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Note

Rapid and simple purification of the ^{125}I-labeled α-1 adrenergic radioligand 2-[β-(4-hydroxylphenylethyl)aminomethyl] tetralone (BE 2254) using reversed-phase high-performance liquid chromatography

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The ^{125}I-labeled α-1 adrenergic antagonist HEAT {2-[β-(4-hydroxylphenylethyl)aminomethyl] tetralone, BE 2254} has been found to be a useful radioligand for the quantitation of α-1 adrenoceptors in a variety of tissues [1—3] because of its high affinity and high specific activity. However, iodinated HEAT (I-HEAT) is somewhat labile, and undergoes decomposition under alkaline conditions at room temperature to form iodotyramine and non-iodinated residues. The resulting iodotyramine interferes with binding assays by causing a high background of non-specifically bound radioactivity. Thus, we find that repurification at regular intervals is necessary to maintain the usefulness of this ligand.

The usual method utilized for the purification of I-HEAT is paper chromatography [4, 5]. We have found that this technique results in a low recovery of purified I-HEAT, involves an undesirable exposure hazard owing to the need for large paper sheets and relatively large volumes of solvents, and requires long chromatographic times. We now report the development of a method for the rapid single-step purification of I-HEAT using high-performance liquid chromatography (HPLC). This method resolves I-HEAT from non-iodinated HEAT, its decomposition products, and by-products of its radioiodination.

EXPERIMENTAL

The conditions used for iodination of HEAT are the same as those reported by Engel and Hoyer [4], except that the amount of Na^{125}I was reduced by

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half. The iodination product (and non-iodinated HEAT) is extracted from the reaction mixture with three portions (300 μl each) of ethyl acetate. A pipette tip is used to facilitate separation of the layers, and the organic (upper) layers were recovered and pooled. The volume is reduced by evaporation under a fine stream of anhydrous nitrogen in a fume hood to approximately 30 μl. Distilled water (30 μl) is added and the residual ethyl acetate driven off with the nitrogen stream. Methanol (70 μl) containing 1% (v/v) 50 m*M* Tris—HCl (pH 6.5) is then added to the aqueous residue, producing a solution which is approximately 70% methanol, 30% water, and which is suitable for HPLC.

HPLC purification of HEAT

A Waters Model 204 liquid chromatograph consisting of two delivery systems (a Model 6000A and a Model M45), a Model U6K injector, and a Model 680 automated gradient controller were used. HEAT was monitored by absorbance at 254 nm using a Waters Model 450 variable-wavelength detector. An isocratic reversed-phase HPLC system (C_{18}, 10 × 0.5 cm, 10-μm column, Alltech) was employed for all separations. The mobile phase was methanol—water—diethylamine, pH 6.5 (70:30:0.04) and a flow-rate of 0.5 ml/min was used. Between runs, the column was washed with 100 ml water followed by 100 ml of 100% methanol containing 0.04% diethylamine (pH 6.5) to remove any remaining free iodine and decomposition products.

[^{125}]HEAT binding to myometrial membrane particulates

A membrane particulate fraction was prepared from rabbit myometrium as described by Roberts et al. [6]. Protein was quantitated by the method of Bradford [7] as modified for membrane proteins [6] using bovine serum albumin as standard.

Aliquots of membrane particulate fraction (40 μl) in 50 m*M* Tris, 4 m*M* magnesium chloride, pH 7.4 containing 50—70 μg of protein are added to 20 μl [^{125}I]HEAT (25—1600 p*M* final concentration in saturation studies, 80 p*M* in competition studies) in 50 m*M* Tris, 4 m*M* magnesium chloride, pH 7.4 and 20 μl of 1 m*M* hydrochloric acid to start the binding reaction. Non-specific binding is estimated by substituting Prazosin · HCl (Pfizer), 40 μ*M* in 1 m*M* hydrochloric acid for the 1 m*M* hydrochloric acid in the reaction. Incubation is carried out for 30 min at 30°C and terminated by the addition of 5 ml of ice cold 50 m*M* Tris, 4 m*M* magnesium chloride, pH 7.4 containing 10% (v/v) polyethylene glycol (average MW 400) followed by filtration under low vacuum on Whatman GF/C filters. Washing of filters is completed by the addition of three 5-ml vols. of the same cold buffer solution. Radioactivity is quantitated by γ-emission counting (Packard Instruments) at 72% efficiency.

