for CSF QUIN were found: 0 to 1 years, 31±15 n*M* QUIN (mean±standard deviation); 1.1 to 3 years, 26±15 n*M*; 3.1 to 14 years, 14±9 n*M*. © 1997 Elsevier Science B.V.

Keywords: Quinolinic acid

1. Introduction

Quinolinic acid (QUIN) is an excitotoxin that acts at the NMDA glutamate receptor [1]. It is believed to be important in the pathogenesis of the neural lesions in a number of human diseases [2–5]. QUIN is synthesised in astrocytes but readily enters the extracellular compartment, particularly when produced in excess [6]. There is no extracellular metabolism nor active transport of QUIN in cerebral tissue [7]. Therefore, when produced in excess QUIN would be expected to accumulate in the extracellular compartment, which includes cerebrospinal fluid (CSF).

QUIN has previously been measured in CSF by gas chromatography–electron capture negative-ion mass spectrometry (GC–ECNI–MS) [8,9]. These sensitive methods, however, require mass spectrometry instrumentation which is not as widely available as that for electron impact mass spectrometry (EI–MS). A gas chromatography–electron impact mass spectrometry (GC–EI–MS) method has

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been used to quantify CSF QUIN but large sample sizes and pre-purification steps are required [10].

In order to measure CSF QUIN in a paediatric population, we developed a simple GC–MS method which took advantage of the ready availability of EI–MS but which also approached the reported sensitivity achieved with ECNI–MS [9].

2. Experimental

2.1. Materials

2,3-Pyridine dicarboxylic acid (quinolinic acid) and 2,6-pyridine dicarboxylic acid (dipicolinic acid; 2,6-PDA) were purchased from Aldrich (Gillingham, UK). *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)-trifluoroacetamide (M*t*BSTFA), with 1% *tert.*-butyldimethylchlorosilane (*t*BDMCS), was obtained from Pierce and Warriner (Chester, UK). HPLC-grade acetonitrile was obtained from BDH Laboratory Supplies (Poole, UK). All water used was purified through a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA).

2.2. Derivatisation

Aliquots (5 to 200 μl) of CSF and aqueous standard solutions, to all of which 250 fmoles of 2,6-PDA in aqueous solution (50 μl) had been added, were lyophilised in 1-ml/2-ml screw cap glass reaction vials. *tert.*-Butyldimethylsilylation was achieved by the addition of M*t*BSTFA/*t*BDMCS (40 μl) and dry acetonitrile (40 μl). The vials were tightly capped (PTFE-faced silicone seals), vortexed for 30 s and heated in an oven at 65 to 70°C for 2 h. Vials were allowed to cool to room temperature and the reaction mixture transferred to autosampler vials for analysis.

2.3. Gas chromatography

Analysis was performed with an HP 5890 Series II Plus gas chromatograph equipped with an HP 7673 autosampler (Hewlett–Packard, Bracknell, UK). Derivatised samples (2 μl; still in derivatising reagent) were injected using the fast-injection mode of the autosampler into a split/splitless injector equipped with a single-tapered injection port liner insert heated to 240°C. Separation was achieved using a fused-silica capillary column (30 m×0.25 mm I.D.) cross-linked with 0.25-μm film thickness of methyl silicone (HP-1; Hewlett–Packard). Carrier gas was helium and the flow rate was temperature programmed (0.8 ml min^{-1} at an oven temperature of 120°C) with an initial pressure pulse (1.6 ml min^{-1} held for 2 min). The oven temperature programmme was as follows: 120°C for 2 min (the spilt valve was then opened), 20°C min^{-1} to 210°C, 1 min at 210°C, 2°C min^{-1} to 234°C, 30°C min^{-1} to 300°C, 2 min at 300°C.

2.4. Mass spectrometry

Mass spectra were obtained using a HP 5970 quadrupole mass-selective detector (Hewlett–Packard). Mass spectra were recorded at an electron energy of 70 eV with an ion source temperature of 170°C and an interface temperature of 300°C. To obtain spectra of the peaks corresponding to the *tert.*butyldimethylsilyl (*t*BDMS) derivatives of QUIN and 2,6-PDA (internal standard), the mass spectrometer was operated in scan mode. Mass spectra were recorded by scanning m/z 50 to 450 at 1.5 scans per s. For quantitation the MS was operated in selected-ion monitoring mode (SIM) at 1.5 scans per s and a dwell time of 100 ms per mass. The initial ion currents measured were m/z 338 (quantitation ion) and 380 (candidate qualification ion). For quantitation the area of the QUIN-*t*BDMS ion current peak area was compared to that of the internal standard ion current peak area, both at m/z 338. Data were acquired and analysed using MS ChemStation (Hewlett–Packard) on a Transcom 486DV2-80 PC.

