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Note

Determination of adiphenine, diphenylacetic acid and diethylaminoethanol by high-performance liquid chromatography

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Adiphenine, known for its atropinic properties, can also stimulate the liberation of anterior pituitary glycoprotein hormones[1–3]. The pharmacodynamic properties were first reported 40 years ago[4] and the drug has been used as an antispasmodic and anticholinergic agent in clinical studies. The disposition of adiphenine labelled with ^{14}C at two positions was previously investigated in rats and mice after i.v. administration[5]. As a next step, we are studying the selective fixation on the brain and central nervous system observed in previous studies and attempting to elucidate the mechanism of action of this compound by an increased knowledge of cerebral metabolism.

For this experiment, it was necessary to be able to detect, rapidly and accurately, adiphenine and its previously identified main metabolites, diethylaminoethanol and diphenylacetic acid. Brown and co-workers[6–8] studied the degradative fate of anticholinergic compounds, and in particular of adiphenine, by ion-pair high-performance liquid chromatography (HPLC). This system did not allow a convenient separation of adiphenine from its two main metabolites in brain homogenates of injected rats.

We describe here two HPLC systems that we have developed, using a reversed phase and silica, for the identification of tissue metabolites.

The procedures described can be applied to the determination of the three compounds in tissue extracts. No pre-treatment or derivatization is required prior to the analysis. The entire procedure needs 30 or 45 min per sample. We are currently using these methods in our laboratory for metabolic studies concerning [^{14}C]- and [^{3}H]adiphenine.

EXPERIMENTAL

Apparatus

The method was developed using an Altex (Chromatem, Touzart et Matignon, France) HPLC system equipped with two C 380 solvent delivery pumps and an Altex 420 solvent programmer coupled to a UV absorbance detector operated at 254 nm

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and/or to a Berthold LB 5026 radioactivity detector and to a double-channel chart recorder.

Reagents

All solvents were of analytical reagent grade. Methanol supplied by Merck (Darmstadt, G.F.R.) was redistilled twice before use.

The labelled compounds used were 2-diethylaminoethyl[1-^{14}C]diphenyl acetate hydrochloride ([^{14}C]adiphenine hydrochloride), 2-diethylamino[2-^{3}H]ethyldiphenyl acetate hydrochloride ([^{3}H]adiphenine hydrochloride), [2-^{14}C]- or [2-^{3}H]N,N-diethylaminoethanol and [1-^{14}C]diphenylacetic acid.

[^{14}C]- or [^{3}H]adiphenine hydrochloride was obtained by the reaction of diphenylacetic acid with [^{14}C]- or [^{3}H]2-chloroethyl-N,N-diethylamine and potassium carbonate in acetonitrile. It was purified by low-pressure liquid chromatography on a silica column. Its structure was confirmed by its melting point and its IR and NMR spectra (the specific radioactivities were 2.73 and 15 mCi/mmol, respectively.)

[2-^{14}C]- or [2-^{3}H]N,N-diethylaminoethanol was synthesized in our laboratory by reaction of ethyl bromide with [^{14}C]- or [^{3}H]2-aminoethanol in tetrahydrofuran under reflux (the specific radioactivities were 3.5 and 15 mCi/mmol, respectively.)

[1-^{14}C]Diphenylacetic acid was synthesized by radioactive carbonation of diphenylmethyllithium[9] (the specific radioactivity was 2.75 mCi/mmol).

The radiochemical purities of all labelled species were $>97\%$.

Procedure

A 30 × 0.47 cm I.D. column was packed either with LiChrosorb RP-18, 10 μm, or with LiChrosorb Si 100, 10 μm (Merck).

On LiChrosorb RP-18, the mobile phase consisted of a non-linear elution gradient, prepared by mixing two solvents, (A) 0.027 *M* aqueous ammonia and (B) 0.027 *M* ammonia in methanol, as follows: from A (100) up to A plus B (70:30) in 6 min, 5 min linear, up to A plus B (30:70) in 15 min, 15 min linear, up to B (100) in 2 min, linear. For LiChrosorb Si 100, the mobile phase consisted of a non-linear gradient from two solvents, (A) dichloromethane–diethylamine (100:0.2) and (B) dichloromethane–ethanol–diethylamine (80:20:0.2), as follows: from A (100) for 4 min to A plus B (50:50) in 13 min, 13 min linear. For both systems the flow-rate was 2 ml/min and all separations were performed at ambient temperature. Samples were injected into the column through a continuous flow loop injector.

In view of the basic mobile phase (pH >8) used, the stability and lifetime of the columns were tested. Between experiments the column was kept in an aqueous phase at a pH below 8. The columns were equilibrated before each experiment. The stability was checked by the detection of a standard solution containing known weights of the three compounds. It is reasonably possible to use the same column for 8 weeks without any change in performance.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of the three standard compounds on RP-18 and Fig. 2 the separation on silica. On the former support, the more polar compounds emerge first, and the order of retention is reversed compared with that on silica.

