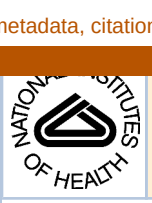

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LC–MS/MS determination of carbamathione in microdialysis samples from rat brain and plasma

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Abstract

A selective liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed for the determination of *S*-(*N*, *N*-diethylcarbamoyl) glutathione (carbamathione) in microdialysis samples from rat brain and plasma. *S*-(*N*, *N*-Diethylcarbamoyl) glutathione (carbamathione) is a metabolite of disulfiram. This metabolite may be responsible for disulfiram's effectiveness in the treatment of cocaine dependence. ~~An analytical method using liquid chromatography–tandem mass spectrometric (LC–MS/MS) was developed to determine carbamathione *in vivo* using microdialysis sampling from rat brain and plasma.~~ Chromatographic separations were carried out on an Alltech Altima C-18 (50 mm long × 2.1 mm i.d., 3 μm particles) analytical column at a flow rate of 0.3 ml/min. Solvent A consisted of 10 mM ammonium formate, methanol, and formic acid (99:1:0.06, v/v/v). Solvent B consisted of methanol, 10 mM ammonium formate and formic acid (99:1:0.06, v/v/v). A 20 min linear gradient from 95% aqueous to 95% organic was used. Tandem mass spectra were acquired on a Micromass Quattro Ultima "triple" quadrupole mass spectrometer equipped with an ESI interface. Quantitative mass spectrometric analysis was conducted in positive ion mode selected reaction monitoring (SRM) mode looking at the transition of *m*/*z* 407–100 and 175 for carbamathione and *m*/*z* 392–263 for the internal standard *S*-hexyl glutathione. The simultaneous collection of microdialysate from blood and brain was used to monitor carbamathione concentrations centrally and peripherally. Good linearity was obtained over a concentration range of 0.25–10,000 nM. The lowest limit of quantification (LLOQ) was determined to be 1 nM and the lowest limit of detection (LLOD) was calculated to be 0.25 nM. Intra- and inter-day accuracy and precision were determined and for all the samples evaluated, the variability was less that 10% (R.S.D.).

Keywords

Disulfiram; Carbamathione; Microdialysis

1. Introduction

A selective and physiologically relevant liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed for the determination of *S*-(*N*, *N*-diethylcarbamoyl)

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glutathione (carbamathione) in microdialysis samples from rat brain and plasma. Carbamathione is a metabolite of disulfiram [1], a drug that has been used for over 60 years for the treatment of alcohol abuse since the discovery that disulfiram sensitized individuals to ethanol [2]. The pharmacological basis for disulfiram's action is its inhibition of liver low K_m mitochondrial aldehyde dehydrogenase ($ALDH_2$). In patients treated with disulfiram, unpleasant symptoms characterized by headache, nausea, and vomiting are produced if ethanol is ingested. Disulfiram requires bio-activation to *S*-methyl *N*, *N*-diethylthiolcarbamate sulfoxide (DETC-MeSO) for its inhibition of $ALDH_2$ [3,4]. *In vivo*, DETC-MeSO is further oxidized to the sulfone which is carbamoylated forming carbamathione, a glutathione adduct (Fig. 1). Carbamathione is a partial NMDA glutamate antagonist and does not inhibit $ALDH_2$ [1]. More recently, disulfiram has been found to be effective in the treatment of cocaine dependence [5], and this effect is independent of ethanol use [6]. We have hypothesized that carbamathione is responsible for disulfiram's efficacy in cocaine dependence, whereas the disulfiram metabolite DETC-MeSO, an $ALDH_2$ inhibitor, is responsible for the aversive action of disulfiram. Carbamathione may therefore prove to be useful as a pharmacological agent in the treatment of cocaine dependence with the advantage that it lacks $ALDH_2$ inhibitory activity.

To further investigate the mechanism of action of carbamathione in cocaine dependence, a selective and reproducible method to determine carbamathione in both ~~brain dialysate and plasma~~ is needed. Many of the methods used to detect drug candidates, including DETC-MeSO and other disulfiram metabolites in brain or plasma require time-consuming clean-up procedures [3]. LC–MS/MS is an excellent technique for the separation and analysis of various drug compounds. In addition, mass spectrometric detection can provide the detection limits needed to detect low level metabolites of drug compounds. Brain microdialysis provides a unique approach for studying changes in brain biochemistry, including minimizing potential tissue damage and interrogating near real time metabolism. As an analytical tool, the dialysis membranes used in the microdialysis studies have a molecular weight cut-off ranging from 5000 to 50,000 Da thus reducing the need for complex protein removal before analytical measurement. The aim of these studies was to develop a LC–MS/MS method for the detection of carbamathione in microdialysis samples from both brain and plasma.