2.5. CSF collection

CSF was obtained from a reference population of 37 infants and children admitted to the neurological clinic for diagnostic purposes; those suffering from infective or inflammatory disorders and inborn errors of pterin and amine metabolism were excluded. Lumbar CSF was obtained from patients under

sedation or light anesthesia in a standardised way. The CSF was frozen at the bedside by immersion in liquid nitrogen and stored at −70°C until analysis. The first 2 ml was used for other purposes whilst the next 1 ml fraction was used in this study.

2.6. Intra-assay and inter-assay variability and statistical analysis

Standards were prepared by the addition of an aqueous solution of QUIN and 2,6-PDA (10 pmol of each) to aliquots (50 μl) of ventricular CSF and derivatised as described above. Intra-assay and inter-assay variances in the QUIN concentration were calculated from 24 injections of the standard over a 5 day period using analysis of variance (ANOVA); the coefficients of variance were derived from these.

Linear regression equations were compared using Student's *t*-test [11]. Relationships between CSF QUIN concentration and age were examined using Pearson product–moment correlation and ANOVA.

3. Results and discussion

3.1. Derivatisation

M*t*BSTFA is a powerful *t*BDMS donor capable of *tert.*-butyldimethylsilylating active protic functions, including carboxylic, hydroxyl, amino and thiol moieties [12]. The two carboxylic groups of QUIN and 2,6-PDA, the internal standard, were silylated by M*t*BSTFA, in the presence of the catalyst *t*BDMCS, forming di-*t*BDMS esters of both compounds. Because QUIN has been reported to lose a carboxylic acid group (forming nicotinic acid) following rapid heating above 70°C [13], this was avoided although the reaction proceeded to completion quicker at higher temperatures, i.e. up to 100°C.

3.2. Spectrometry

Analysis of *t*BDMS derivatives by GC–EI–MS yields a predominant and characteristic $[M-57]^+$ fragment ion, resulting from the loss of one *tert.*-butyl function $[C(CH_3)_3]$ from the molecular ion. It is the formation of this major fragment ion, containing the entire skeleton of the parent molecule, which endows this method with high sensitivity and specificity. For quantitation of QUIN in CSF, the required sensitivity has only previously been achieved using ECNI–MS of the dihexafluoroisopropyl ester [8] or the dipentafluorobenzyl ester [9]. ECNI–MS generally results in less fragmentation compared to ionisation by EI–MS. However, ECNI–MS instrumentation, in addition to being less widely available than that for EI–MS, requires greater instrument maintenance. We have now shown that the sensitivity reported with ECNI–MS can be approached by EI–MS using the *t*BDMS ester derivatives. A further advantage of our method is that the reaction is performed in a single vial and does not require pre-purification of QUIN nor sample clean-up following derivatisation.

The sole moieties of QUIN which were derivatised were both carboxylic groups. Fig. 1 shows a typical mass spectrum of QUIN–*t*BDMS and its molecular fragmentation pattern. The predominant base peak of the spectrum was *m/z* 338, corresponding to the characteristic $[M-57]^+$ fragment ion, and which was chosen for quantitation. The other two dominant ion fragments were *m/z* 73 and 147 interpreted as $[(CH_3)_3Si^+]$ and $[(CH_3)_3SiO^+{=}Si(CH_3)_2]$, respectively, and were not monitored since they were derived from the *t*BDMS function and did not possess any part of the parent molecule. The second most abundant fragment ion which was interpreted as possessing the QUIN molecule was *m/z* 380 $[M-15]^+$ corresponding to the loss of a methyl group (CH_3). However, the intensity of *m/z* 380 relative to *m/z* 338 was typically about 2% while the intensity of the molecular ion *m/z* 395 $[M^+]$ was very weak (typical relative intensity <0.5%). The intensities of these potential qualifying ions were too small to be measured in samples where the amount of injected QUIN was less than 250 fmoles, equivalent to 50 n*M* QUIN in aqueous standards and samples.

