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Short Communication

Measurement of GABA in rat brain microdialysates using *o*-phthaldialdehyde–sulphite derivatization and high-performance liquid chromatography with electrochemical detection

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Abstract

A number of recent studies have detected γ-aminobutyric acid (GABA) in brain microdialysates by measuring an electroactive *o*-phthaldialdehyde (OPA)–alkylthiol derivative using HPLC with electrochemical detection. A particular problem of this approach is the stench of the thiol reagents involved, and the poor stability of the electroactive GABA derivative. Here we report that these practical disadvantages can be overcome by substitution of the OPA–alkylthiol reaction with an OPA–sulphite reaction. This provides a simple, sensitive and reliable means for determination of GABA in microdialysates of rat brain.

1. Introduction

The intracerebral perfusion technique, microdialysis, is becoming the method of choice for measuring release of the neurotransmitter γ-aminobutyric acid (GABA) in the brain *in vivo*. The detection of the very low levels of GABA in microdialysates has been determined using HPLC in combination with either fluorescence or electrochemical detection [*e.g.* refs. 1–4]. Although GABA itself is neither fluorescent nor electroactive it can be made so by pre-column derivatization. The most common derivatization methods use a reaction between GABA and *o*-phthaldialdehyde (OPA) in the presence of an alkylthiol reagent to produce an isoindole derivative that is both fluorescent and electroactive [5–7].

Despite these developments, particular problems with the OPA–alkylthiol reaction include the stench of the thiol reagents and the often poor stability of the isoindole derivative, particularly that produced by GABA [5,8]. Jacobs, however, has reported that substitution of the alkylthiol reagent with sulphite generates an isoindole derivative which is both odourless and stable [9]. Recently, this approach was utilised successfully to determine GABA in biological samples, specifically *post-mortem* human brain tissue [10]. Here we have utilised the OPA–sulphite reaction to detect GABA in rat brain microdialysates.

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2. Experimental

2.1. Liquid chromatography

The HPLC system consisted of an LKB 2150 pump (Pharmacia Biotech, Milton Keynes, UK), a Rheodyne 7125 injector (20-μl loop, Anachem, Luton, UK), a Rainin Dynamax guard column (Microsorb 5-μm C_{18} particles, 15 × 4.6 mm I.D., Anachem) and a Rainin Dynamax analytical column (Microsorb 5-μm C_{18} particles, 250 × 4.6 mm I.D., Anachem). The electrochemical detector was a Bioanalytical Systems LC-4/4A (Biotech Instruments, Luton, UK) utilising a glassy carbon working electrode (+0.85 V *vs.* Ag/AgCl). Detector output current was monitored using either a Milton Roy 4000 integrator (Milton Roy, Shannon, Ireland) or a Tekman TE 200 stripchart recorder.

The mobile phase was prepared by mixing 4 parts (v/v) of HPLC-grade methanol with 6 parts of aqueous buffer containing 0.1 *M* sodium dihydrogen phosphate and 0.1 m*M* EDTA. The mobile phase was adjusted to pH 4.4 (ranging from 3.6 to 5.2 in certain experiments) with concentrated HCl. Before use the mobile phase was filtered (Whatman 50 hardened filter paper) using vacuum assistance. All solutions were prepared with water purified by a water purification system (Elgastat Spectrum, Elga, High Wycombe, UK). Isocratic separation was achieved at room temperature using a standard mobile phase flow-rate of 1.0 ml/min.

2.2. OPA reagent

The derivatizing reagent was prepared by adding the following in order: 22 mg OPA (Sigma, Poole, UK), 0.5 ml of 1 *M* sodium sulphite (Aldrich, Gillingham, UK), 0.5 ml of ethanol and 9 ml of sodium borate buffer, pH 10.4 (0.4 *M* boric acid adjusted to pH 10.4 with 5 *M* sodium hydroxide). Note that addition of ethanol results in the formation of a precipitate which rapidly dissolves on addition of the borate buffer. The reaction mixture was made up freshly each day.

