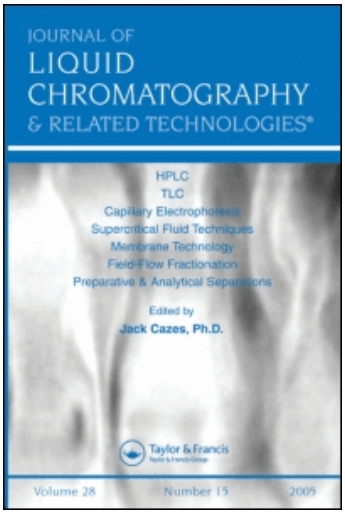

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Preparation and Purification of Tritiated Dextromethorphan

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PREPARATION AND PURIFICATION OF TRITIATED DEXTROMETHORPHAN

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ABSTRACT

Tritiated dextromethorphan (^{3}H-DM) was prepared from dextrorphan and purified by reverse-phase high performance liquid chromatography (HPLC). The tritiated drug was found to be homogeneous and identical to authentic unlabeled DM by both HPLC and thin layer chromatography. The specific radioactivity of ^{3}H-DM was 26 Ci/mmole.

INTRODUCTION

Dextromethorphan (DM) is a non-narcotic antitussive which suppresses cough by an action on the central nervous system. Although little is known concerning the mechanism of action of this drug, the structural similarity of DM to codeine has suggested that DM acts at the same central sites as codeine to suppress cough. However, Cavanagh et al. (1) have recently demonstrated that naloxone antagonizes the antitussive effects of codeine and other opiate analgesics but does not antagonize the antitussive activity of DM. These findings indicate different modes of action for the cough suppressant effects of DM and codeine.

Since DM does not elicit its antitussive effects by an opiate receptor interaction, as evidenced by the lack of effect of naloxone, the possibility exists that this drug acts at other specific central nervous system sites which can be detected by an appropriate binding assay procedure. To study such a possibility, we have prepared tritium labeled DM (^{3}H-DM) of high specific activity, and, as initially reported (2-4), we have found specific high affinity binding sites for DM in guinea-pig brain which may be related to the antitussive effects of the drug.

The present report describes the preparation of ^{3}H-DM and its purification by reverse-phase high performance liquid chromatography (HPLC). Since it is necessary that the radioligands used in binding studies be at the minimum 95 percent pure (5), it was essential for us to develop simple chromatographic systems in which we could obtain pure ^{3}H-DM in good yield. Dixon et al. (6) have described a method to purify ^{3}H-DM using column chromatography and thin layer chromatography (TLC) but the purity and recovery of ^{3}H-DM achieved by their procedures were not stated.

MATERIALS AND METHODS

Dextrorphan tartrate and dextromethorphan hydrobromide were generously supplied by Hoffman-La Roche (Nutley, NJ). N-methyl-N'-nitro-N-nitroso guanidine and the diazomethane-generating apparatus were obtained from Aldrich Chemical Co. (Milwaukee, WI). n-Nonylamine was purchased from Sigma (St. Louis, MO). Acetonitrile and n-propanol were HPLC grade (Burdick and Jackson) and all other reagents were Fisher ACS reagent grade. Acetic acid, acetonitrile, triethylamine and n-propanol were distilled over ninhydrin prior to use.

Deionized water (Hydro Systems, Durham, SC) was used in the preparation of all aqueous buffers.

