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A SENSITIVE RADIORECEPTOR ASSAY FOR ATROPINE IN PLASMA

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Abstract—The development of a sensitive, relatively simple radioreceptor assay for atropine in plasma is reported. It is based on the ability of anti-muscarinic compounds to compete with tritiated quinuclidinyl benzilate for muscarinic receptor binding sites. As little as 300 fmol (3×10^{-10} M) atropine could be reliably assayed, the extraction procedure permitting the estimation of 3 pmol (0.9 ng) atropine per ml of plasma. The method has been applied to the determination of plasma concentration-time profiles after i.m. administration of atropine to man, and could probably be extended to other anti-muscarinic drugs.

Antimuscarinic drugs like atropine (± hyoscyamine) have long been used clinically, both acutely in, for instance, pre-operative medication and in the treatment of anticholinesterase pesticide poisoning, and chronically in the treatment of conditions such as peptic ulcer and Parkinsonism. However, although widely used clinically there is no simple, sensitive method available to assay the low levels of anti-muscarinic compounds found in plasma after therapeutic doses.

Although a radioimmunoassay for atropine has been reported [1, 2], it was apparently more sensitive to the pharmacologically inactive isomer than to the active isomer, (-)hyoscyamine. Radiolabelled atropine has been used for pharmacokinetic studies in rat [3], dog [4, 5] and man [6], *inter alia*, but, unless preliminary separations were undertaken, atropine could not be distinguished from its inactive metabolites.

This paper reports the development of a method for the estimation of atropine in plasma, based on its ability to compete with tritiated quinuclidinyl benzilate ([^{3}H]QNB) for muscarinic receptor binding sites. The procedure has been used to monitor plasma levels of atropine in man.

MATERIALS AND METHODS

Materials

^{3}H-QNB (*l*-quinuclidinyl [phenyl-4(n)-^{3}H] benzilate, 43 Ci/mmole) and [G-^{3}H] atropine (376 mCi/mmole) were supplied by the Radiochemical Centre, Amersham. Atropine sulphate, atropine methyl nitrate and hyoscine hydrobromide were obtained from Sigma (London) Ltd. Dexetimide, ethopropazine hydrochloride and orphenadrine citrate were kindly donated by Janssen Pharmaceuticals Ltd., May and Baker Laboratories and Riker Laboratories, respectively. Aprophen was synthesized by Mr. G. L. Sainsbury of this Establishment.

Methods

Preparation of receptor material. Porcine brain minus cerebellum was homogenized in 10 vol. ice-cold 0.25 M sucrose in 10 mM Tris–citric acid buffer, pH 7.4, using a Teflon–glass Potter–Elvejhem homogenizer. The homogenate was centrifuged at $1{,}000\,g_{av}$ for 10 min at 2°, and the resultant supernatant was decanted and centrifuged at $30{,}000\,g_{av}$ for 30 min. The pellet from the second centrifugation was re-suspended in 10 vol. water; this suspension was frozen for 16 hr, then thawed and re-centrifuged at $30{,}000\,g_{av}$ for 60 min. The final receptor pellets were stored at −20°, and re-suspended in 50 mM sodium phosphate buffer, pH 7.4 to a concentration of approximately 5 mg protein/ml, as required. Both the receptor pellets and portions of the pig brain were stored for up to one year at −20°, without significant loss of activity.

Binding assay. All constitutents in the binding assay were made up to a final volume of 1 ml with 50 mM sodium phosphate buffer, pH 7.4 [7]. To duplicate incubation tubes (7 cm × 1 cm diam), with or without atropine, were added phosphate buffer and 50 μl of receptor preparation. This solution was left to pre-incubate for 10 min, then 50 μl ^{3}H-QNB were added to give a final concentration of 0.8 nM.

Incubations were for 10 min at room temperature (approx 20°), then 2 ml ice-cold phosphate buffer was added to each tube. The samples were immediately filtered through Whatman GF/B glass fibre discs under vacuum using a Millipore multiple filtering manifold. The assay tubes were rinsed with 4 ml cold phosphate buffer, and the filter discs washed *in situ* with the rinsings, followed by 2 × 10 ml cold buffer. The filters were then dispersed in 10 ml of Scintillator 299 or Picafluor 15 (Packard) for counting in a Packard Tri Carb Liquid Scintillation Spectrometer.