2. Experimental

2.1. Chemicals and reagents

Carbamathione was synthesized using methods previously developed [1,7]. The structure of carbamathione was confirmed by mass spectrometry and NMR (^1H, D_2O). Exact mass determination of $[M+H]^+$ $C_{15}H_{27}N_4O_7S$ was 407.1594 ± 0.0014 (n = 4) which was 2.3 ppm from the expected mass. The NMR chemical shifts were δ 1.14 m 6H ($(CH_3CH_2)_2N$–), δ 2.11 m 2H (Glu-β, β′), δ 2.45 m 2H (Glu-γ, γ′), δ 3.15 m 1H (Cys-β), δ 3.37 m, 5H ($(CH_3CH_2)_2N$–, Cys-β), δ 3.81 t 1H (Glu-α), δ 3.85 s 2H (Gly-α,α′), δ 4.55 t, 1H (Cys-α). The purity of the carbamathione synthesized was determined by HPLC with UV detection at 215 nm. A sample of 1000 nM concentration gave a chromatogram with S/N > 100 and there was no other detectable peak. LC/MS injections of the standard revealed no other MS detectable peaks during the LC gradient.

The internal standard (IS) *S*-hexyl glutathione was purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol, ammonium formate and formic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA). Ketamine was purchased from Fort Dodge Animal Health (Fort Dodge, IA, USA) and xylazine was purchased from Lloyd Laboratories (Shenandoah, IA, USA). Nanopure water was prepared by a Water Pro Plus purification system (Labconco, Kansas City, MO, USA). Sodium chloride, potassium chloride, calcium chloride and magnesium chloride were purchased from Fisher Scientific (Fairlawn, NJ, USA).

2.2. Instrumentation

The HPLC system consisted of a Waters 2695 pump/injector and a reversed phase C-18 column. The mass spectrometer was a Micromass Quattro Ultima "triple" quadrupole (hexapole collision cell) equipped with an electrospray ionization (ESI) interface. The software program that provided the data platform for spectral acquisition, spectral presentation and peak quantification was MassLynx.

2.3. Method

2.3.1. HPLC system—The chromatographic separations were performed on an Alltech Altima C-18 (50 mm × 2.1 mm i.d., 3 μm particles) analytical column (Alltech Associates Inc., Deerfield, IL, USA) at a flow rate of 0.3 ml/min with an analysis time of 35 min. Solvent A consisted of 10 mM ammonium formate, methanol and formic acid (99:1:0.06, v/v/v). Solvent B consisted of methanol, 10 mM ammonium formate and formic acid (99:1:0.06, v/v/v). The chromatograph was developed with a 20 min linear gradient from 95% aqueous to 95% organic followed by a 15 min re-equilibration.

2.3.2. MS system—Tandem mass spectra were acquired on a Quattro Ultima "triple" quadrupole instrument (Micromass Ltd., Manchester, UK). The electrospray source block was 80 °C and probe desolvation temperature 200 °C. Argon collision gas was set to attenuate the beam by 15% (2e–3 mbar on a gauge near the collision cell). The cone voltage was 35 V. Quadrupoles 1 and 3 were tuned to a resolution of 0.9 amu FWHH. Mass spectrometric analysis was conducted in positive ion mode and set-up in selected reaction monitoring (SRM) mode. Data processing was performed on MassLynx 4.1 version software.

2.4. Stock solutions and spiked samples

Ringer's solution, which was used as perfusate for the microdialysis probes, consisted of 145 mM sodium chloride, 2.8 mM potassium chloride, 1.3 mM calcium chloride and 1.2 mM magnesium chloride. A stock solution of 1 mM carbamathione (M.W. 406 g/mol) was prepared in 10 mM ammonium formate. The internal standard (IS), *S*-hexyl glutathione, was prepared as a 1 mM stock solution in 1 M ammonium hydroxide. Standard curves for carbamathione at concentrations of 0.25, 0.5, 1, 5, 10, 25, 50, 100, 1000, 5000 and 10,000 nM were prepared. Calibration standards were prepared by serially diluting the carbamathione stock solution with drug-free dialysate (Ringer's solution). Fifteen microliters of IS (5 nM) was added to 15 μl of the calibration standard sample and vortexed. This mixture was then diluted with 30 μl of water and injected onto the LC column directly.

