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PITFALLS IN DEMONSTRATING AN ENDOGENOUS LIGAND OF IMIPRAMINE RECOGNITION SITES

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Abstract—The recognition sites for the 5-hydrotryptamine (5-HT) uptake inhibitors imipramine and paroxetine may represent receptors for a presently unknown endogenous ligand, whose function would be to modulate 5-HT uptake. Attempts to isolate such a factor from rat brain tissue are described, following a published procedure. It is shown that chromatographic fractions found to inhibit the binding of [^{3}H]imipramine and [^{3}H]paroxetine to rat brain membranes consisted of material essentially unretained by the reverse-phase HPLC column, and they were of high osmolarity. Thus, the activity was probably unspecific in nature, and the presence in rat brain of the factor has not been unequivocally demonstrated.

In addition to the classical agonists or antagonists acting on the well established receptors for neurotransmitters, several classes of drugs act at specific recognition sites which might represent receptors for presently unknown endogenous ligands. Potential drug-characterised receptors of current interest include: digoxin (modulation of Na^+–K^+ ATPase) [1], benzodiazepine (modulation of GABA-ergic transmission) [2], and imipramine or paroxetine (modulation of uptake of 5-HT) [3]. Whether there should exist a new endogenous ligand for each of these systems is a matter of controversy [4], but, in the absence of convincing theoretical arguments for or against the presence of the postulated ligands, the direct experimental approach of fractionating tissue or body fluids appears to be justified.

High affinity binding sites for [^{3}H]imipramine have been described in blood platelets and in brain from several species, including man. This recognition site is associated with the uptake system for 5-HT [5, 6], but it is different from the substrate recognition site for 5-HT [7, 8]. The association of 5-HT with its recognition site could be modulated by imipramine or other uptake inhibitors through an allosteric interaction [6, 9]. This regulatory mechanism may be altered in some patients with affective disorders, since in most studies a decrease in the density of [^{3}H]imipramine binding sites has been observed in blood platelets from untreated severely depressed patients [see 10, 11 for discussion]. One possible explanation for this result would be the existence of a circulating factor, which would possess high affinity for the imipramine recognition site. This hypothesis has stimulated research aimed at the identification of endogenous ligands acting at the level of the modulatory site of 5-HT uptake.

Recently, several authors have demonstrated the presence in brain tissue or plasma of substances which displace the specific binding of [^{3}H]imipramine and inhibit 5-HT uptake [9, 12–16]. In the publications by Barbaccia *et al.* [9, 12, 13], rat brains were homogenised in aqueous acetic acid, and then the extracts subjected to reverse phase HPLC. These authors reported two peaks of imipramine-displacing activity, the second of which was due to endogenous 5-HT, which is known to have weak activity in this system [17]. We have attempted to repeat this investigation, and have found that the first peak of activity described by Barbaccia *et al.* [13], which is not 5-HT, is likely to be non-specific in nature.

MATERIALS AND METHODS

Thirty rats were killed by cervical dislocation and the brains rapidly removed. The tissue was homogenised in 200 ml 1 M acetic acid at 90°, using an Ultraturnax homogeniser. After centrifugation (10 min, 48,000 *g*, 4°), the supernatant was collected, and the pellets washed with another 100 ml acetic acid. The extract was then freeze-dried at temperatures below −5°. The residue was taken up in 100 ml of 95% ethanol, and after centrifugation the supernatant was evaporated to dryness under a stream of nitrogen (waterbath at 37°).

The residue was taken up for HPLC in 300–500 μl of the eluant, and filtered (BAS microfilters; 0.2 μm). The filtrate was injected either in two equal portions, or else all at once. Two HPLC columns (250 × 4.6 mm; total volume 8.3 ml) were packed with μ-Bondapak C-18 (10 μm), and connected in series. Elution at 1 ml/min and 0° was performed with a pyridine acetate buffer (pyridine 0.4 M, acetic acid 0.125 M; pH 5.8), and fractions of 1 ml were collected. The elution volume of 5-HT (27–31 ml) was established immediately before and after running the sample by injecting authentic 5-HT (u.v. detection at 290 nm) and on one occasion by co-injection of [^{14}C]5-HT with a brain extract.