RESULTS

The separation of ^{125}I-labeled HEAT from non-iodinated HEAT is shown in Fig. 1A. At the flow-rate employed (0.5 ml/min), native HEAT had a retention time of approximately 8 min while I-HEAT was eluted 14 min after injection. Thus unlabeled HEAT, which is present in great excess in the extract of the iodination mixture, can be easily and completely resolved from I-HEAT. The

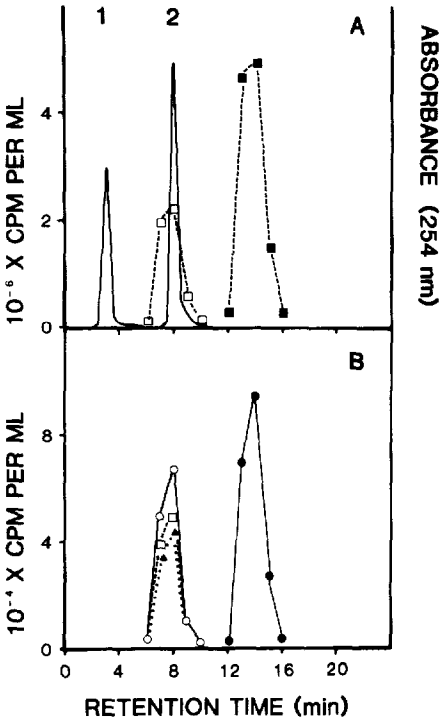

Fig. 1. HPLC separation of HEAT and related compounds. Separatons were performed using a C_{18} reversed-phase column (10 × 0.5 cm, 10 μm) eluted isocratically at a flow-rate of 0.5 ml/min using a solvent consisting of methanol—water—diethylamine, pH 6.5 (70:30:0.04). Panel A: the solid line represents the absorbance profile for tyramine (1) and HEAT (2). The dashed line represents the radioactive profile obtained with ^{125}I-labeled tyramine (open squares) and ^{125}I-labeled HEAT (closed squares). Panel B: ^{125}I-labeled tyramine and ^{125}I-labeled HEAT were isolated by HPLC as in panel A. Aliquots of each were treated under mild acidic or basic conditions as described in the experimental section prior to rechromatography. The samples are as follows: (□) I-tyramine after base treatment; (▲) I-tyramine after acid treatment; (○) I-HEAT after base treatment; and (●) I-HEAT after acid treatment.

major radioactive product formed upon the decomposition of ^{125}I-labeled HEAT is ^{125}I-labeled tyramine. Fig. 1A also shows the elution profiles for tyramine and ^{125}I-labeled tyramine. ^{125}I-labeled tyramine elutes in a position concurrent with native HEAT at a retention time of about 8 min. Thus, ^{125}I-labeled HEAT is readily resolved from both the non-iodinated compound and its major decomposition product.

In order to confirm the above results, the following experiment was performed. I-HEAT is unstable under mildly alkaline conditions but is stable under mildly acid conditions [3]. In contrast, I-tyramine is apparently stable under either of these conditions. I-HEAT and I-tyramine were isolated as described above and the solvent was removed under a stream of nitrogen. Aliquots of each were treated for 1 h in the presence of either dilute ammonium hydroxide (pH 9) at 50°C or dilute hydrochloric acid (pH 3) at −20°C (the recommended storage temperature for I-HEAT). Following this treatment, the pH of each solution was adjusted to 6.5 and the samples were run again on the HPLC system described above. As shown in Fig. 1B, treatment

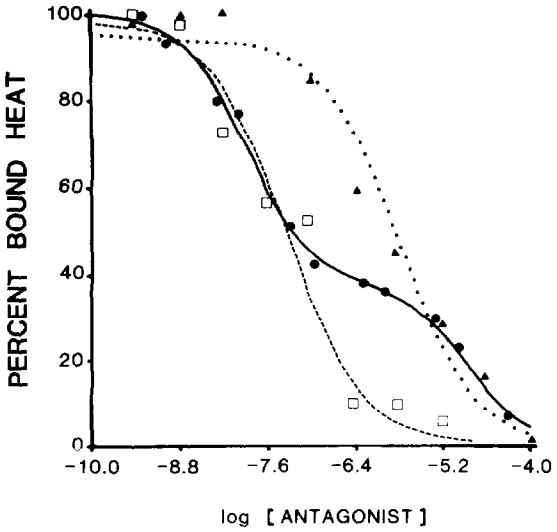

Fig. 2. Competition curves for I-HEAT binding to myometrial membranes. Myometrial membrane particulate fractions were prepared as described in Experimental and incubated with I-HEAT (80 p*M*) in the presence of the unlabeled antagonists. Phentolamine • HCl (●): equilibrium binding affinity constant of the inhibitor (K_i) 1—6.9 n*M*, 40.4%; K_i 2—2400 n*M*, 40%. Prazosin • HCl (□): K_i 1.0 n*M*. Yohimbine • HCl (▲): K_i 1100 n*M*.