2.5. Accuracy and precision

The intra-day accuracy and precision were calculated by analyzing five replicates of Ringer's solution containing carbamathione at three concentrations: 5, 100 and 5000 nM. The inter-day accuracy and precision were determined by analyzing the three concentrations on five different runs.

2.6. Stability

The stability of carbamathione in Ringer's solution was evaluated under different temperature and storage conditions. Samples of carbamathione were subjected to room temperature, −20 °C, and three freeze–thaw cycles. All stability studies were conducted at three concentrations of carbamathione (5, 100 and 5000 nM) with three determinations each.

2.7. Matrix effects assessment

The post-column infusion method was used to provide a qualitative assessment of matrix effects and to identify chromatographic regions most likely to experience matrix effects. Briefly, an infusion pump was used to deliver a constant amount of carbamathione into the LC stream entering the ion source of the mass spectrometer. The mass spectrometer was run in SRM mode to follow the infused analyte. Blank dialysate was injected on the LC column under the conditions previously described for the assay. Since the carbamathione was infused into the mass spectrometer at a constant flow, a steady ion response was obtained as a function of time. Any endogenous compound that eluted from the column and caused a variation in ESI response of the infused analyte would be seen as a suppression or enhancement in the response of the infused carbamathione. Matrix effects were also assessed by comparison of standards in Ringer's and in water at three concentrations (5, 100 and 5000 nM).

2.8. Sample collection

2.8.1. Microdialysis probes—The simultaneous collection of dialysate from blood and brain offers a useful approach to monitor drug concentrations centrally and peripherally during drug administration. The procedure used for this study involved the implantation of two microdialysis probes: one into a specific brain region and the other into the jugular vein. Brain microdialysis probes (BR-2) with 2 mm membranes were purchased from Bioanalytical Systems Inc. (West Lafayette, IN, USA). The vascular probes were fabricated in-house [8]. The relative recovery of carbamathione through the microdialysis probe was estimated by delivery experiments. This approach to calibrating a microdialysis probe *in vivo* involves determining the amount of carbamathione that diffuses out of the membrane relative to the amount in the perfusate (i.e., the probe's delivery) and assume that the same relation exists for the amount of analyte that will diffuse into the probe relative to the concentration in the sample (i.e., the probe's recovery) [9]. This delivery experiment was carried out for each microdialysis probe *in vivo* by perfusing 1000 nM carbamathione in Ringer's solution through the probe and determining how much diffused through the membrane.

2.8.2. Surgical techniques—All experiments were carried out in accordance with IACUC animal protocols. Male Sprague–Dawley rats weighing 300–400 g were used for all experiments. The rats were housed in temperature controlled rooms with access to food and water *ad libitum* prior to surgery. The rats were anesthetized by isofluorane inhalation followed by an i.p. injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture. Booster doses of ketamine (1:4 dilution with Ringer's solution) were administered by i.m. injections. The animals remained anesthetized throughout the experiments and microwaveable heating pads were used to maintain the animals' body temperature.

2.8.2.1. Vascular probe implantation: The jugular vein of the rat was isolated by making an incision over the right shoulder of the rat and carefully pulling the fat and tissue out of the way. Once isolated, a small nick was made in the vein with spring scissors held almost perpendicular to the vein. The vascular probe was then inserted into the vein to the vena cava. The jugular was tied above and below the probe to hold the probe in place. The probe was then sutured to the surrounding muscle to prevent it from slipping out of the vein. The incision was then closed with stitches.

2.8.2.2. Brain probe implantation: The brain probe was implanted following the vascular probe implantation. The rat was given a booster dose of ketamine (1:4 dilution with Ringer's solution) i.m. to maintain adequate anesthesia. The hair on the top of the skull of the rat was shaved and disinfected by swabbing the area with ethanol and betadine. The rat was then placed in a stereotaxic apparatus with the incisor bar set at 3.3 mm from the interaural line. A midline 1-in. incision was made through the skin at the top of the skull parallel to the sagittal suture.