3.3. Chromatography

A typical SIM chromatogram illustrating the resolution of the *t*BDMS ester derivatives of QUIN and the internal standard from other peaks in normal CSF is shown in Fig. 2. Both QUIN and the internal standard produced a single peak and are resolved

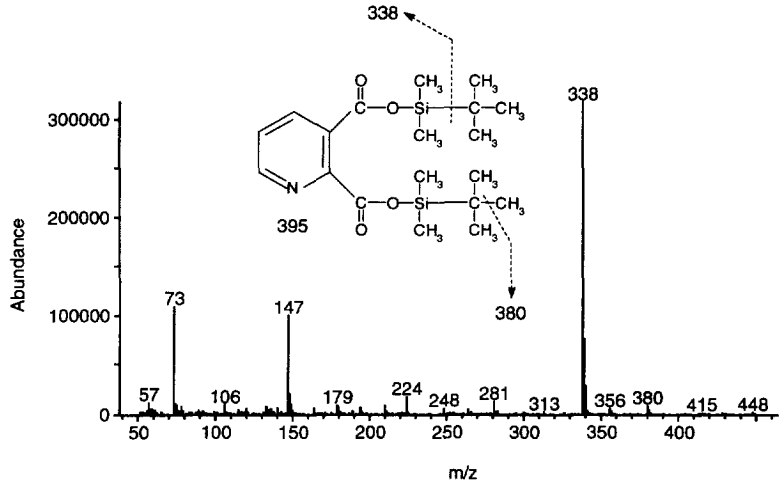

Fig. 1. Mass spectrum obtained by electron-impact ionisation of the di-*t*BDMS ester of 2,3-pyridine dicarboxylic acid (QUIN-*t*BDMS) derived from an 8 pmol QUIN standard. The molecular fragmentation patterns of the base peak (*m/z* 338) and of *m/z* 380 are shown and are derived from the loss of the $[C(CH_3)_3]$ and (CH_3) moieties, respectively, from the molecular ion *m/z* 395 $[M^+]$. Background ion signals which elute immediately prior to QUIN-*t*BDMS were subtracted from the spectrum.

sufficiently from other endogenous compounds to permit accurate quantitation. The values and ratios of the fragment ions were consistent between QUIN recorded from a standard and that from CSF, indicating that QUIN was the sole compound present in CSF at the retention time of the QUIN standard.

3.4. Standard curve, sensitivity, intra-assay and inter-assay variances

Calibration curves based on ion current ratios for QUIN:2,6-PDA were linear over at least the range from 2 n*M* to 1 m*M* QUIN with 50 n*M* 2,6–PDA.

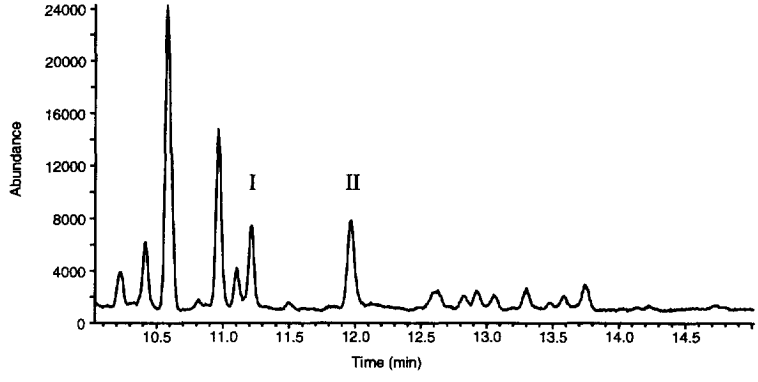

Fig. 2. Selected ion (*m/z* 338) gas chromatogram of normal CSF collected from a 6 year old child and silylated with M*t*BSTFA. The *t*BDMS derivative of QUIN (I; R_t, 11.2 min) is separated from that of the internal standard (2,6-PDA; II; R_t, 11.9 min), and endogenous compounds. 2,6-PDA was added to give a final concentration of 50 n*M*. The concentration of CSF QUIN calculated from the standard curve was 18.3 n*M*.

The concentrations in CSF samples were calculated by reference to a standard curve (2 to 200 n*M* QUIN; 50 n*M* 2,6–PDA). Analysis of injections of 10 fmol of QUIN, equivalent to 2 n*M* aqueous standard solution, resulted in a peak-to-peak *S/N* of 16:1. Intra-assay coefficient of variation (CV) was 11.1% and inter-assay CV was 13.1%.

3.5. Stability of tBDMS derivatives

Periodic repeat analysis of several samples kept at room temperature indicated that there was no change in the amount of QUIN-*t*BDMS or 2,6-PDA-*t*BDMS within 24 h. However, while QUIN-*t*BDMS continued to remain stable, the amount of 2,6-PDA-*t*BDMS detected gradually decreased after 24 h. Therefore, all samples were analysed within 24 h of derivatisation.