Fractions were evaporated to dryness under vacuum (Savant "Speedvac"), and taken up in H_2O, as indicated in the figures. Osmolarity of the reconstituted fractions was determined by the freezing-point depression method (Knauer Semi-Micro Osmometer).

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The [^{3}H]imipramine binding assay was performed as described elsehwere [17]. Briefly, male Sprague–Dawley rats (180–220 g) were killed by decapitation, and the brains were immediately removed and placed on ice. Cerebral cortex was dissected and homogenised in 50 vol. of ice-cold buffer (Tris–HCl pH 7.4, 50 mM; NaCl, 120 mM; KCl, 5 mM) for 2 × 30 sec with a polytron homogeniser at setting 5. The homogenate was centrifuged at 30,000 *g* for 10 min, the pellet was resuspended in the same buffer, at the same concentration, and then recentrifuged. The pellet was finally resuspended in buffer at a protein concentration of 3.5 mg/ml as measured using the method of Peterson [18]. The membranes were either used fresh or stored frozen at −80° for up to 2 weeks. [^{3}H]Imipramine binding inhibition was determined by incubating for 60 min at 0° aliquots of the membrane suspension (final concentration approximately 1.6 mg protein/ml) with 5 nM [^{3}H]imipramine and aliquots (usually 20 μl) of the HPLC fractions in H_2O, in a final volume of 250 μl. Specific binding, defined as that inhibited in the presence of 100 μM desipramine, represented 60–70% of the total binding at 5 nM [^{3}H]imipramine. Incubations were terminated by rapid dilution with 4 ml of cold buffer, and immediate filtration through Whatman GF/F glass fibre filters.

For [^{3}H]paroxetine binding experiments [19], aliquots of membrane suspension were incubated with 0.2 nM [^{3}H]paroxetine at 20° for 60 min, in a final volume of 1 ml, in the presence or absence of aliquots (100 μl) of the HPLC fractions. The incubation buffer was the same as for [^{3}H]imipramine binding. The specific binding of [^{3}H]paroxetine was defined as that inhibited in the presence of 10 μM fluoxetine. Incubations were terminated by rapid dilution with 4 ml of ice-cold buffer, and immediate filtration through Whatman GF/C glass fibre filters. Filters were routinely pretreated with 0.05% polyethylenimine before use. The filters were washed with 3 × 4 ml of ice-cold buffer, dried, and the radioactivity retained measured by liquid scintillation spectrometry (toluene containing 5 g/l PPO and 0.1 g/l POPOP).

RESULTS

The deproteinised extract obtained by homogenising the rat brains in hot acetic acid gave, after lyophilisation, a yellowish residue, most of which was insoluble in ethanol. The ethanol-insoluble fraction presumably consisted mainly of inorganic salts. Evaporation of the ethanolic supernatant yielded a much smaller residue, most of which dissolved in the pyridine–acetate buffer used as HPLC eluent. The chromatographic fractions were reconstituted in water (after evaporation of the buffer), and tested for inhibition of [^{3}H]imipramine binding. Typical profiles are shown in Figs. 1–3. In some cases, the osmolarity of the reconstituted fractions was also measured (Fig. 3).

Inhibition of specific [^{3}H]imipramine binding was consistently found in fractions having the same elution volume as 5-HT (Figs. 1, 2). This volume decreased from 31 to 27 ml over a period of a few months, due to deterioration of the column packing. Taking into account the IC_{50} of 5-HT on [^{3}H]imipramine binding (≃1 μM [7]) and the concentration of 5-HT in rat brain (426 ng/g [20]), the amount of 5-HT in this peak corresponds to a recovery of somewhat less than 10%. The losses were no doubt due in part to the use of heat to denature the tissue. The amount of what appeared to be specific binding (that is binding displaceable by 100 μM desmethylimipramine) was also altered in the region 6–9 ml, but the effects were more varied than for the 5-HT region. On some occasions, inhibition was seen (Fig. 1), but this could not always be reproduced,