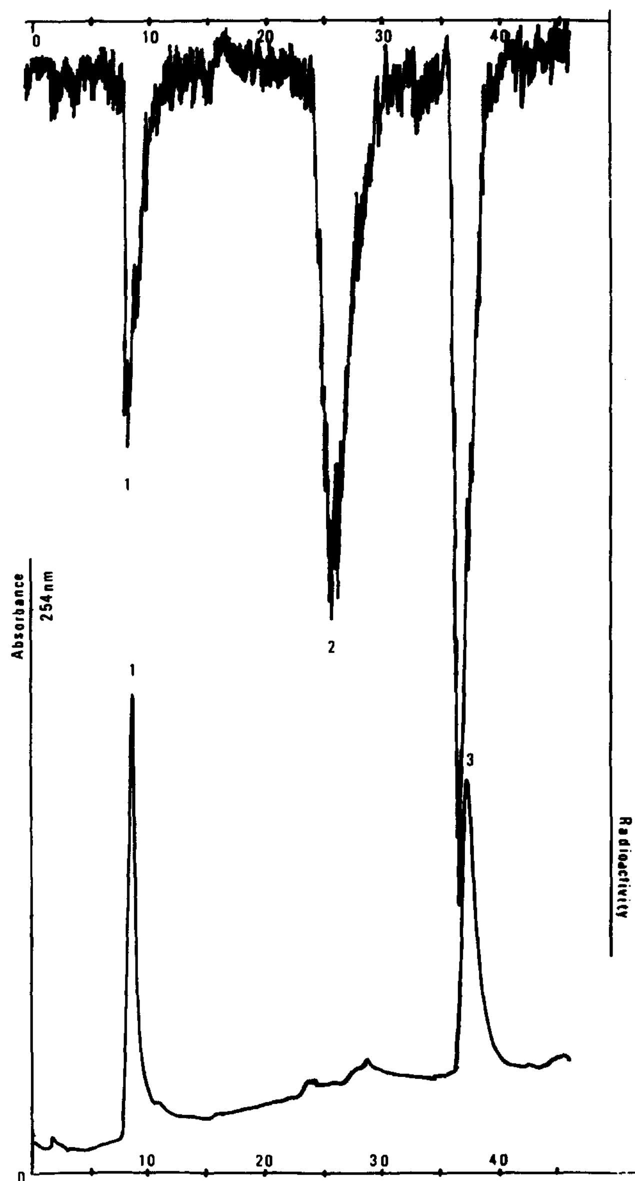

Fig. 1. Separation of adiphenine (3; t_R = 37.0 min), diethylaminoethanol (2; t_R = 25.8 min) and diphenylacetic acid (1; t_R = 12.5 min) on 10-μm LiChrosorb RP-18. Mobile phase: non-linear elution gradient prepared by mixing 0.027 *M* aqueous ammonia and 0.027 *M* ammonia in methanol. Flow-rate: 2.0 ml/min. Column: 30 × 0.47 cm I.D.

Adiphenine could only be eluted from the column as a base, and not in the chloride form, so we had to use a basic medium. We did most of our assays with labelled compounds owing to our particular identification of radioactive tissue metabolites. Each of them was present at a very low concentration undetectable by UV methods.

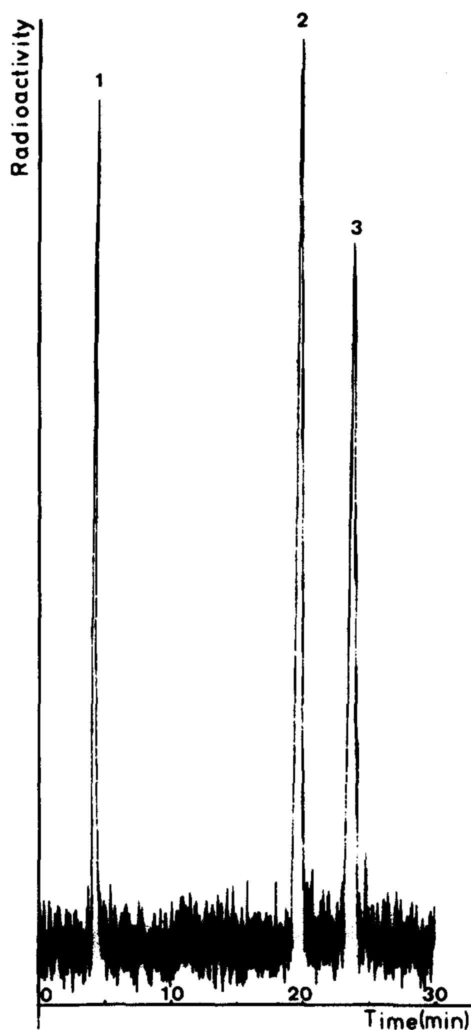

Fig. 2. Separation of adiphenine (1; t_R = 4.5 min), diethylaminoethanol (2; t_R = 19.5 min) and diphenylacetic acid (3; t_R = 23.5 min) on 10-μm LiChrosorb Si 100. mobile phase: non-linear gradient from two mobile phases consisting of dichloromethane–diethylamine and dichloromethane–ethanol–diethylamine. Other conditions as for Fig. 1.

With these two HPLC separation procedures and with the double labelling of adiphenine, we were able to obtain a precise idea of the brain radioactivity of rats after i.v. injection of adiphenine. Two minutes after a dose of 4 μmol per rat, we detected, per gram of extracted brain, either 49.3 nmol of unchanged compound ([^{3}H]adiphenine) and 11.7 nmol of diethylaminoethanol or 50.7 nmol of unchanged compound ([^{14}C]adiphenine) and 10.3 nmol of diphenylacetic acid.

The methods, which are highly accurate and reproducible, allowed us to identify brain radioactivity. We are now able to establish a kinetic picture of adiphenine in rats. This is of great help in metabolic studies currently being carried out in our

laboratory to elucidate the mechanism of action of this anticholinergic drug at the cerebral level.

We plan to extend this methodology to the identification of radioactivity in other target organs and in particular to the hypophysis and adrenal glands.

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