Chromatography

The HPLC equipment consisted of a Milton-Roy Simplex minipump, model 396-31 (Riviera Beach, FL) and a Rheodyne #70-10 sample injection valve with a 1 ml loop (Rainin Instrument Co., Inc., Ridgefield, NJ); gradients were generated by an LKB Ultragrad 11300 gradient maker (Rockville, MD). Separations were performed on an Altex Lichrosorb RP-18 column (10 μm particle size, 4.6 x 250 mm) or an Altex Ultrasphere-cyano column (5 μm particle size, 4.6 x 250 mm). UV determinations of the column effluents were made with a Beckman System 25 spectrophotometer and radioactivity was determined in a Beckman LS-230 liquid scintillation counter using a phase-combining scintillant (Liquiscint) from National Diagnostics (Somerville, NJ). The presence of n-nonylamine in the column effluents was monitored by an automated fluorescamine-based detection system (7) using a Spectra/Glo Model FL-1B Fluorometer (Gilson Medical Electronics, Inc., Middleton, WI) coupled to a Linear dual channel recorder (Irvine, CA). During the preparation of mobile phase solvents, aqueous buffers were filtered through Millipore HA 0.45 μm filters and organic solvents through millipore FH 0.5 μm filters. Prior to use all solvents were thoroughly degassed.

TLC was carried out on Whatman LK5D silica gel G plates and DM standards were visualized with iodoplatinate.

Fluorescence Spectroscopy

Fluorescence spectroscopy of labeled and unlabeled DM was performed with a Perkin-Elmer Model 650-10S spectrofluorometer coupled to a Perkin-Elmer recorder (Norwalk, CT).

Preparation of Diazomethane

Diazomethane was generated by the action of alkali on N-methyl-N'-nitro-N-nitroso guanidine using the procedure and apparatus described by Fales et al. (8).

Preparation of ^{3}H-DM from dextrorphan

Iodination of dextrorphan [(+)-3-hydroxy-17-methylmorphinan] was carried out by the method of Grewe et al. (9). Briefly, 255 mg of dextrorphan tartrate were dissolved in water and the free base (0.6 mmole) obtained by the addition of 1 N sodium hydroxide. A solution of iodine (0.6 mmole) in potassium iodide (0.9 mmole) was added dropwise and with constant stirring to the dextrorphan solution. After 10 minutes the solution was bubbled with 100% CO_2 and filtered. The precipitate was washed twice with water, dried under vacuum and taken up in chloroform. The chloroform was then evaporated at 50°C under a stream of N_2 and iodinated dextrorphan obtained by crystallization from methanol. 60 mg of the iodinated compound were sent to New England Nuclear Corp. (Boston, MA) and tritiated by catalytic reduction with 25 Ci of tritium gas to a specific activity of 60.4 mCi/mg. Methylation of ^{3}H-dextrorphan to form tritiated (+)-3-methoxy-17-methylmorphinan (^{3}H-DM) was carried out by reacting 2-8 mCi of ^{3}H-dextrorphan with diazomethane in ether for 30 minutes at 0°C. At the end of the reaction, ether and residual diazomethane were removed by a stream of N_2 and the components of the reaction mixture subjected to HPLC as described in the next section. The yield of ^{3}H-DM varied between 20 and 50 percent.

RESULTS AND DISCUSSION

HPLC systems were initially designed to achieve a good separation of DM from dextrorphan. Because DM is more lipophilic than dextrorphan, such a separation on an

octadecylsilane column (RP-18) could be readily obtained. However, DM exhibited long retention times with asymmetric peaks and severe tailing and this behavior was observed even when ion-pairing reagents were present in the mobile phase. Several investigators who observed similar phenomena during ion-pair HPLC of hydrophobic cationic drugs found that retention time and peak symmetry could be dramatically improved when long-chain alkyl ammonium compounds were included in the mobile phase (10,11). This approach was investigated and the nine-carbon ammonium compound, n-nonylamine, was incorporated into the mobile phase. The results were quite satisfactory in that retention time of DM was significantly reduced and peak shape greatly improved.

As Fig. 1 illustrates, an excellent separation of DM from dextrorphan was obtained using a 2-30% acetonitrile gradient in 0.1 M acetic acid, 0.01 M n-nonylamine. In addition, this HPLC system also resolved ^{3}H-DM from several tritiated side products of the methylating reaction (Fig. 1). The radioactive profile of an aliquot from a typical diazomethane reaction mixture shows not only a distinct peak of radioactivity eluting in the same position as unlabeled DM but also two other radioactive peaks with significantly greater retention times. Recovery of radioactivity in this system was greater than 90 percent.