The adventitious tissue covering the skull was removed using cotton swabs. The bregma line was exposed for use as a reference point. Two 1 mm diameter holes were drilled 2 mm anterior and posterior to the insertion site of the guide cannula. Two stainless steel anchor screws (1 mm diameter, 2 mm length) were inserted into these holes. The coordinates of the insertion site relative to bregma were –4.8 mm posterior, –1 mm lateral and –7.2 mm ventral, according to the rat stereotaxic atlas [10]. The insertion site was marked with a marker. A 1 mm diameter hole was drilled through the skull at the insertion site with a high-speed dental drill. An intracerebral guide cannula was lowered into the ventral tegmental area (VTA) using a micromanipulator attached to the stereotaxic apparatus. The guide cannula was placed by the BR-2 microdialysis probe.

2.8.3. Dosing and sampling—After probe implantations, tygon tubing was used to attach the end of each probe to a Hamilton 1-ml syringe mounted a CMA 100 microinjection pump from Bioanalytical Systems Inc. (West Lafayette, IN, USA). After probe implantations, the probes were perfused with Ringer's solution at a flow rate of 1 μl/min and allowed to equilibrate for 1 h. Subsequently, basal perfusate samples were collected every 15 min for 2 h in PCR tubes (Fisher Scientific, Fairlawn, NJ, USA.). For dosing purposes, 0.15 M carbamathione was prepared in 0.15 M sodium bicarbonate solution. After the i.p. administration of carbamathione, perfusate samples were collected for an additional 3 h. At the end of the experiment, the rat was sacrificed by placement in an isofluorane chamber for approximately 30 min.

3. Results and discussion

3.1. Method development

In the initial stages of developing a method of detection for carbamathione, several solvents were tested in order to determine the optimal mobile phase. Carbamathione dissolves readily in solution containing ammonium formate and sodium bicarbonate but does not dissolve in pure methanol, acetonitrile or water. Thus sodium bicarbonate was used to dissolve carbamathione for administration to animals and ammonium formate was incorporated into the HPLC mobile phase. Methanol was selected as the organic constituent of the mobile phase with formic acid added to improve the signal strength. *S*-Hexyl glutathione was selected as an internal standard since it was a non-endogenous glutathione adduct. Selected reaction monitoring (SRM) acquisitions were used for sensitivity and extended dynamic range. The SRM of three transitions were recorded, m/z = 100 and m/z = 175 as products of m/z = 407.4 at 25 eV CE ($[M+H]^+$ carbamathione) and m/z = 263 as the product of m/z = 392.4 at 10 eV CE ($[M+H]^+$ *S*-hexyl glutathione) (Fig. 2). Quantification of carbamathione was based on the sum of the integrations of the SRM traces from the two product ions. The cone voltage and collision energy were optimized for carbamathione and the IS by continuously infusing a mixture of the two. The optimum cone voltage was 35 V.

3.2. Matrix effects results

The post-column infusion of 1000 nM carbamathione challenged by the injection of blank dialysate demonstrated no significant matrix interference. The comparison of standards prepared in Ringer's and in water supported this assessment since there was no significant difference in the SRM peak areas.

3.3. Method validation

3.3.1. Selectivity—The selectivity of the method was tested by analyzing drug-free brain and plasma dialysate from five different rats. Each dialysate sample was tested using the HPLC–MS/MS conditions proposed to ensure no interference with carbamathione and the IS. No other endogenous peaks were observed (Fig. 3).

3.3.2. Linearity, accuracy and precision—The calibration curves were prepared in concentrations ranging from 0.25 to 10,000 nM. The carbamathione calibration curve was constructed by plotting the peak area ratio of carbamathione to the IS versus the concentration of carbamathione. The calibration curve was characterized by two different linear segments. The regression equation was $y = 0.1416x + 0.0813$ ($r = 0.9998$, $n = 5$) over the range of 0.25–25 nM and $y = 0.1410x + 3.0845$ ($r = 0.9985$, $n = 5$) over the range of 50–10,000 nM. The calibration data revealed good linear correlations. The lowest limit of detection (LLOD) was estimated as the amount of carbamathione that resulted in a signal three times the noise (S/N ≥ 3). The LLOD was calculated to be 0.25 nM. The lowest limit of quantification (LLOQ) with acceptable accuracy and precision (<10%) was 1 nM. Intra-day accuracy and precision were determined by analyzing five replicates at three different concentration levels. Inter-day accuracy and precision were determined by analyzing samples at the three concentration levels, five times at each concentration (Table 1). For all the samples evaluated, the variability was less that 10% (R.S.D.).

