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Mass spectrometric analysis of carisoprodol and meprobamate in rat brain microdialysates

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Abstract

We report the evaluation of several mass spectrometry-based methods for the determination of carisoprodol and meprobamate in samples obtained from the rat brain by *in vivo* intracranial microdialysis. Among the techniques that aspire to perform analyses without chromatographic separation and thereby increase throughput, chip-based nanoelectrospray ionization and the use of an atmospheric pressure solids analysis probe fell short of requirements because of insufficient detection sensitivity and hard ionization, respectively. Although direct analysis in real time (DART) provided the required soft ionization, shortcomings of a tandem mass spectrometry-based assay also included inadequate detection sensitivity and, in addition, poor quantitative reproducibility. Therefore, liquid chromatography coupled with atmospheric-pressure chemical ionization tandem mass spectrometry was developed to determine carisoprodol and meprobamate from artificial cerebrospinal fluid as the medium. No desalting and/or extraction of the samples were necessary. The assay, combined with *in vivo* sampling via intracranial microdialysis, afforded time-resolved concentration profiles for the drug and its major metabolite from the nucleus accumbens region of the brain in rats after systemic administration of carisoprodol.

Keywords

In vivo intracranial microdialysis; carisoprodol; meprobamate; nanoelectrospray ionization; direct analysis in real time; atmospheric pressure solids analysis probe; LC–MS/MS; atmospheric-pressure chemical ionization

Introduction

Carisoprodol (N-isopropylmeprobamate, Soma[®]) is a centrally-acting skeletal muscle relaxant frequently indicated for the alleviation of lower back pain and short-term treatment of acute musculoskeletal conditions.^[1,2] Carisoprodol is metabolized relatively rapidly in the liver to the barbiturate sedative hypnotic, meprobamate,^[3] and the latter would seem to account for both its therapeutic effects and abuse potential.^[4] Meprobamate (Miltown[®],

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Supporting Information

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Equanil[®]) was commonly used in the treatment of anxiety before its classification as a Schedule IV controlled substance in the USA.^[5] Based on a propensity to cause abuse, tolerance and physical dependence similar to meprobamate,^[6] carisoprodol also was placed more recently to Schedule IV.^[7] While it has been widely accepted that the therapeutic actions and abuse potential of carisoprodol can be attributed to its metabolism to meprobamate,^[5] recent studies have confirmed that carisoprodol has meprobamate-independent intrinsic effects,^[8–10] and is itself is an active compound with significant barbiturate-like actions in the central nervous system (CNS).^[11]

One approach to discern how carisoprodol itself and meprobamate contribute to rewarding effects that confer abuse, and dependence, would be to compare the time course of each at relevant sites in the CNS. Sampling via *in vivo* intracerebral microdialysis has been an attractive approach to gain insight in distribution of drugs and drug metabolites to the brain.^[12,13] The microdialysis technique offers the possibility to obtain a large number of sample fractions from a single animal with temporal resolution of a few minutes and study duration of several hours. Accordingly, it is desirable to have an analytical method with corresponding throughput available to match the sampling rate of the experiment. Mass spectrometric detection appears to be attractive for this purpose due to its speed, sensitivity, selectivity and ease of use. Due to minimal or no sample preparation, methods that could eliminate online chromatographic separations and thereby increase sample throughput by using ambient ionization and flow-injection analysis^[14,15] have been proposed to increase productivity. For brain microdialysates obtained from pharmacokinetics and drug metabolism studies, this approach has appeared to be especially promising because the protein-free matrix could be considered “simple” compared to biological fluids such as plasma.^[16] Here, we report the evaluation of three sample introduction and ionization methods for the prospective analysis of carisoprodol and meprobamate in rat brain microdialysates without chromatographic separation: chip-based nanoelectrospray ionization (nanoESI),^[17] as well as contact surface desorption/ionization using direct analysis in real time (DART)^[18] and atmospheric pressure solids analysis probe (ASAP).^[19]