When an entire reaction mixture was subjected to HPLC using the system just described, the elution volume corresponding to the DM peak was collected as four individual fractions (similar to #20,21,22 and 23 in Fig. 1) which were not pooled. Aliquots of each of these fractions were analyzed for radiochemical purity by rechromatographing on the same HPLC system and by TLC in two solvent systems: (chloroform:methanol:ammonium hydroxide, 75:25:0.3; ethanol:acetic acid:water, 60:30:10). The results from these chromatographic analyses indicated that the earliest

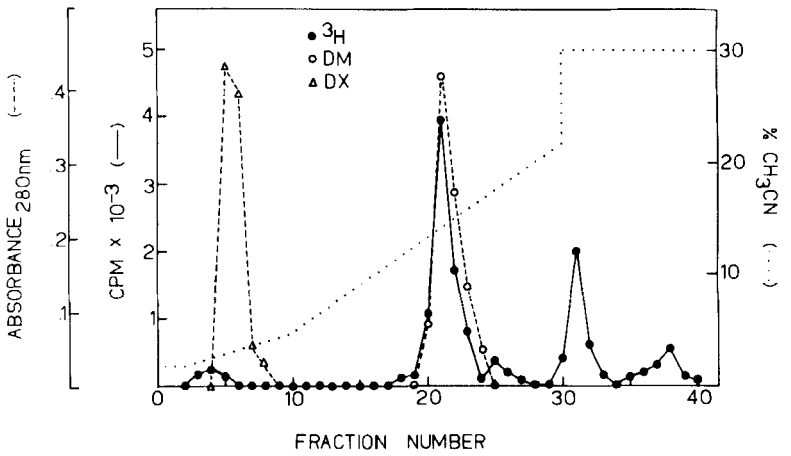

FIGURE 1. Reverse-phase HPLC of ^{3}H-DM prepared by methylation of ^{3}H-dextrorphan. An aliquot from a typical diazomethane reaction mixture was applied to an RP-18 column and the radioactivity eluted with a 2-30% acetonitrile gradient in 0.1 M acetic acid, 0.01 M n-nonylamine. Two ml fractions were collected at a flow rate of 0.67 ml/min. Recovery of radioactivity was 91 percent. Also shown is the elution profile for 0.7 μmoles of unlabeled dextrorphan (DX) and 0.7 μmoles of unlabeled DM.

eluting fraction (#20) was impure. During the HPLC analysis only 60 percent of the radioactivity of fraction #20 co-eluted with the peak of unlabeled DM. TLC analysis yielded a similar estimation of purity since it was observed that less than 50 percent of the radioactivity of fraction #20 migrated with unlabeled DM. In contrast to the heterogeneous nature of the radioactivity in the early DM peak fraction (#20), the radioactivity in the next three fractions (#21-23) appeared homogeneous and behaved identical to that of authentic unlabeled DM during HPLC and TLC analysis.