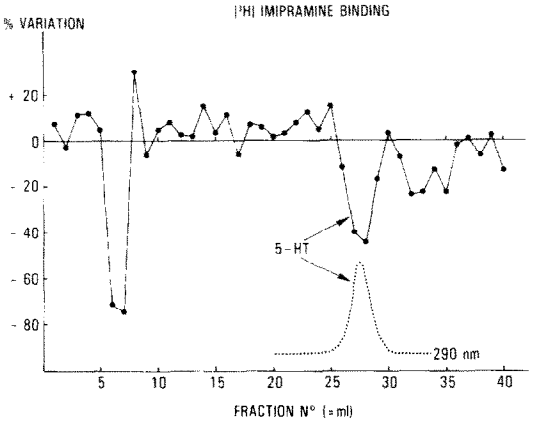

Fig. 1. Inhibition of [^{3}H]imipramine binding by fractions obtained by HPLC separation of a rat brain extract prepared according to Barbaccia *et al.* [12, 13]. The amount of extract was equivalent to 15 brains. Fractions were taken up in 150 μl H_2O and 20 μl were tested. The trace marked 290 nm was redrawn from a recording of an injection of authentic 5-HT obtained immediately after chromatography of the tissue extract. Ordinate: percentage variation of binding; Abscissa: fraction number (fractions of 1 ml). For full experimental details, see Materials and Methods.

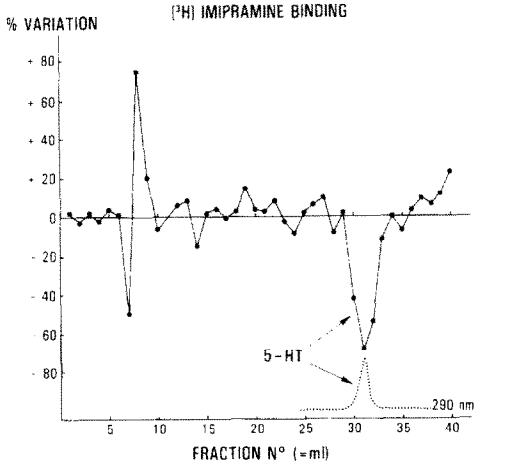

Fig. 2. Inhibition of [^{3}H]imipramine binding by chromatographic fractions obtained as in Fig. 1, except that the amount of extract was equivalent to 30 rat brains, and the fractions were taken up in 120 μl H_2O of which 20 μl were tested.

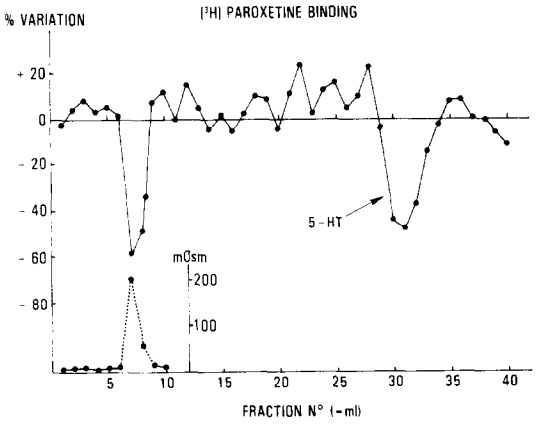

Fig. 3. Inhibition of [^{3}H]paroxetine binding by fractions obtained as in Fig. 1. Aliquots (50 μl) of fractions 1–10 were evaporated, and the osmolarity determined after reconstitution in 50 μl H_2O. The remainders of the fractions were reconstituted in 140 μl H_2O, of which 100 μl were tested.

and there was often a more complex profile of inhibition and augmentation. An example of this is shown in Fig. 2, for which the conditions were the same as for Fig. 1, except that twice the amount of brain extract was run. Similar profiles were obtained using the [^{3}H]paroxetine binding assay (Fig. 3).