Carisoprodol and meprobamate concentrations have also been determined by chromatographic methods combined with mass spectrometric (MS) detection. Gas chromatography–mass spectrometry (GC–MS) without derivatization has a rather high limit of detection (2 µg/mL)^[20] and, therefore, derivatization has been employed to increase volatility and assay sensitivity for these analytes after their extraction from clinical samples.^[21] Carisoprodol and meprobamate can be conveniently separated by reversed-phase (RP) liquid chromatography (LC), and LC-MS methods published using electrospray ionization (ESI) for their determination in human urine and plasma,^[22] bovine serum,^[23] as well as equine urine and serum^[24] have reached limit of quantitation (LOQ) from 2 µg/mL on a single quadrupole instrument^[22] down to 0.25 – 5.0 ng/mL on a triple quadrupole tandem mass spectrometer.^[23] Due to the option of avoiding tedious offline extraction from small volumes followed by derivatization and, instead, simply using online desalting on an RP column, LC appears to be an ideal choice to the hyphenated MS analyses of carisoprodol and meprobamate in samples obtained through *in vivo* intracranial microdialysis. In our studies, a linear ion trap mass spectrometer was used to achieve the necessary sensitivity and selectivity for the reported measurements.

Experimental

Chemicals

Artificial cerebrospinal fluid (aCSF) was obtained from Harvard Apparatus (Holliston, MA, USA). All other drugs, chemicals, analytical standards, reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mass spectrometry

Mass spectra were recorded using a linear ion trap (LTQ, Thermo, San Jose, CA, USA) in the m/z range of 50–300. To perform tandem mass spectrometry (MS/MS), the precursor ions were isolated for collision-induced dissociation (CID) in the LTQ. Specific MS conditions and, if applicable, set-up for MS/MS acquisitions are detailed in the sections below.

Chip-based nanoESI-MS

Nanoflow infusion was performed by a TriVersa Nanomate (Advion, Ithaca, NY, USA) chip-based nanoelectrospray system operated at 0.5-psi nitrogen pressure and 1.5-kV nanoESI voltage in positive ion mode using an ESI Chip with 400 microfabricated nanoelectrospray emitters.^[25] Samples were prepared in water/methanol (1:1, v/v) solutions containing 0.25% acetic acid, and 5 μ L aliquots were deposited into the wells of the microtiter plate placed in the Nanomate. NanoESI and MS data acquisition were carried out by ChipSoft (Advion, version 8.1.0) and the Xcalibur (Thermo, version 2.0) software, respectively. Ion transfer capillary temperature was maintained at 200 °C.

DART

The DART source was a model 100 device (IonSense, Saugus, MA, USA) operated with helium as working gas at a flow rate of 12 L/min and temperature of 300 °C. The discharge needle voltage was set to 3800 V and the voltages of electrode 1 and electrode 2 to +400 V and +500 V, respectively. Samples were prepared by depositing 3 μ L from methanolic solution onto the outer surface of sealed end of glass melting point determination tubes (100 mm $\times$ 1.5 mm o.d., Sigma-Aldrich) followed by air drying. Mass spectra were acquired by bringing the sealed end of the tube with the deposited sample into the stream of helium gas and thereby sweeping the desorbed and ionized molecules into the heated capillary of the atmospheric pressure interface of the instrument.

The $[M+H]^+$ ions of carisoprodol (m/z 261.2), meprobamate (m/z 219.1) and diethyl acetamidomalonate internal standard (ISTD, m/z 218.1) were subjected to collision-induced dissociation (CID) in the MS/MS-based experiments. Meprobamate and the ISTD were fragmented using a common precursor isolation window of m/z 218.6 $\pm$ 1.5 Th, collision energy of 20%, and activation time of 30 ms, but using m/z 158.1 and m/z 176.1 as product ions, respectively, in selected reaction monitoring (SRM). Carisoprodol was detected with precursor isolation window of m/z 261.2 $\pm$ 1.0 Th, collision energy of 20%, activation time of 30 ms, and relying on m/z 176.1 as product ion in the SRM.

