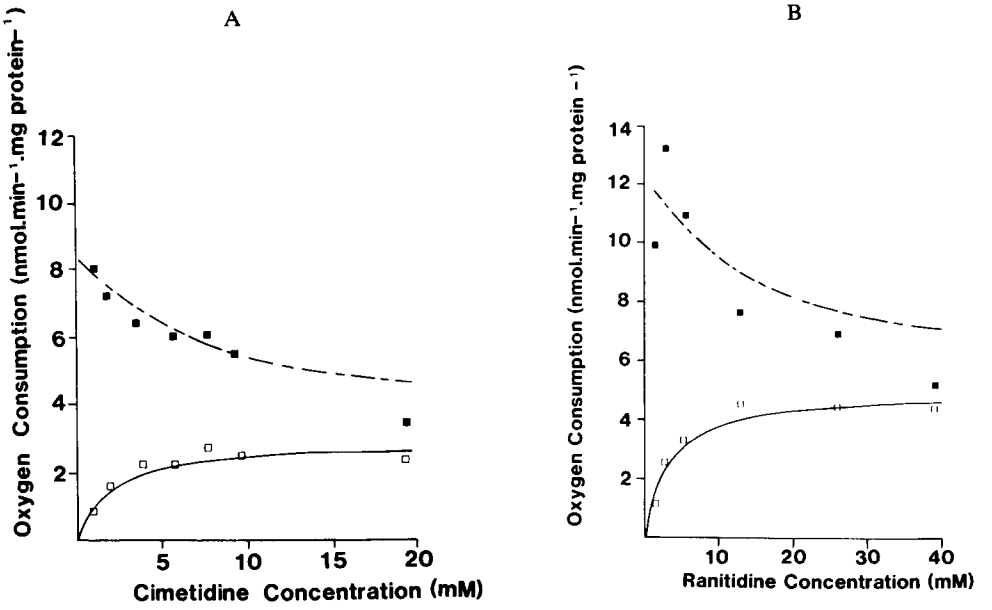

Fig. 3. Total oxygen consumption as a consequence of varying cimetidine or ranitidine concentation in the presence and absence of a fixed methimazole concentration (294.5 μM): (A) Cimetidine plus methimazole: ——— predicted, ■ ■ observed; Cimetidine alone: □ □; (B) Ranitidine plus methimazole: ——— predicted, ■ ■ observed; Ranitidine alone: □ □.

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Natural occurrence of *trans*-gamma hydroxycrotonic acid in rat brain

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γ-Hydroxybutyric acid (GHB) is a naturally occurring substance endowed with potent neuropharmacological and neurophysiological properties [1]. Recently, much evidence has accumulated delineating its role as a possible neurotransmitter (for a review see [2]). While the synthetic pathway of GHB is well established [3, 4], the mechanism of its degradation remains controversial. Its rapid breakdown via the Krebs cycle has been considered [5]. The generation of *trans*-γ-hydroxycrotonic acid (*trans*-HCA) following β-oxidation has also been suggested [6]. Moreover, the existence of GHB and *trans*-HCA in renal tissue has been recently reported [7]. We demonstrated the presence of *trans*-HCA in brain employing capillary gas chromatography, coupled with chemical ionization mass spectrometry, using ammonia as reagent gas.

Materials and methods

Wistar rats, weighing 120–150 g, were stunned by a blow to the head and decapitated. In less than 2 min, the brain was homogenized with the internal standard (β-methyl

trans-HCA) in 4 vol. of 0.1 M formic acid maintained at 0°. After centrifugation (20 min, 5000 *g*) the supernatant was subjected to derivatization using the same procedure that is used for pure compounds.

Trans-HCA was prepared from *trans*-γ-bromocrotonic acid, and β-methyl *trans*-HCA (β-Me *trans*-HCA) was obtained from β-methyl *trans*-γ-hydroxybutenolide [8] by $NaBH_4$ reduction in basic medium.

The internal standard, and *trans*-HCA were derivatized to form pentafluorobenzyl-ester O-trimethylsilyl derivatives as follows: An aliquot (10 μl) of aqueous solution containing 10 μg of *trans*-HCA and/or β-Me *trans*-HCA was dried and treated with 10 μl of a mixture of pentafluorobenzyl bromide (PFB-Br) (Pierce Chemical Company) in acetonitrile (8:92, v/v), and 5 μl of a mixture of diisopropylethylamine in acetonitrile (5:95, v/v); the reaction was carried out for 1 hr at 0°. Excess reagent was removed under a stream of nitrogen, and then 20 μl of a mixture of *N*-*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Merck) in acetonitrile (50:50, v/v) was added and the reaction was allowed to proceed for 30 min at room temperature. Excess BSTFA was removed under a stream of nitrogen, then 40 μl of acetonitrile was added, and then the solution was submitted to mass spectrometry.