3.6. 2,6-PDA as the internal standard

2,6-PDA, a structural isomer (type II internal standard) of QUIN, proved to be a suitable and reliable internal standard. 2,6-PDA was resolved from the endogenous compounds detected in lumbar puncture CSF, ventricular CSF and standard solutions of QUIN at *m/z* 338 and *m/z* 380. No peaks were detected at the retention time of the internal standard in the selected ion (*m/z* 338 and *m/z* 380) gas chromatograms of either normal CSF or QUIN standard solutions which lacked the addition of the internal standard. For the quantification of QUIN in brain and CSF, Heyes and Markey [14] recommended the (^{18}O)isotopmer of QUIN, in favour of the previously used structural isomer 2,4-PDA. They reported that the dihexafluoroisopropanol (HFIP) derivative of (^{18}O)QUIN behaves more physicochemically like QUIN-HFIP than does 2,4-PDA-HFIP and results in optimum reproducibility of the QUIN:IS ratio. While (^{18}O)QUIN is advantageous for the quantitation of QUIN in brain tissue which requires pre-derivatisation extraction of QUIN, we sought to quantify QUIN only in CSF and used a cheaper alternative, 2,6-PDA. In our method there was no post-derivatisation product concentration or extraction and the only sample preparation required prior to derivatisation was lyophilisation of aqueous standard solutions and CSF samples. Therefore, any physicochemical differences between QUIN and 2,6-PDA did not affect the reproducibility of the QUIN:IS ratio. Furthermore, we found that the response of the *t*BDMS derivatives of QUIN and 2,6-PDA in varying concentrations (10 to 1000 n*M*) were identical with respect to both slope (mean (95% CL) difference 0.002 (−4.14 to 4.22), $p>0.1$) and intercept (mean difference 0.08 (−4.51 to 4.67), $p>0.5$). These results indicate that there is no significant variation in the ionisation and fragmentation abundance of these geometric isomers.

3.7. CSF QUIN concentrations in a paediatric reference population

The concentration of QUIN in CSF in a paediatric reference population is age-related (Fig. 3). CSF samples were taken in a standardised manner from 37 infants and children with various neurological and metabolic diseases not expected to affect tryptophan metabolism. There is a highly significant inverse effect of age (ANOVA on the logarithmic regression $F_{1,36}=14.5$, $p=0.0005$). Because a fixed volume of CSF was taken from each patient in our study, regardless of size, the effect of age may be due to the length of the child. This possibility was examined using multiple regression and partial correlation analysis. The effect of age is entirely accounted for by length ($F_{1,35}=16.7$, $p=0.0002$), and once this is

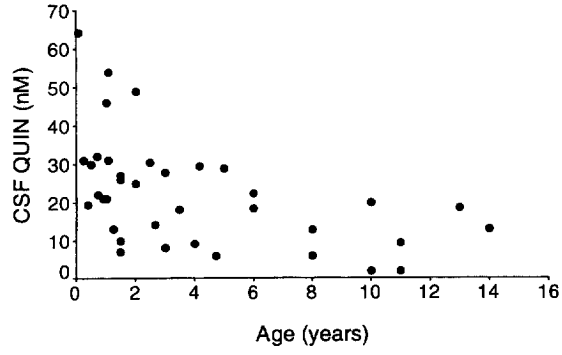

Fig. 3. The effect of age on CSF QUIN concentration in a reference population of 37 infants and children with normal tryptophan metabolism.

Table 1
Paediatric age related preliminary reference ranges for quinolinic acid in cerebrospinal fluid

Age (years)	Number	CSF QUIN concentration (n*M*)		
		Mean	SD	Range
0 to 1.0	7	31	15	20 to 64
1.1 to 3.0	15	26	15	7 to 54
3.1 to 14	15	14	9	2 to 29

taken into account there remains a non-significant direct effect of age which has also been noted in adult reference populations [15]. This finding implies a rostro-caudal gradient of QUIN down the neuraxis and that lumbar CSF QUIN is mostly derived from the brain. Although CSF QUIN concentrations would be more accurately corrected for length, clinical practice suggests age-related norms are more useful.

To construct preliminary age-related reference ranges, the children were divided into 3 groups: 0 to 1 years, 1.1 to 3 years and 3.1 to 14 years. CSF QUIN concentrations were significantly different between the groups (ANOVA $F_{2,34}=5.26$, $p=0.01$). Table 1 shows the age-related reference ranges, in the older children these are comparable to previously published adult reference values [10,15].

In conclusion, we have described a GC–EI–MS method for the quantitation of QUIN in CSF suitable for a clinical or academic environment, providing a simple, relatively cheap and more widely available alternative to the established GC–ECNI–MS methods. In addition, no sample clean-up or pre- or post-derivatisation is necessary and QUIN is added to the growing list of small organic molecules which can be derivatised by M*t*BSTFA. Finally, in establishing paediatric reference ranges we found an age-related relationship for CSF QUIN.

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