ASAP

The ASAP probe (M&M Mass Spec Consulting, Newark, DE, USA) was mounted on the side of the atmospheric-pressure ionization source fitted with the APCI vaporizer and discharge electrode (needle). The vaporizer and heated capillary temperatures were set to 350 °C and 175 °C, respectively, the discharge current to 4 μ A, sheath gas (nitrogen) flow rate to 25 units and auxiliary and sweep gas flow rates to 5 units. As described in the DART section, analytes were deposited onto the outer surface of sealed end of glass melting point determination tubes from methanolic solutions. Then, the air-dried samples were introduced into the corona discharge zone of the APCI source adjacent to the orifice of the heated capillary using the ASAP probe.

LC-APCI-MS/MS

The study was conducted using a Surveyor HPLC gradient pump with a Micro AS autosampler (Thermo, San Jose, CA, USA). The separation was run on a Discovery HS C18 50 mm $\times$ 2.1 mm column with 5 μ m sorbent particles (Supelco, Bellefonte, PA, USA) under isocratic conditions with water/acetonitrile/acetic acid 68:32:0.5 (v/v/v) as a mobile phase at flow rate of 0.25 mL/min. The injection volume was 5 μ L. Online desalting was used with the divert valve mounted on the LTQ directing the effluent to waste for 0.7 min after injection.

Again, the $[M+H]^+$ ions of carisoprodol (m/z 261.2), meprobamate (m/z 219.1) and the ISTD (m/z 218.1) were used as precursor ions of MS/MS scans. Meprobamate and the ISTD were fragmented in a single scan event (fragmentation window m/z 218.6 $\pm$ 1.5 Th, collision energy 20%, activation time 30 ms) using m/z 158.1 and m/z 176.1 as product ions for meprobamate and the ISTD, respectively. Carisoprodol was monitored through its own SRM scan (precursor-ion isolation window of m/z 261.2 $\pm$ 1.0 Th, collision energy of 20%, activation time of 30 ms, detecting the analyte using the major fragment of m/z 176.1 in the SRM chromatograms.

The LC/APCI/MS/MS method was validated in terms of sensitivity, accuracy, precision and linearity using 5 μ L injections of solutions containing carisoprodol and meprobamate standards and 10 ng/mL of ISTD. Limit of detection (LOD) and LOQ were assessed as a concentration that yielded peaks with signal-to-noise ratio (S/N) of 3 and 10, respectively. Calibration was performed for both analytes dissolved in artificial cerebrospinal fluid and in the 1 ng/mL to 1000 ng/mL concentration range. To determine precision and accuracy of the method, solutions containing 10 ng/mL of both analytes and the ISTD were injected in groups of six replicates five times within one day in two-hour intervals (intraday reproducibility) and in six consecutive days (day-to-day reproducibility). The observed relative errors and relative standard deviations (RSD) were calculated from arithmetic means of the six-replicate groups.

In vivo intracerebral microdialysis in rats

Samples were collected from four male Sprague-Dawley rats (Hsd:Sprague Dawley[®]SD[®], 250–300 g body weight, purchased from Harlan Laboratories, Indianapolis, IN) using CMA/12 probes (CMA Microdialysis, Torshamnsgatan, Sweden) according to a protocol

described earlier in detail^[26,27] and modified to sample from the nucleus accumbens region of their brain.^[28] Carisoprodol was administered intraperitoneally (i.p.) at a single dose of 100 mg/kg body weight. At a probe perfusion rate of 2 μ L/min using aCSF, microdialysates were collected every 5 min in the first hour after carisoprodol administration, then every 20 min for 2 hours. The procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center before the initiation of the study. The samples were prepared by mixing with equal volume of 20 ng/mL aqueous ISTD solution to achieve 10 ng/mL concentration of ISTD in the solutions to be analyzed.