The sample (1–2 μl) was placed on the needle of an all-glass type Ros injector before volatilization into a fused silica capillary column (25 m × 0.22 mm i.d., about 10^5 theoretical plates). Helium was used as carrier gas. Mass spectra and selected ion monitoring data were obtained using a GC/MS FINNIGAN 4021 type instrument which was used in the CI mode. Ammonia, at a pressure of 0.20 torr in the ion source, was used as reagent gas, in order to produce prominent $(M + NH_4)^-$ quasi-molecular ions with the derivatized compounds, thus increasing the sensitivity and specificity of detection (Figs. 1 and 2).

Results and discussion

Walkenstein [6] suggested that *trans*-HCA may be present in the brain as a GHB catabolite. During the examination of organic acids in renal tissue by GC/MS, Niwa

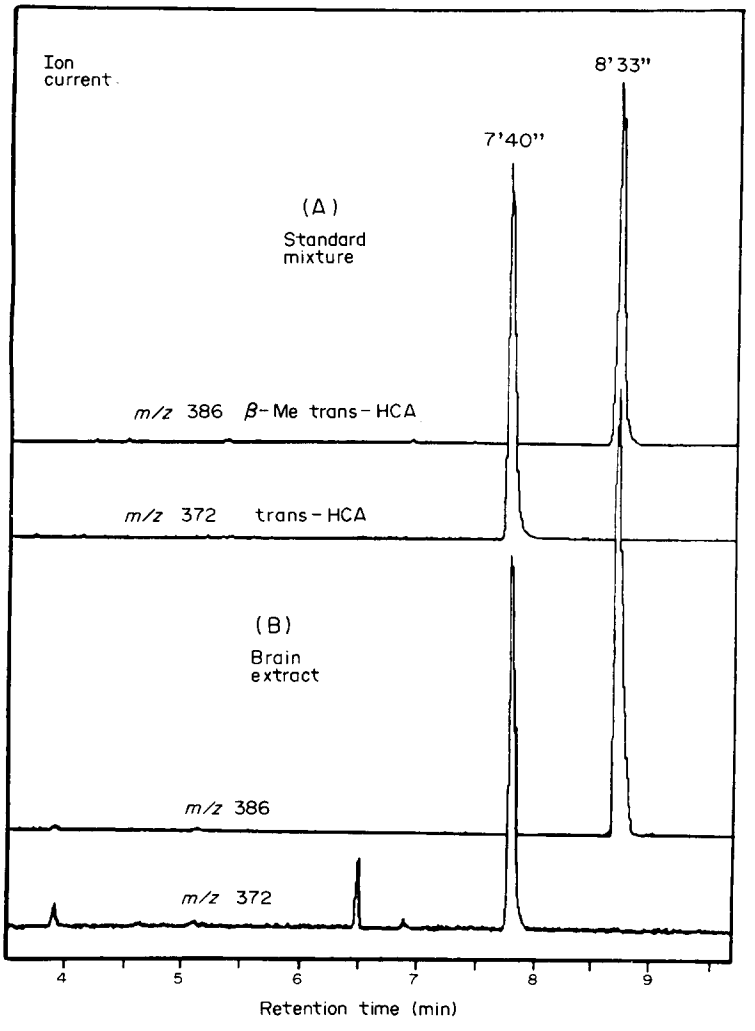

Fig. 1. Plot of the selected ion monitoring (SIM) traces of quasimolecular ions of *trans*-HCA and β-Me *trans*-HCA (pentafluorobenzyl-ester O-trimethylsilyl derivatives). A, authentic compounds; B, brain extract (1500 ng of β-Me *trans*-HCA was added to the brain as internal standard before extraction and derivatization). Conditions were: Column oven: temperature programmed from 150 to 220° at 4°/min; injector 240°; GC/MS interface 250° and ion source 200°. Retention times of authentic and expected compounds were identical. All control samples did not contain interfering peaks at the expected retention times.