2.3. GABA derivatization

GABA in the microdialysates was derivatized by addition of 2 μl of the above OPA reagent to 20 μl of microdialysate. The reaction was allowed to proceed for *ca.* 25 min at room temperature before direct injection of the reaction mixture onto the chromatograph. GABA in the standard solutions was derivatized by addition of 10 μl of the above OPA reagent to 1 ml of GABA standard (Sigma).

Assays were calibrated daily by injection of 1 pmol of GABA standard (20 μl of $5 \cdot 10^{-8}$ *M*) made up freshly in artificial cerebrospinal fluid (CSF).

2.4. Microdialysis procedure

The microdialysis procedure was carried out in freely moving rats as described previously [11]. In brief, single cannula dialysis probes (3 mm tip length, Hospal dialysis membrane) were implanted stereotaxically into the nucleus accumbens or striatum of halothane-anaesthetised rats (male Sprague-Dawley, 270–300 g). Approximately 24 h following surgery, rats were connected to a perfusion system for freely moving animals (CMA 120, CMA/Microdialysis, Stockholm, Sweden) and perfused continuously (1 μl/min) for up to 6 h with artificial CSF (140 m*M* NaCl, 3 m*M* KCl, 1 m*M* $MgCl_2$, 1.2 m*M* $CaCl_2$, 1.2 m*M* Na_2HPO_4, 0.27 m*M* NaH_2PO_4, 7.2 m*M* glucose, pH 7.4). Perfusates were collected in small Eppendorf tubes every 25 min.

The recovery of GABA across the dialysis membrane was determined *in vitro* by immersing the tip of the microdialysis probe (perfused continuously at 1 μl/min) in artificial CSF containing $5 \cdot 10^{-7}$ *M* GABA at 37°C. Three 25-min perfusates were collected and the recovery was expressed as the mean concentration of GABA in perfusate/concentration of GABA in external medium (x 100). Probes were soaked in artificial CSF at 37°C for 24 h prior to the experiment.

3. Results and discussion

The reaction of exogenous GABA with the OPA–sulphite reagent generates an electroactive product that, under our chromatographic conditions, elutes after *ca.* 6 min (Fig. 1). This product increased linearly in proportion with