A separate in vitro experiment was carried out to determine the microdialysis recovery. A solution containing 200 ng/mL of meprobamate and 20 ng/mL of carisoprodol in aCSF was dialysed at 2 μ L/min to collect four 20-min samples in succession after 1-h probe equilibration, and the concentrations of analytes were then determined in the dialysed solution and in the collected microdialysates. The recovery was defined as the ratio or percentage of these concentrations (microdialysate/dialysed solution).

Results and discussion

After systemic administration, carisoprodol enters the bloodstream and is distributed throughout the body, including the brain.^[29,30] N-Dealkylation of carisoprodol catalyzed by the cytochrome P450 (CYP) isoform CYP2C19 in the liver results in meprobamate,^[3,30] which also has the ability to enter the central nervous system.^[31] As the midbrain-forebrain-extrapyramidal circuit with its focus in the nucleus accumbens has been linked mechanistically to substance abuse,^[32] psychoactive drug and metabolite concentrations measured from this site were expected to yield the most relevant information regarding mechanistic and behavioral correlates studied in experimental animals.^[10,11]

NanoESI, DART and ASAP were evaluated regarding their applicability of chromatography-free MS to carisoprodol and meprobamate analyses in microdialysis samples. Desalting of brain microdialysates is essential for nanoESI; however, it can be done rapidly as part of sample preparation by using ZipTips.^[17] DART and ASAP may tolerate inorganic salts as sample constituents without significant adverse impact on MS analyses of small organic compounds.^[33,34] Nevertheless, desalting will prevent source contamination, which could reduce frequency of cleaning due to formation of nonvolatile inorganic deposits affecting assay performance. Therefore, we also employed acidified aqueous/methanolic sample solutions (containing 10 μ g/mL of the analyte) for the initial “go/no go” evaluation of these methods to determine carisoprodol and meprobamate in rat brain microdialysates.

In addition to focusing on signal intensity attributable to the analytes in the initial evaluations, soft ionization was also considered an important criterion to enable mass spectrometric analyses without chromatographic separation. Specifically, the lack of in-source fragmentation of the molecular ions simplifies mass spectra and facilitates the determination of analytes present at low concentration through MS/MS-based methods.^[35] As demonstrated in Fig. 1, only DART was worth further consideration based on these requirements. Chip-based nanoESI performed poorly for carisoprodol, because the recorded

mass spectrum featured ions unrelated to the analyte (emanating from contaminants of the solvents and/or sample). ASAP was found to be a relatively “hard” (energetic) method of ionization, because extensive fragmentation diminished the relative abundance of the molecular ions ($[M+H]^+$ at m/z 261 and 219) to merely 0.4 and 0.1 percent, respectively, with a small fragment ion at m/z 55 (plausibly $C_4H_7^+$) representing the base peak in the recorded ASAP mass spectra of both carisoprodol and meprobamate.