Binding of ^{3}H-QNB in the presence of 10^{-6} M atropine was carried out to assess non-specific binding, which was less than 5 per cent.

Assay for atropine in plasma. Plasma proteins were precipitated by adding two volumes of methanol, and allowing the mixture to stand overnight at 2°. The samples were centrifuged at 500 *g* for 10 min, and aliquots, typically 300 μl, of the supernatant evaporated to dryness in the assay tubes under a gentle stream of warm nitrogen.

A standard curve was prepared by adding known amounts of atropine sulphate to the dried plasma extract derived from a blood sample taken from each subject 15 min before drug administration. The curve was plotted on log probit graph paper (Fig. 1), and the concentration of atropine in the test samples determined from it, taking into account the ten-fold dilution in the assay tubes.

Human studies. Blood samples* from indwelling intravenous cannulae were collected into EDTA or heparinized (10 I.U./ml) tubes at various times after the intramuscular injection into the lateral aspect of the upper thigh of a combination of atropine sulphate (2 mg) and *N*-methylpyridinium-2-aldoxime methane sulphonate (500 mg) in a 2 ml volume. Injections were given at 10.00 hr, 2 hr after a standardised light breakfast. The blood samples were centrifuged at 1,000 *g* for 10 min, and the plasma stored at 4° until required.

Atropinesterase activity of human plasma. Heparinized whole blood was spun at 1,000 *g* for 10 min at 2°. 2 ml of the plasma was made 10^{-7} M with respect to atropine and incubated at 37°. Samples (10 μl) were taken after 5 min and after 30 min for the estimation of atropine using the standard binding assay described above.

RESULTS

After clinical doses of atropine, plasma levels were expected to be of the order of 10^{-8} M, and it was anticipated therefore that 100 μl plasma samples would be required to give approximately 50 per cent inhibition of binding, when the final incubation volume was 1 ml. Preliminary experiments, however, indicated that as little as 10 μl of human plasma in a final incubation volume of 1 ml inhibited binding of ^{3}H-QNB to muscarinic receptors by 10–20 per cent with 50 μl inhibiting binding by more than 50 per cent and 100 μl by 70 per cent (Table 1).

Table 1. Inhibitory effect of human plasma on QNB binding

Plasma sample	Vol (μl)	% Inhibition
1	50	57
1	100	71
2	50	57
2	100	70
3	50	59
3	100	69

Plasma samples from three untreated volunteers were added to phosphate buffer, and incubated with the brain receptor preparation (equivalent to 400 μg protein) and [^{3}H] QNB (2 nM) in a final volume of 1 ml. Results are the mean of duplicate determinations.

* Samples were obtained through the courtesy of Dr. R. I. Gleadle of this establishment.

When control plasma samples were subjected to centrifugation using a 'Centriflo' cone (Amicon Corp) with a molecular weight cut-off of 50,000, there was no factor inhibiting ^{3}H-QNB binding present in the ultrafiltrate. As there was no evidence of atropinesterase activity in the human plasma samples, it was considered likely that plasma inhibited QNB binding to CNS receptors by providing competing binding sites on plasma proteins. It was decided, therefore, to remove proteins by precipitating them with methanol. This procedure reduced binding of [^{3}H]-QNB by 100 μl of control plasma from 70 per cent to 10–15 per cent. The overall recovery of atropine was assessed as follows. Control plasma samples were adjusted to contain various concentrations of atropine from 5.10^{-9} to 10^{-7} M, and the plasma proteins were precipitated with two volumes of methanol. The samples were centrifuged and 300 μl aliquots from the supernatant were evaporated to dryness and the atropine estimated using the standard binding assay. The standard curve obtained when atropine was added to plasma prior to extraction was not significantly different from that observed when the atropine was added to the dried plasma extract. The mean recovery at each concentration was in excess of 95 per cent, and the recovery at 10^{-8} M atropine in plasma was 104 per cent (S.D. = ± 3.6, $n = 4$). In a supplementary experiment, the recovery of ^{3}H-atropine (2.75×10^{-7} M) from plasma, after the precipitation of protein with methanol, was found to be 103 per cent (± 2.5, $n = 4$).