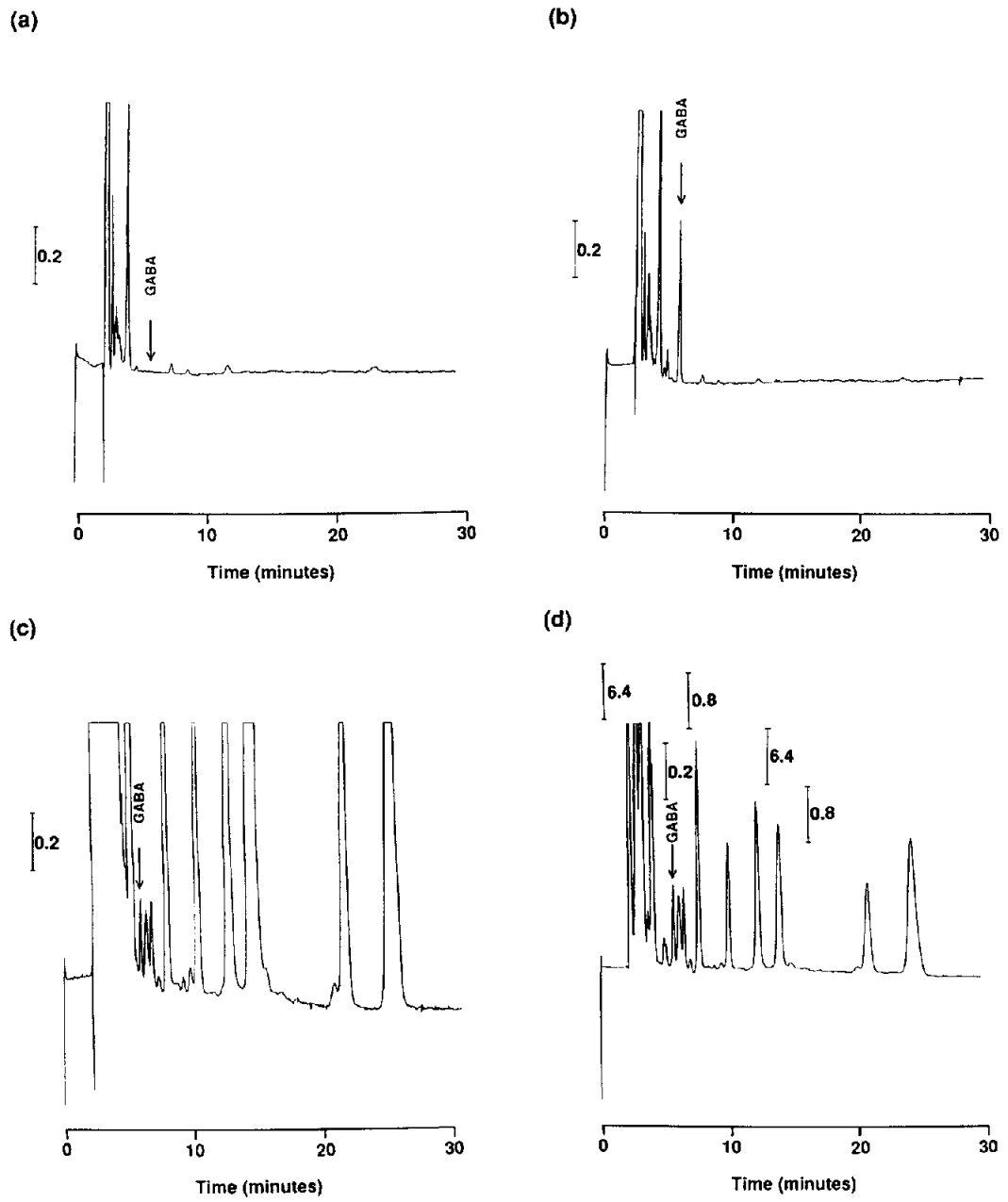

Fig. 1. Original chromatograms showing separation of OPA–sulphite derivative of GABA. (a) OPA reagent added to artificial CSF. (b) OPA reagent added to artificial CSF containing 1 pmol GABA. (c) OPA reagent added to microdialysate from rat nucleus accumbens. (d) Chromatogram represented in (c) but with altered attenuation. Note that chromatograms (a), (b), and (c) are all at the same attenuation. Mobile phase; 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, 40% (v/v) methanol, pH 4.6. Detection; glassy carbon electrode, +0.85 V *vs.* Ag/AgCl. Chromatograms are originals recorded by a Milton Roy CI4000 integrator. Load volume 20 μl. Verticle calibration bars represent detector output current (nA).

increasing amounts of GABA (least squares fit; $y = 0.919x + 0.079$, $R^2 = 1.00$), which is consistent with the OPA–sulphite derivatization of GABA to an electroactive isoindole product as previously identified [9]. The hydrodynamic voltammogram (Fig. 2) yielded a half-wave potential of *ca.* +0.74 V *vs.* Ag/AgCl (pH 4.4, phosphate buffer).

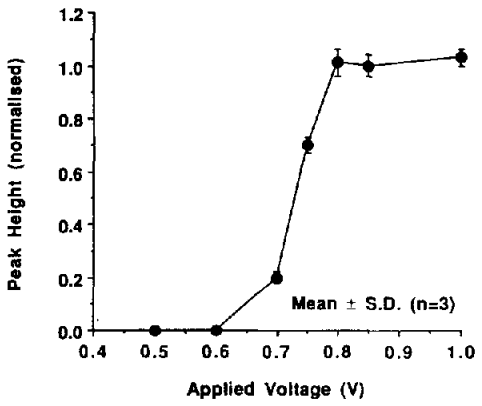

Fig. 2. Hydrodynamic voltammogram of GABA OPA–sulphite derivative. Mobile phase as described in methods (pH 4.4, flow-rate 1.0 ml/min).