For a pilot evaluation of an MS/MS-based quantification without chromatography, DART was first optimized by varying parameters of the ion source while ionizing same amounts (9 ng) of carisoprodol and meprobamate to ensure that the system was tuned for the best possible response. Considering the features of the linear ion trap used in our experiments, diethyl acetamidomalonate ($[M+H]^+$ at m/z 218.1) was evaluated as an ISTD despite the mere 1.0 Th difference between the m/z of its precursor ions from that of meprobamate ($[M+H]^+$ at m/z 219.1). The first ^{13}C -isotopic peak of diethyl acetamidomalonate is isobaric with meprobamate; hence, this overlap is non-ideal and should be avoided generally when choosing an ISTD. However, meprobamate and diethyl acetamidomalonate could be isolated together for CID-MS/MS, while the resulting series of product-ion scans could be transformed into two separate SRM traces thanks to the distinct masses of corresponding major fragments (m/z 158.1 for meprobamate and m/z 176.1 for diethyl acetamidomalonate). This latter observation supported our consideration of diethyl acetamidomalonate as an acceptable ISTD even for meprobamate. Figure 2 shows three replicates of desorption/ionization followed by CID-MS/MS after depositing 3 ng of carisoprodol and meprobamate to the glass probes. These were the lowest quantities of the analytes that yielded reliable signal. Nevertheless, it was apparent from the SRM peak areas that the repeatability was not satisfactory for accurate quantification, even without evaluating the coefficient of variation (CV) of the responses, at 1 $\mu g/mL$ analyte levels in sample solutions without preconcentration. Therefore, our implementation of DART-MS/MS was not suitable to determine carisoprodol and meprobamate in rat brain microdialysates. Possible improvement could consider additional pumping applied to the atmospheric pressure inlet on the mass spectrometer to compensate for the increased vacuum load because of the use of high-flow helium, which has been reported to increase detection sensitivity by 10 to 100 times.^[36] Automated sample introduction into the DART beam with high precision may also improve reproducibility of the method.^[36] The application of these custom measures has been beyond the scope of our work; therefore, we decided to develop an LC-MS/MS assay for the analysis of carisoprodol and meprobamate in rat brain microdialysates.

Compared to ESI,^[37] we found that APCI afforded higher detection sensitivity for LC-MS analyses of carisoprodol and meprobamate. APCI also is less prone to matrix effect for dilute samples than ESI.^[38] Full-scan APCI mass spectra of the analytes and product-ion spectra of their protonated molecules are shown in Fig. 3. Meprobamate and carisoprodol were separated well on a short octadecylsilica reversed-phase column under isocratic conditions with an acidified water/acetonitrile mobile phase. Similarly to other assays we developed,^[27,39] online desalting also could be done conveniently by diverting the effluent to waste and thereby avoiding laborious sample preparation such as liquid-liquid extraction also applied to *in vivo* microdialysates.^[40] Meprobamate and diethyl acetamidomalonate

eluted with retention time difference of about 0.1 min. Again, the resulting series of product-ion scans could be transformed into two separate SRM chromatograms thanks to the distinct masses of corresponding major fragments (m/z 158.1 for meprobamate and m/z 176.1 for diethyl acetamidomalonate). Therefore, we developed MS/MS-based quantitation using diethyl acetamidomalonate as an ISTD added to the microdialysates at 10 ng/mL concentration. In Figure 4, SRMs resulting from three replicate 5- μ L injections of meprobamate and carisoprodol solutions, 1 ng/mL each in aCSF, with ISTD concentration of 10 ng/mL are displayed. These analyte concentrations were equal or close to the LOQs based on the S/N values obtained. Therefore, the sensitivity and reproducibility of the measurements demonstrated that LC–APCI-MS/MS was suitable for an accurate determination of carisoprodol and meprobamate concentrations in aCSF as a matrix. Performance of the developed assay is summarized in Table 1. Overall, LOD/LOQ, reproducibility and accuracy met requirements of experimental studies for samples collected by *in vivo* microdialysis from the rat brain. Calibration plots are shown in Figure S-1 in the online Supporting Information. Higher detection sensitivity for meprobamate was due to its higher response factor (approximately 50% higher than that of carisoprodol), partly because it eluted earlier than carisoprodol and, thus, afforded narrower peaks upon isocratic elution, as shown in Fig. 4. Further improvement of the method may include the application of ultrahigh performance liquid chromatography (UPLC), which has been reported to reduce an assay's cycle time to one minute upon measuring heroin and its metabolite from the extracellular fluid of the rat brain using *in vivo* microdialysis sampling.^[41]