Because of the very high affinity of QNB for the muscarinic sites, it was found desirable to pre-incubate the receptor and atropine for 10 mins, before adding the radioligand. Also, the temperature and time of incubation with the radioligand present were found to have a pronounced influence on the sensitivity of the assay. The sensitivity to atropine was doubled when samples were incubated at 0° for 60 min or at 20° for 10 min, when compared to 20° for 60 min.

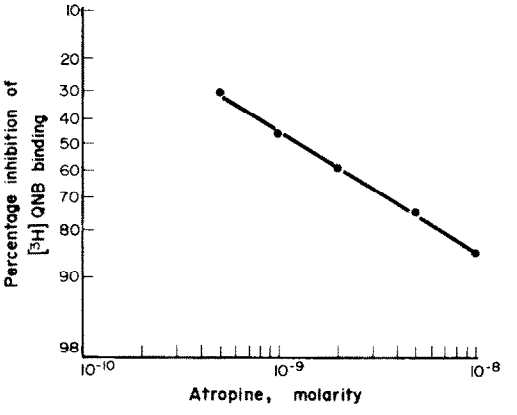
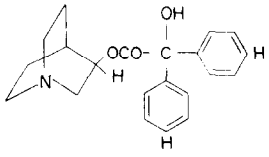
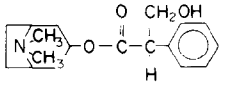
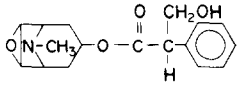
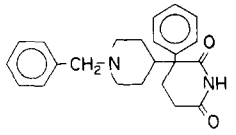
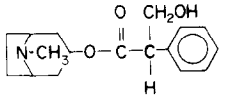
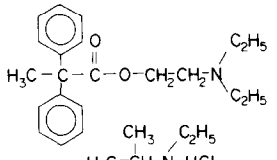
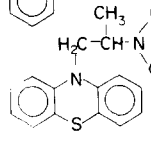
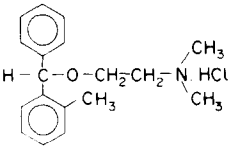


Fig. 1. Sensitivity and linearity of the radioreceptor assay for atropine. The final molarity of [^{3}H]QNB was 8×10^{-10}, and each incubation contained receptor material equivalent to 200 μg protein. The percentage inhibition of [^{3}H]QNB binding is plotted on a probit scale against the log concentration of atropine. Each point is the mean of duplicate determinations which differed by less than 5 per cent.

Table. 2. Displacement of [^{3}H] QNB binding to pig brain membranes *in vitro* by various anti-muscarinic compounds

Relative potency of some antimuscarinc compounds

Compound	Formula	IC_{50}(nM)
[^{3}H] QNB		
Atropine methyl nitrate		0 4
Hyoscine		0.5
Dexetimide		0 54
Atropine		1.0
Aprophen		1.6
Ethopropazine		10
Orphenadrine		60

[^{3}H] QNB (0.8 nM) was incubated with brain membranes (equivalent to 200 μg protein) in the presence of various concentrations of the antimuscarinic compound, in duplicate, at 20° for 10 min. The IC_{50} value is the concentration of the drug at which specific QNB binding was inhibited by 50 per cent. Non-specific binding was estimated in the presence of 2×10^{-6} M unlabelled atropine.

Using 200–400 μg protein and with 0.8 nM [^{3}H] QNB in a final incubation volume of 1 ml, a standard curve for atropine showed the assay to be sensitive to 300 fmol (Fig. 1).