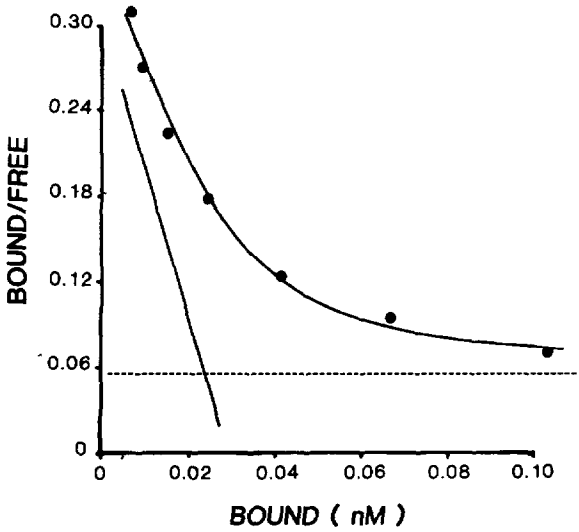

Fig. 3. Saturation curve for I-HEAT binding to myometrial membranes. Myometrial membranes were incubated with I-HEAT as described in Experimental. Total binding data was then analyzed with the following results: equilibrium binding affinity constant (K_d) = 96.6 ± 17.3 p*M* (S.E.M.); equilibrium binding site concentration (B_{max}) = 74.2 ± 10.3 fmol/mg of membrane protein. Non-specific binding is indicated by the dashed line parallel to the abscissa (bound/free = 0.0055 ± 0.0005).

of I-tyramine with either acid or base has no effect on its relative retention time. Similarly, treatment of I-HEAT with acid did not alter its retention time. Treatment of I-HEAT with mild base, however, resulted in the generation of a radioactive compound which cochromatographed with I-tyramine. Further

treatment of this degradation product with either acid or base had no effect on its elution position.

To confirm the usefulness of the ligand isolated by the described procedure, competition studies of the binding of I-HEAT to myometrial membrane preparations were conducted and are shown in Fig. 2. I-HEAT binds to these preparations in a manner consistent with α-1 adrenergic potencies. Fig. 3 shows a typical saturation isotherm for I-HEAT binding arrayed according to Scatchard [8]. The best-fit curve was calculated from untransformed data by fitting highest-affinity binding using a computer-based iterative non-linear curve fitting program [9]. I-HEAT binds with high affinity (equilibrium binding affinity constant, K_d = 97 pM) and identifies approximately 70 fmol of α-1 binding sites per mg of myometrial membrane protein.

DISCUSSION

We have described a simple one-step HPLC method for the rapid purification of I-HEAT. This method is especially useful for the initial purification of I-HEAT following iodination, since it readily resolves labeled and unlabeled HEAT. I-HEAT is a high-affinity and high-specific-activity radioligand which has been found to be quite useful for the quantitation of low concentrations of α-1 adrenergic receptors in brain [1], pituitary [3], and smooth muscles such as vas deferens [2] and, as shown here, for myometrium.

The usefulness of this ligand can be compromized by the accumulation of the decomposition product iodotyramine, which increases the level of non-specific binding in the assay. The purification method we have described is simple and rapid enough for the routine repurification of I-HEAT at regular intervals, thereby extending the useful life of this ligand. While storage of I-HEAT at −20°C or −80°C under acidic conditions retards the formation of iodotyramine, we find that some decomposition still occurs.

Although I-HEAT is available commercially, its cost and lack of stability may limit its usefulness for the detection of low concentrations of α-1 adrenoceptors. The procedure described in this communication facilitates the preparation of this ligand at a very low cost. Furthermore, the ability to rapidly repurify the ligand from its degradation products will both extend its useful life and maintain the high signal-to-noise ratio needed for the accurate measurement of low levels of α-1 adrenoceptors.

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