Sampling by microdialysis from the extracellular fluid of the rat brain represents a dynamic non-equilibrium system that is not characterized by an explicit link between the concentration of analytes in the tissue and that in the microdialysate collected.^[12] Therefore, a separate *in vitro* experiment was carried out to estimate a “recovery” percentage for sampling, which turned out to be 20% for both carisoprodol and meprobamate. Figure 5 summarizes the concentration–time profile of the drug and its metabolite based on quantitative LC–APCI-MS/MS assays of *in vivo* microdialysates from the nucleus accumbens of rats administered with 100 mg/kg i.p. carisoprodol and considering these recovery estimates. Our preliminary results obtained by using four experimental animals have shown that carisoprodol reached its highest concentration ($c_{\max}$) 20 min after injection ($t_{\max}$), while meprobamate afforded $t_{\max}$ of about 2 h. For about 40 min, carisoprodol concentrations in the extracellular fluid of the nucleus accumbens were higher than or equal to those of meprobamate. On the other hand, $c_{\max}$ of the metabolite was about 5 times higher than that of the parent drug.

Conclusions

Chip-based nanoESI and ASAP fell short of requirements because of insufficient detection sensitivity and hard ionization, respectively, for the development of rapid mass spectrometry-based quantitation without chromatographic separation for the analyses of carisoprodol and meprobamate in rat brain microdialysates. Although DART was promising, subsequent evaluation of an MS/MS-based assay also revealed shortcomings of inadequate detection sensitivity and poor quantitative reproducibility. On the other hand, LOD and LOQ of an LC–APCI-MS/MS method we developed for both analytes were found to be equivalent

to or improved substantially with a concomitant decrease in the assay's cycle time compared to previously published methods. Owing to the relative cleanliness of aCSF as a medium, extraction step(s) could be avoided, and the samples were injected directly on the column with desalting performed online. Combined with *in vivo* intracerebral microdialysis sampling, the method was applicable to obtain time-resolved concentration profiles for carisoprodol and its major metabolite from the nucleus accumbens region of the brain in rats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chip-based nanoESI, DART and ASAP mass spectra of (A) carisoprodol and (B) meprobamate.

Figure 2.

DART-MS/MS analysis of meprobamate (3 ng, upper trace) and carisoprodol (3 ng, middle trace) in triplicate using diethyl acetamidomalonate as an ISTD (1.5 ng, lower trace). The insets indicate the SRMs and the fragmentation of $[M+H]^+$ based on which the compound was detected (m/z given as nominal mass). Carisoprodol-to-ISTD peak area ratio: 0.075 ± 0.032 (CV: 42.1%); meprobamate-to-ISTD peak area ratio: $0.076 \pm 0.060.96$ (CV: 78.5%); carisoprodol-to-meprobamate peak area ratio: $1.525 \pm 1.360.96$ (CV: 89.2%).

Figure 3. APCI mass spectra and CID-MS/MS product ion spectra of the [M+H]⁺ ions of (A) carisoprodol and (B) meprobamate.

Figure 4.

Three replicate 5- μ L injections of meprobamate (1 ng/mL, upper traces), carisoprodol (1 ng/mL, middle traces) for LC-APCI-MS/MS analysis using diethyl acetamidomalonate as ISTD (10 ng/mL, lower traces) and SRMs indicated (m/z given as nominal mass).

Carisoprodol-to-ISTD peak area ratio: $1.43 \cdot 10^{-2} \pm 1.03 \cdot 10^{-3}$ (CV: 9.8%); meprobamate-to-ISTD peak area ratio: $2.60 \cdot 10^{-2} \pm 1.89 \cdot 10^{-3}$ (CV: 7.3%).

Figure 5. Carisoprodol and meprobamate concentrations in the extracellular fluid of the nucleus accumbens of rats determined by *in vivo* microdialysis followed by LC–APCI–MS/MS assay after administration of 100 mg/kg i.p. carisoprodol.

Table 1

Figures of merit summary for the determination of carisoprodol and meprobamate in aCSF solutions by LC–APCI-MS/MS.

	carisoprodol	meprobamate
LOD (ng/mL)	0.25	0.15
LOQ (ng/mL)	1	0.25
Intraday reproducibility (RSD, %)	2.3	4.3
Day-to-day reproducibility (RSD, %)	7.2	12.7
Accuracy (relative error, %)	−16.0	11.6