3.3.3. Stability—Stability data are shown in Table 2. Carbamathione was determined to be stable under different temperature and storage conditions. Carbamathione standards that were made up in Ringer's were subjected to short term room temperature conditions for 2 and 4 h, long term storage conditions for 10 and 20 days (−20 °C), and to three freeze–thaw cycles. All the samples evaluated displayed variability of less that 10% (R.S.D.).

3.3.4. Microdialysis probe relative recoveries—Based on the delivery experiments, the relative recovery was determined to be 72.3 ± 3.51% for the plasma vascular probe and 11.4 ± 1.79% for the brain probe. The concentrations of carbamathione determined in physiological samples were corrected for the relative recovery of the probe used.

3.4. Carbamathione administration

This newly developed and validated method has been used successfully in the determination of carbamathione in microdialysis samples from rat brain and plasma simultaneously after the ~~i.p.~~ administration of 20 mg/kg carbamathione (Fig. 4). Sampling intervals of 15 min was achieved.

4. Conclusion

A selective LC–MS/MS method for the determination of carbamathione from *in vivo* microdialysis sampling of rat blood and brain has been described. The separation of carbamathione was carried out on a C-18 column with a methanol gradient. The analyte was detected by tandem mass spectrometry in positive ion mode. MRM experiments were used to monitor the ions of the analyte. A good linearity was obtained over a concentration range of 0.25–10 μM. The LLOQ was determined to be 1 nM and the LLOD was 0.25 nM. The method has been successfully applied to a biological system and used in the determination of carbamathione in microdialysis samples in a reproducible manner.

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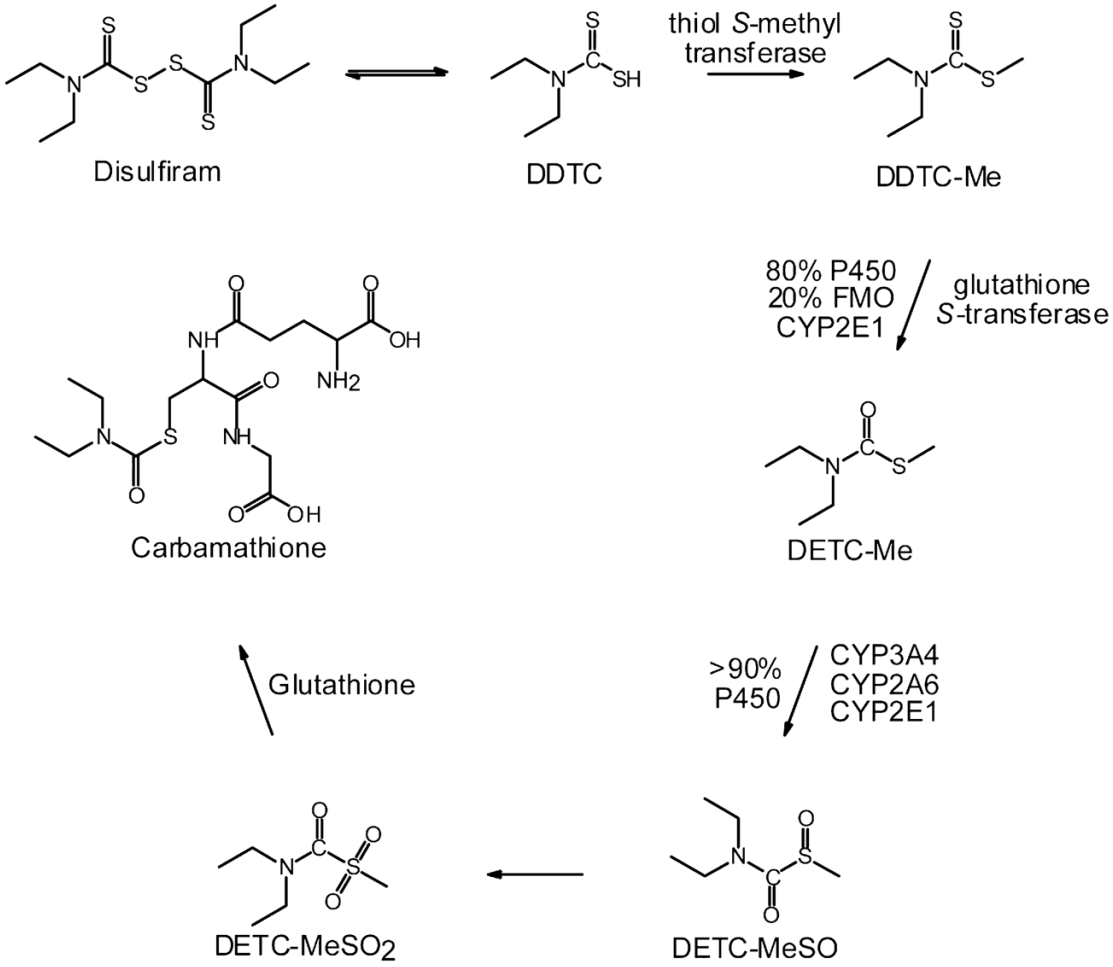