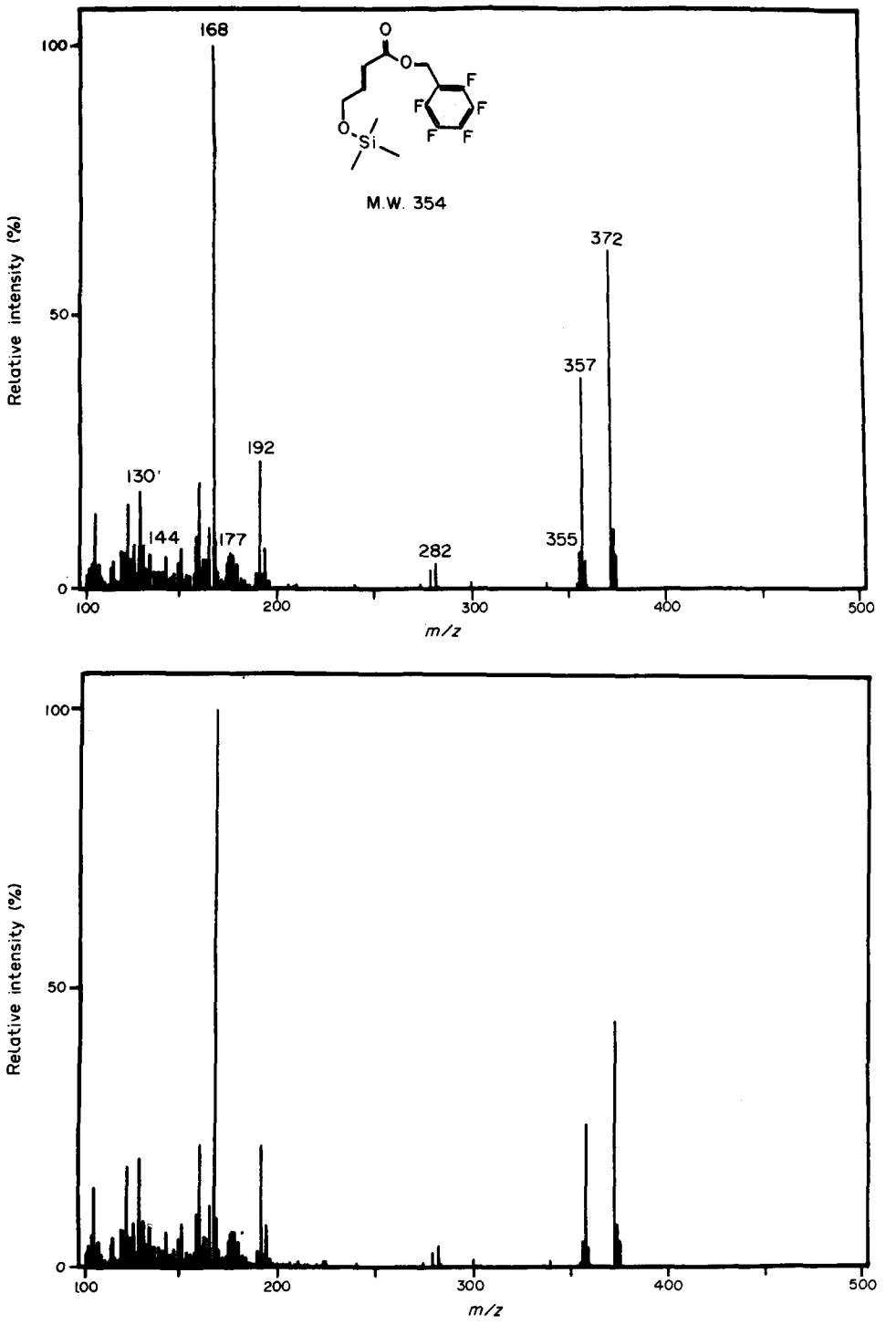

Fig. 2. Ammonia chemical ionization mass spectra for pure *trans*-HCA (pontafluoro benzyl-ester *O*-trimethylsilyl derivative—upper spectrum), and of brain extract (retention time 7'40"—lower spectrum). The pattern displays relatively intense peaks at m/z 372 $[M + NH_4]^+$, m/z 357 $[M\text{-}CH_3 + NH_4]^+$, m/z 355 $[M + H]^+$.

[7] showed the existence of *trans*-HCA, as well as that of GHB, in this tissue. Rat brain *trans*-HCA was measured quantitatively by computing the areas under the mass chromatogram produced with the derivatized tissue extract containing the internal standard. The mass spectrometer was tuned in order to detect only the ionic species at m/z 386 and 372 (respectively $[M + NH_4]^+$ ions from derivatized β-Me *trans*-HCA and expected *trans*-HCA). Accurate quantitative analysis was achieved by using as a reference an adequate calibration curve previously obtained on the derivatized authentic *trans*-HCA and β-Me *trans*-HCA. Using our analytical procedure we demonstrated the presence of

trans-HCA in rat brains and found its endogenous concentration in whole brain to be 22 ± 6 nmoles/g (wet wt) of tissue (mean ± S.E.M.).

This concentration is about 10 times higher than that of γ-hydroxybutyrate. An alternative source of this unsaturated compound in brain other than γ-hydroxyburate could be considered. However, it has not been possible to show the existence in brain of a unsaturated derivative of GABA (i.e. γ-amino-crotonic acid).

Thus, *trans*-HCA is present in brain tissue, as well as in kidney tissue [7], and might represent an intermediate of GHB catabolism in brain [6].

Recently, we have demonstrated that synthestic *trans*-HCA interferred with GHB transport and binding in brain membranes *in vitro* [9, 10]. It seems likely that *trans*-HCA might play an important role as a substance that interfers with the functional role of GHB.

In summary, using capillary gas chromatography ammonia chemical ionization mass spectrometry, we demonstrated for the first time the presence of trans-4-hydroxycrotonic acid in brain. A concentration of 22 ± 6 nmoles/g (wet wt) of *trans*-4-hydroxycrotonic acid was found in rat brain. This short chain unsaturated organic acid might represent an intermediate in 4-hydroxybutyrate metabolism. It is noteworthy that trans-4-hydroxycrotonic acid is also an important ligand for the 4-hydroxybutyric acid binding site in brain [9]. The presence of this unsaturated compound in brain has the potential of opening up a new area of neurobiological research, particularly if it is neuroactive.

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