Since the elution volume of the first peak of activity corresponds to non- or slightly-retained material (the void volume of this type of column is about 80% of the total volume), these fractions would be expected to contain all the salts and other polar compounds present in the extract. Osmolarity measurements (Fig. 3) show that this was indeed the case, and therefore that the effects of these fractions on ligand binding were not necessarily due to a specific effect on the [^{3}H]paroxetine recognition site.

The column used by Barbaccia *et al.* [13] was larger than those available to us, having a total volume of 14.3 ml compared to 8.3 ml for ours. However, as the packing material was identical, elution volumes (especially of non-retained substances) should be closely proportional to the respective column volumes. Hence it is probable that those early-eluting fractions (numbers 12–14) found by Barbaccia *et al.* [13] to inhibit specific binding of [^{3}H]imipramine, correspond to those (7–8) found in our experiments to be of high osmolarity. Taking into account the dilution used (see Materials and Methods), the assay medium for a fraction of 200 mOsm (fraction 7, Fig. 3) would contain, in addition to the buffer, a mixture of compounds at a final concentration of 50–100 mM ([^{3}H]imipramine binding), or 100–200 mM ([^{3}H]paroxetine binding) the exact values depending on whether the fraction contained predominantly non-ionised compounds or fully dissociated salts.

Most of our experiments have been carried out with the HPLC columns at 0° in order to improve the retention of polar compounds and hence their separation from non-retained material. Raising the temperature to 20° halved the retention volume of 5-HT, but did not affect that of the earlier active fractions having high osmolarity (results not shown). This is a further indication that the peak of inhibitory activity in these early fractions represents essentially unretained material.

DISCUSSION

In order to demonstrate the existence of an endogenous ligand for a drug recognition site, an extract must be obtained which satisfies three criteria: (1) known endogenous ligands (in the present case 5-HT) must be shown to be absent or present in negligible concentrations; (2) other endogenous compounds, or compounds deliberately or inadvertently introduced during the extraction procedure, must not have been concentrated to the extent that they interfere with the assay system; (3) the extraction procedure must not have provoked the transformation of relatively inactive endogenous compounds into active derivatives.

The procedures described by Barbaccia *et al.* [13] clearly satisfy the first criterion, as 5-HT is well separated from the peak of the presumed endocoid. However, we have shown that the fractions in which [^{3}H]imipramine and [^{3}H]paroxetine binding displacing activity was located correspond practically to the void volume of the reverse-phase column, and hence consist of a mixture of all those constituents of the initial extract which are too polar to be retained. Some of these constituents will certainly have been concentrated with respect to the initial amount of tissue, and in addition, many inorganic and organic cations will probably be present as the acetates. The effects of such concentrations on a binding assay would be unpredictable, and probably dependent on the composition of the mixture. However, osmotic considerations alone would render these fractions unsuitable for assays of 5-HT uptake in either synaptosomes or brain slices. It is unlikely, therefore, that criteria (2) and (3) above have been satisfied in the experiments by Barbaccia *et al.* [13], and thus, the biological activity of these early fractions corresponding to the peak of activity on [^{3}H]imipramine binding and [^{3}H]5-HT uptake is likely to be non-specific.

Conversely, though, the presence of a polar imipramine-displacing substance, can not be excluded by these results, and speculation that a glycyl–tryptoline conjugate [13] could be the endogenous ligand is interesting in this context, because such compounds are hardly if at all retained on reverse-phase columns. There exist few methods of obtaining salt-free extracts of such polar bases from tissue; the most general approach is cation exchange chromatography, but this requires elution with either concentrated buffers of limited volatility, or else strong acids which would degrade labile metabolites.

We have also explored the possibility that an imipramine-displacing substance could be more *strongly* retained than 5-HT on reverse-phase chromatographic columns. Experiments using different methods of deproteinisation (perchloric acid, acetonitrile/$MgSO_4$) and different column packings (Merck Lichro Prep C-18, Spherisorb ODS) have revealed no significant activity in any fractions from bovine, sheep or rat brain, and also from human urine. In agreement with Barbaccia *et al.* [13], this excludes the possibility that a simple tryptoline could

be the endogenous ligand, at least as far as the rat brain is concerned.