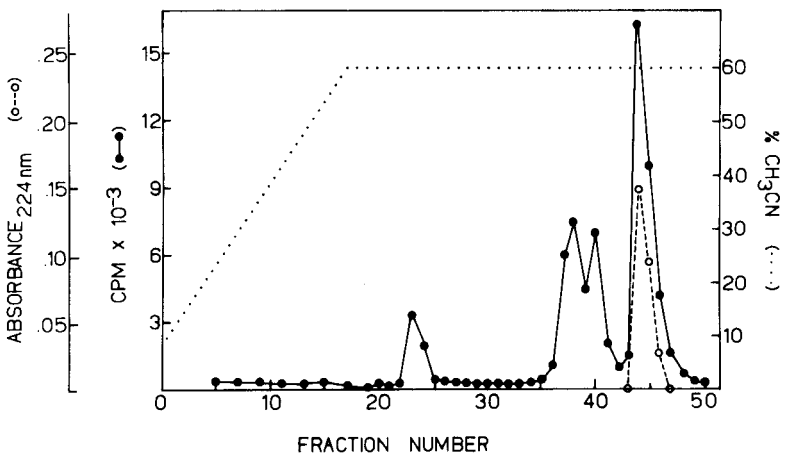

FIGURE 2. Reverse-phase HPLC of the radioactivity of fraction #20. An aliquot of fraction #20, mixed with 50 nmoles of unlabeled DM, was applied to an Ultrasphere-cyano column and eluted with a 10-60% acetonitrile gradient in 7.5 mM H_3PO_4, 10 mM triethylamine, pH 4.0. One ml fractions were collected at a flow rate of 0.6 ml/min. Recovery of radioactivity from the column was 99%.

The radioactive homogeneity of fractions #21-23 was further established by reverse-phase HPLC on an Ultrasphere-cyano column using a 10-60% acetonitrile gradient in 7.5 mM H_3PO_4, 10 mM triethylamine. The resolving power of this system is demonstrated in Fig. 2 where it can be seen that three tritiated species in addition to ^{3}H-DM are present in fraction #20. In agreement with the results obtained by TLC analysis, ^{3}H-DM accounts for less than 50 percent of the radioactivity of this fraction. When fractions #21-23 of the ^{3}H-DM peak were analyzed by this same HPLC system, one homogeneous peak of radioactivity co-eluting with authentic unlabeled DM was found for all three fractions. Based on these results as well as the

previous analyses by HPLC and TLC, ^{3}H-DM in the three later peak fractions was considered radiochemically pure and used for subsequent binding studies without further purification.

Having achieved the purification of tritium labeled DM, it was necessary to remove n-nonylamine from the ^{3}H-DM preparations since it interfered with the binding assay. Although n-nonylamine could be removed by evaporation under reduced pressure, this procedure resulted in significant losses of the tritiated drug. Therefore, n-nonylamine was separated from ^{3}H-DM by reverse-phase HPLC on an octadecylsilane column using a 10-40% n-propanol gradient in 10 mM KH_2PO_4, pH 4.0. Recovery of ^{3}H-DM during this procedure was greater than 95 percent.

Tritiated DM was stored at 4°C in the propanol-phosphate buffer with no chemical deterioration in one year. The concentration of labeled drug was determined by fluorescence spectroscopy using excitation and emission wavelengths of 230 nm and 305 nm respectively. A specific activity of 26 Ci/mmole was calculated for the final ^{3}H-DM product. During the fluorescence determinations it was observed that the excitation and emission spectra of radioactive DM were identical to that of authentic unlabeled DM.

In conclusion, we have described the preparation of tritium labeled DM and the chromatographic procedures used to obtain ^{3}H-DM in radiochemically pure form. Our results have clearly demonstrated that reverse-phase HPLC is an excellent method to purify ^{3}H-DM. By incorporating n-nonylamine into the mobile phase buffer, a significant portion of the ^{3}H-DM peak eluted from the RP-18 column during the first HPLC step was pure. Even though n-nonylamine was an interfering substance in our binding assay and had to be removed from the ^{3}H-DM preparation by an additional HPLC step, the overall methods we have reported are fast, simple and yield excellent recoveries.

Since TLC is a standard method for purifying radioligands used in binding studies, it is perhaps relevant to mention that this chromatographic procedure was our preliminary method for isolating ^{3}H-DM. However, we found that as a routine preparative procedure, TLC provided neither the recovery nor the purity of ^{3}H-DM that was subsequently achieved by HPLC. Nevertheless, TLC served as a useful analytical procedure for assessing the purity of ^{3}H-DM isolated by HPLC.

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