Addition of further amounts of OPA–sulphite reagent to the samples did not increase the amount of reaction product, indicating that the reagent is in excess. We found that the reproducibility of the reaction was satisfactory: intra- and inter-assay coefficients of variation for determination of 1 pmol GABA were 4.6% (n = 11) and 9.6% (n = 8), respectively. Furthermore, small amounts of GABA could be quantified with the approximate limit of detection (twice baseline noise) being 25–50 fmol. These findings fit well with those of Pearson *et al.* [10] who used the OPA–sulphite reaction in combination with HPLC with electrochemical detection to determine GABA in *post-mortem* human brain tissue.

The high sensitivity of the OPA–sulphite derivatization method allowed us to detect GABA in brain microdialysates of the awake rat (Fig. 1). GABA levels in the microdialysates were essentially constant over 5–6 h of perfusion. The mean ± S.D. (n) levels of GABA in microdialysates of rat nucleus accumbens and striatum were 0.49 ± 0.23 (15) and 0.35 ± 0.29 (13) pmol/20 μl, respectively. These measurements are in good agreement with comparable data obtained using OPA–alkylthiol reactions combined with HPLC and electrochemical or fluorescence detection [1–4]. The mean ± S.D. % recovery of GABA across the microdialysis probe (see Experimental) was 30.5 ± 1.7 (n = 4 probes).

One advantage immediately gained by the use of the OPA–sulphite reaction over the commonly used OPA–alkylthiol reaction to derivatise GABA, was the lack of stench that follows the use of thiol reagents (*e.g.* β-mercaptoethanol, *tert.*-butylthiol). Thus, the OPA–sulphite derivatization procedure can be employed in busy laboratory environments on the open bench, without the need for containment of equipment in specially ventilated spaces.

A further recognised problem of the derivatization of primary alkylamines by the OPA–alkylthiol reaction is the often poor stability (few minutes life-time) of the isoindole reaction products, and particularly that of GABA [8]. The data in Fig. 3 show that the OPA–sulphite derivatization of GABA proceeds rapidly to

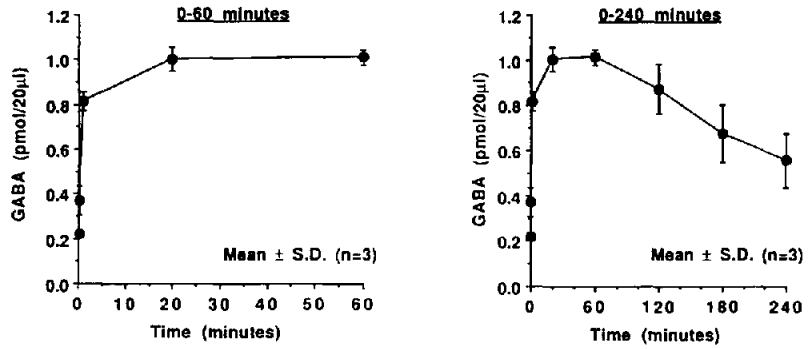

Fig. 3. Time course of GABA derivatization. OPA–sulphite reagent was added to artificial CSF containing 1 pmol GABA at t = 0 and the reaction was allowed to proceed at room temperature for the times indicated.

produce an electroactive product that is stable at room temperature for *ca.* 60 min although this product then degrades over the following hours (and has disappeared within 24 h). It is reported previously that the rate of OPA–sulphite derivatization of the primary alkylamine 2-propylamine is quick and that the resulting electroactive derivative is stable relative to the derivative obtained by the OPA–alkylthiol reaction [9]. In contrast to the OPA–sulphite derivative, underivatized GABA is stable in both artificial CSF and microdialysates at 4°C for at least 4 days (Table 1). In this particular case the appearance of a contaminant in the CSF after 4 days precluded a study of stability at 4°C over a longer period.