There are, nevertheless several other reports suggesting the existence of endogenous substances which inhibit 5-HT uptake and/or [^{3}H]imipramine binding. Rehavi *et al.* [15] described the isolation from rat brain of a fraction which inhibited [^{3}H]imipramine binding to human platelet and rat brain membranes, and also [^{3}H]5-HT uptake into human platelets and rat brain synaptosomes. We have attempted to repeat this procedure, which involves gel filtration followed by chromatography on silica gel, but the only specific biological activity isolated has been due to 5-HT (unpublished observations). As in the reverse-phase HPLC experiments described above, fractions of high osmolarity caused large but variable increases or decreases in specific [^{3}H]imipramine binding.

Angel and Paul [14] have shown that various simple extracts of human plasma potently inhibit [^{3}H]5-HT uptake into rat brain synaptosomes. Brusov *et al.* [16] have described an extensive fractionation procedure for human plasma, which revealed several substances which inhibit [^{3}H]imipramine binding to platelets as well as platelet [^{3}H]5-HT uptake. Perhaps unexpectedly, the retention behaviour of these substances on alumina strongly suggests the presence of a catecholic function.

Brusov *et al.* [16] used as part of their extraction procedure ultrafiltration using Amicon UM-2 membranes. A note of caution is required, since we have found that some but not all batches of membranes of the obsolescent UM series slowly release one or more active compounds into salt solutions (such as Krebs' bicarbonate or Tris buffers), but not into pure water. The contaminant most consistently found with Amicon UM-05 membranes was isolated by reverse-phase HPLC, and tentatively identified by gas chromatography–mass spectrometry as tributylamine. Subsequently, this amine was found to inhibit [^{3}H]imipramine binding with an IC_{50} of 160 μM (C. R. Lee and S. Z. Langer, unpublished observations). Numerous amino-compounds are known to inhibit 5-HT uptake into platelets with low potencies [21], and if they are released by the Amicon UM membranes in sufficient concentrations, an artefactual inhibition of [^{3}H]imipramine binding can be obtained. Other types of ultrafiltration membrane, such as the PM series, appear to be free from this source of artefact.

A very recent paper [22] describes the isolation from human plasma of a 48 kdalton acidic glycoprotein, which potently inhibits [^{3}H]imipramine binding and serotonin uptake. This substance is clearly not serotonectin, the circulating glycoprotein which binds serotonin and modulates platelet uptake of serotonin, because serotonectin does not bind [^{3}H]imipramine, nor does it affect [^{3}H]imipramine binding to platelet membranes [23]. Thus, at least two circulating glycoproteins are involved in the regulation of peripheral serotonin transport. The existence of these glycopeptides by no means excludes the possible existence of an additional endocoid of low molecular weight. Failure to detect such a compound could have been due to limitations in the separation methods discussed above. The isolation of any unknown compound present at concentrations in the low ng/g range is in any case extremely difficult, however favorable the chromatographic properties. Several known or potential metabolites of tryptophan inhibit imipramine binding and 5-HT uptake to some extent; formation of trace quantities of an ephemeral and highly potent inhibitor could easily be envisaged.

In conclusion the presence in brain tissue of an endogenous modulator of 5-HT uptake, other than 5-HT itself, has not yet been convincingly demonstrated, although if this were an unstable or highly polar compound of low molecular weight, it would not yet have been detected. On the other hand, there exists in human plasma a factor of high molecular weight which inhibits [^{3}H]imipramine binding. Whether there also exist in plasma factors of low molecular weight is still an open question. The B_{max} for the [^{3}H]imipramine binding site is decreased in severe untreated depressed patients (see 10, 11), and as this site is closely associated with the 5-HT transporter [7, 8], the characterisation of circulating factors is of great interest to studies of this disease.

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