Fig. 1.
Metabolism of disulfiram.

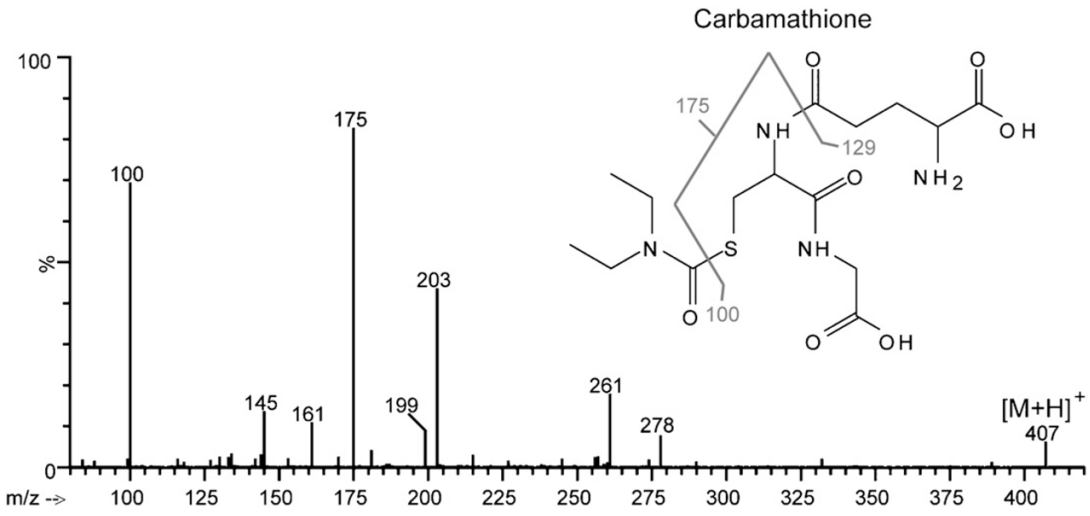


Fig. 2.
Tandem mass spectrum of carbamathione after activation of $[M+H]^+$ at 25 V.