It was previously recognised that OPA–sulphite derivatives are susceptible to decomposition at low pH and concern was noted about the possible on-column hydrolysis of these derivatives in mobile phases of pH below 5–6 [9]. Fig. 4 shows the results of an experiment in which detector response to the GABA derivative produced by the OPA–sulphite reaction is measured in relation to mobile phase pH. The results show that over the pH range 5.0–3.6, the change in detector response is slight. Although there is a tendency for the detector response to reduce at pH below 4, this small effect could be explained by a noted increase in retention time and the associated band-broadening rather than on-column degradation of the derivative. It should be added that whilst we cannot detect on-column degradation of the derivative over our working mobile phase pH, such degradation might be a problem at lower pH values.

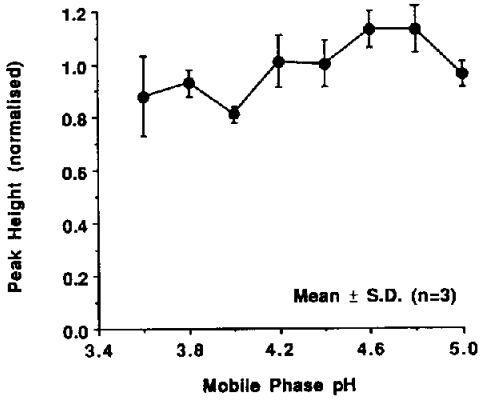

Figure 4. Relationship between detector response to GABA OPA–sulphite derivative and mobile phase pH. Mobile phase as described in methods (flow-rate 1.0 ml/min).

In the present study, isocratic chromatographic conditions were used to separate the electroactive GABA derivative. It is apparent from the chromatograms presented in Fig. 1 that although few peaks arise from derivatization of GABA standards, an abundance of peaks results from the derivatization of the microdialysates. Similar results are obtained when microdialysates derivatized by the OPA–alkylthiol reaction are separated by isocratic conditions comparable to our own [3,4]. We have not been able to identify the source of these peaks although we know that, under the present chromatographic conditions, various amino acids such as glutamate,

Table 1
Stability of GABA (1 pmol) in standard solution and rat brain microdialysate at 4°C over 4 days

Time in storage (h)	Relative response (mean ± S.D.) (%)	
	GABA in standard solution	GABA in microdialysate
0	100 ± 3.0	100 ± 1.8
24	100 ± 4.2	102 ± 14.5
48	91 ± 4.6	97 ± 6.8
96	100 ± 7.4	94 ± 6.6

Load volume 20 μl. Microdialysate was from nucleus accumbens; $n = 3$ for each determination.

aspartate and taurine elute in the solvent front. A wide range of biological substances are likely to be derivatized by the OPA–sulphite reaction, not only amino acids [9,10].

The present chromatographic conditions were selected to optimise sensitivity as well as sample through-put. Although the combination of solvent gradient elution with electrochemical detection would facilitate separation of the derivatives, this option is not practical due to baseline drift during the solvent run (particularly at the high level of sensitivity necessary to detect GABA in brain perfusates). We are confident that our resolution of the GABA derivative is adequate, not only on the basis of the studies presented here but also on the basis of our extensive studies showing that the identified peak changes appropriately in response to standard pharmacological manipulation [12]. For example, the GABA peak increases 2- and 10-fold in response to local application of high potassium (50 mM) and the GABA uptake inhibitor nipecotic acid (0.5 mM), respectively. Furthermore, local application of the sodium channel blocker tetrodotoxin (1 μM) and systemic injection of the GABA synthesis inhibitor 3-mercaptopropionic acid (100 mg/kg), decreases the peak by 40% and 50%, respectively. These results agree very much with data obtained measuring GABA by HPLC with electrochemical detection and OPA–alkylthiol derivatization [3].

In summary, we report that OPA–sulphite derivatization in combination with HPLC and electrochemical detection provides a simple, sensitive and reliable means for determination of GABA in microdialysates of rat brain. This approach has distinct practical advantages over comparable methods utilising OPA–thiol derivatization.

4. Acknowledgements

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5. References

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