The sensitivity of the assay to some other muscarinic antagonists of diverse molecular structure is indicated in Table 2. Both orphenadrine and the phenothiazine, ethopropazine, are used in the treatment of paralysis agitans and other types of Parkinsonism [8] and, as expected, exhibited pronounced antimuscarinic binding activity. Aprophen, which has been used as a spasmolytic and as an analgesic

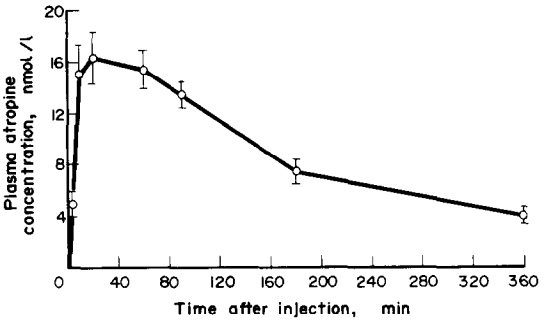


Fig. 2. Plasma concentration–time curves after i.m. injection of atropine sulphate (2 mg) to ten men. Each point is the mean ± S.E.M.

[9], was found to be almost as potent as atropine in the binding assay.

The results of the studies in man on plasma levels of atropine after the intramuscular injection of 2 mg atropine sulphate are shown in Fig. 2. The main peak plasma level of 16.4 nmoles/l was attained within 20 min, although in one subject (results not included) a peak level of 33 nmoles/l was reached within 3 min of injection. Plasma levels of atropine had fallen to approximately 50 per cent of peak levels after 3 hr, and the plasma elimination half-life in the final phase studied was 3.8 hr.

When a repeat determination was performed on plasma samples which had been stored at 4° for 3 months, results for all time periods were within 15 per cent of those obtained on fresh plasma.

DISCUSSION

The development of a sensitive, relatively simple radioreceptor assay for atropine in plasma is reported, in which as little as 300 fmol atropine can be reliably detected (20 per cent inhibition of binding) using the standard assay volume of 1 ml. The sensitivity of the assay can be increased still further by reducing the incubation volume to 200 μl.

Although, routinely, we took 1 ml plasma samples for precipitation with methanol, it is possible to take only 200 μl of plasma, and still perform the assay in duplicate. It was necessary to introduce the methanol extraction step because the plasma proteins interfered with [^{3}H] QNB binding. This procedure reduced binding by control plasma, gave reproducible results, and was regarded as being simpler than subjecting all samples to dialysis. It has the additional merit of being suitable for compounds which are acid or base labile. However, preliminary experiments using equilibrium dialysis indicated that whereas atropine at 10^{-8} M bound less than 20 per cent to plasma proteins, QNB, at the same concentration, was almost completely bound. If, as appears likely, the extent of plasma protein binding of antimuscarinic compounds is correlated with their degree of lipophilicity it may be possible to eliminate the extraction step by using either [^{3}H]atropine or a radiolabelled quaternary salt of, for example, QNB,

atropine or scopolamine*, if such materials of sufficiently high specific radioactivity were to become commercially available. It should also be possible to omit the methanol precipitation step and to assay small volumes of plasma directly when the antimuscarinic plasma concentration is high as may occur with certain treatment schedules or in animal studies.

The assay described here is somewhat more sensitive to atropine, and considerably more sensitive to (-)hyoscyamine, than is the radioimmunoassay reported by Wurzburger *et al.* [2]. The radioimmunoassay [2] does not require an extraction step and, by its very nature, possesses greater specificity than does the radioreceptor assay. Thus with a radioimmunoassay it should be possible to assay one particular antimuscarinic drug in the presence of other antimuscarinic drugs, but using a radioreceptor assay the total antimuscarinic activity of a combination of drugs and their metabolites is determined. Clinically, however, there may be occasions when it would be advantageous to understand the overall contribution to antimuscarinic activity of a combined treatment schedule or of a parent drug and its metabolites. In the case of atropine, the known major metabolites, tropine and tropic acid, are pharmacologically inactive, but this may not be the case with the metabolites of other antimuscarinic drugs. Furthermore, whereas an immunoassay requires the preparation of a different anti-serum for each antimuscarinic drug to be assayed, the radioreceptor assay may be used in the estimation of any drug possessing muscarinic blocking activity (Table 2).

Although porcine tissue was used in the present study, any mammalian brain, for example rat or calf, should provide a suitable source of receptor material.

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* [^{3}H] Methyl scopolamine is now available from NEN Chemicals.

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