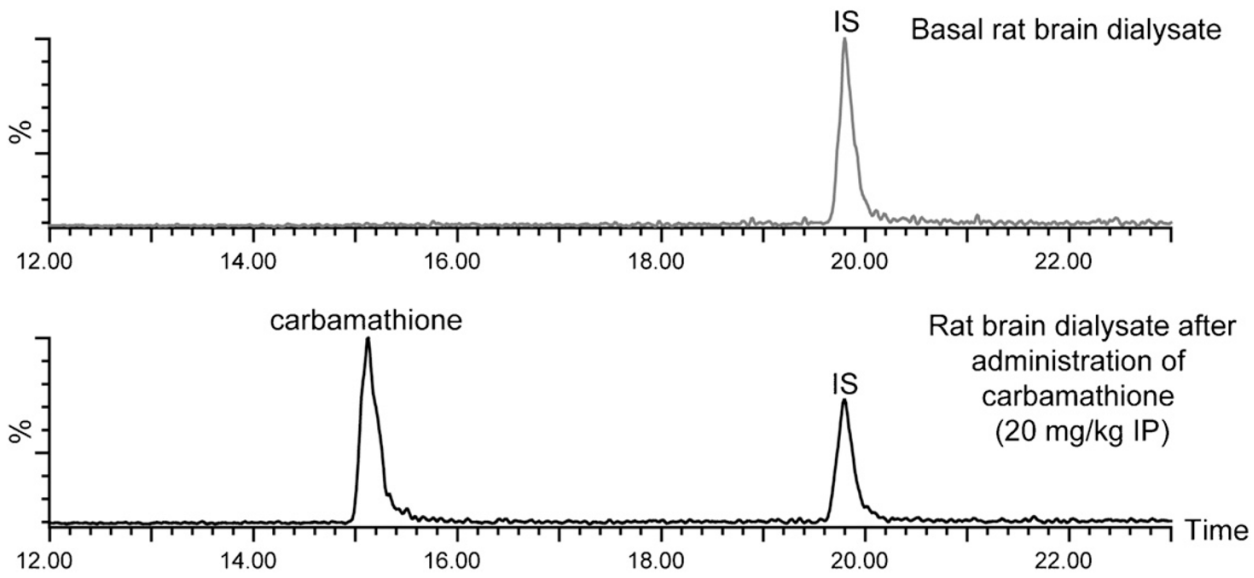
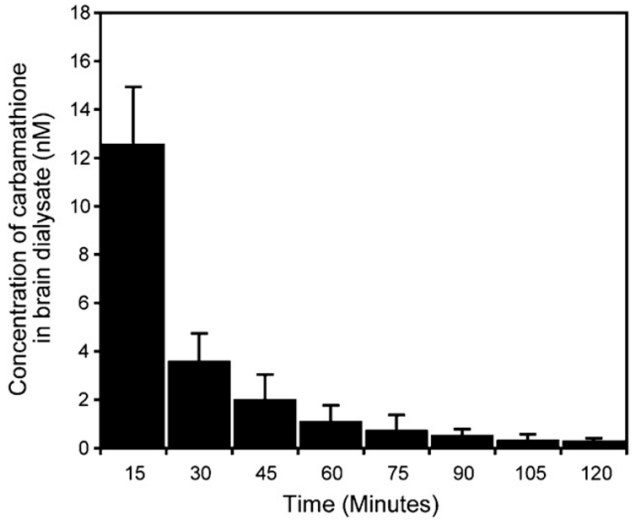


Fig. 3.
HPLC/SRM detection of carbamathione and IS from *in vivo* sampling.



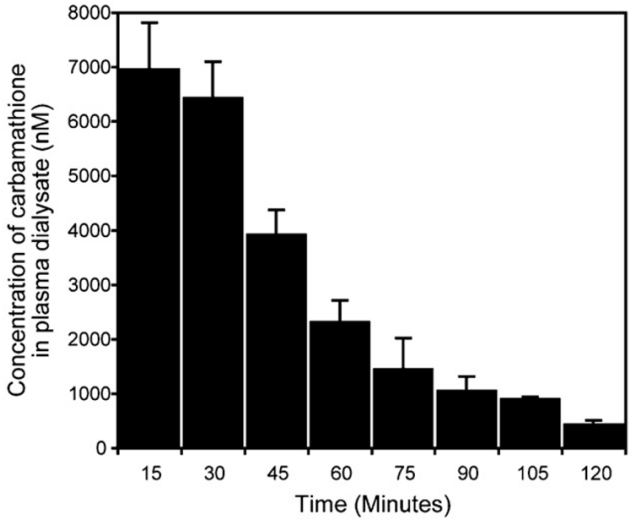


Fig. 4.

Concentration of carbamathione in blood and brain after administration of carbamathione (20 mg/kg ~~IP~~).

Table 1

Intra- and inter-day accuracy and precision for the determination of carbamathione in Ringer's solution (n = 5).

Concentration (nM)		R.S.D. (%)	
Nominal	Mean measured	Intra-day	Inter-day
5	4.780	4.7	7.2
100	103.7	2.2	4.3
5000	4920	3.1	3.8

Table 2

Stability of carbamathione under various conditions (n = 3).

Storage conditions	Concentration (nM)		R.S.D. (%)
	Nominal	Mean measured	
Freeze–thaw stability	5	5.520	8.0
	100	101.3	3.4
	5000	5167	5.5
Stability at room temperature (2 h)	5	4.979	7.0
	100	102.5	5.1
	5000	5041	2.7
Stability at room temperature (4 h)	5	4.779	9.0
	100	95.55	6.9
	5000	5111	6.3
Stability at −20 °C (10 days)	5	4.583	7.4
	100	103.4	2.8
	5000	5016	6.6
Stability at −20 °C (20 days)	5	5.060	8.2
	100	105.8	3.9
	5000	5088	8.7