Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, No. 5, pp. 433–439, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00
© 1988 Pergamon Press plc

Radioreceptor assays of ipratropium bromide in plasma and urine

K. ENSING,* M. POL and R. A. DE ZEEUW

State University, Department of Toxicology, A. Deusinglaan 2, 9713 AW Groningen, The Netherlands

Abstract: Ipratropium bromide (Ipbr) is a frequently used quaternary anticholinergic administered by inhalation in the treatment of chronic obstructive lung diseases. Hardly any pharmacokinetic data are available, which can be useful in the optimisation of anticholinergic therapies. Hence, a radioreceptor assay (RRA) for Ipbr has been developed. The RRA is based on the competition between ^{3}H-*N*-methylscopolamine chloride (^{3}H-NMS) and Ipbr for binding to lyophilised muscarinic receptors from calf brains. The assay has been optimised in respect of incubation conditions and extraction by ion-pair formation with sodium picrate. Detection limits of the drug were 5 ng ml^{-1} in urine and 500 pg ml^{-1} in plasma, after extraction of 2-ml samples.

The method is applicable to monitoring the drug in plasma and urine after therapeutic dosing.

Keywords: *Ipratropium bromide; radioreceptor assay; drug monitoring.*

Introduction

Ipratropium bromide (Ipbr) (Atrovent, Itrop, Boehringer–Ingelheim, FRG) is a *N*-isopropyl derivative of atropine and used in the treatment of chronic obstructive lung diseases. Ipbr antagonises the bronchi-constricting effect of acetylcholine, released by stimulation of the vagus nerve. Ipbr can also antagonise vagal stimulation in other peripheral organs which may cause undesirable side-effects, e.g. tachycardia and reduced salivation. However, because of its quaternary structure, the drug does not pass the blood–brain barrier and lacks central effects.

In clinical practice the drug is generally administered by inhalation in low dosage and for this reason serious side-effects are seldom reported. Nevertheless for a proper understanding of the pharmacological action and to ascertain optimum use of the drug it is necessary to determine pharmacokinetic profiles after inhalation and correlate the data with clinical findings in patients and/or volunteers. Because of its potency, the drug concentrations in plasma are usually in the lower ng ml^{-1} range as indicated by studies with the radiolabelled drug [1].

To determine the drug, a radioreceptor assay (RRA) based on competition between a radiolabelled quaternary ligand (^{3}H-*N*-methylscopolamine chloride; H-NMS) and Ipbr for binding to muscarinic receptors obtained from calf brain is to be developed. The

*Author to whom correspondence should be addressed.

development of the assay for Ipbr in plasma and urine is described. Owing to interference of the endogenous matrix no direct assay in plasma was possible; hence the possibilities of selective ion-pair extraction, re-extraction and concentration of the drug were investigated and optimised in respect of precision and limits of sensitivity of the method.

Materials and Methods

Chemicals

^{3}H-NMS (90 Ci mmol^{-1}) was supplied by NEN (Dreieich, FRG). Ipratropium bromide was kindly donated by Boehringer–Ingelheim (Ingelheim, FRG). Tetrapentylammonium iodide was obtained from Eastman Kodak (Rochester, NY, USA). All other chemicals and solvents of analytical grade were obtained from Merck (Amsterdam, The Netherlands). Polypropylene reaction vials were obtained from Kartell (Milan, Italy).

Sovirel tubes were obtained from Quickfit S.A. (Epernon, France). The 50 mM sodium phosphate buffer (pH 7.4) was prepared from 4 volumes of 50 mM sodium dihydrogen phosphate and 1 volume of 50 mM disodium hydrogen phosphate. Picofluor 30 was used as the scintillation liquid and was obtained from Packard Instruments (Groningen, The Netherlands). Polyethylene counting vials were supplied by Beckmann (Irvine, CA, USA).

Tetrapentylammonium (TPA) solution was prepared by reacting 2.13 g of tetrapentylammonium iodide with 0.637 g of silver oxide in 30 ml of distilled water for 1 h in an ultrasonic water-bath at room temperature. The precipitate was removed by filtration through a glass filter; the filtrate was collected and extracted twice with equal volumes of dichloromethane. The aqueous phase was neutralised with 0.1 M orthophosphoric acid to pH = 7.5 and again extracted with equal volumes of dichloromethane. The TPA concentration in the aqueous phase was determined by ion-pair extraction with picrate into dichloroethane and the solution was diluted to give a final TPA concentration of 2×10^{-3}M. This solution was used in diluted form for the re-extraction of Ipbr.

Preparation of receptor material

Calf brains, freshly prepared or stored at −80°C (without cerebellum) were homogenised in 6 volumes of ice-cold 0.32 M sucrose using a Teflon-glass Potter–Elvejehem homogeniser at 1200 rpm (R.W. 18, Janke and Kunkel, Staufen i. Breisgau, FRG). The homogenate was centrifuged for 10 min at 1000 **g**. The pellet was discarded and the supernatant was centrifuged for 60 min at 100,000 **g**. The latter pellet was resuspended in buffer, equivalent to the original volume of the homogenate, and centrifuged for 30 min at 100,000 **g**. This washing was repeated and finally the pellet was resuspended in 5 volumes of buffer. The receptor preparation was lyophilised for 24 h and the optimum tissue concentration for the RRA was determined experimentally [2].

Radioreceptor assay

To duplicate, Ipbr in buffer was added to polypropylene reaction vials (1.5 ml), in a biofluid or in a suitably prepared aqueous extraction solution, to produce initial concentrations of 10^{-10}–10^{-7}M, corresponding to about 40 pg–40 ng ml^{-1}. Then 25 μl of radiolabelled methylscopolamine was added to produce a final assay concentration of 3×10^{-10}M. Finally a volume of the receptor preparation containing 3 mg of lyophylised receptors was added in order to obtain a 550 μl incubation volume. The

reaction vials were closed, shaken and incubated for 60 min at room temperature, then centrifuged for 15 min at 15,000 g in a Hereaus Christ Biofuge A (Osterode am Harz, FRG). 450 μl of the supernatant was transferred in the polyethylene counting vials, 2 ml of picofluor 30 was added and the mixture vortexed for 5 s before counting in a Beckman 1800 liquid scintillation counter (Irvine, CA, USA). The samples were counted for 40,000 counts with a counting time limit of 5 min.

Extraction and re-extraction of ipratropium

To 10 ml Sovirel tubes, 1 or 2 ml of plasma or buffer containing 10^{-10}–10^{-7}M Ipbr and 100 or 200 μl of 3×10^{-3}M sodium picrate in 50 mM sodium phosphate buffer were added, respectively, to give a final picrate concentration of approximately 3×10^{-4}M. 5 ml of dichloroethane (DCE) was added; the tubes with 1 ml aqueous solution were vortexed for 30 s whereas the 2-ml aqueous samples were rolled for 60 min on a Denley Mixer 5 (Sussex, UK) to avoid the formation of unseparable emulsions. The rolling procedure was also tested for 1-ml aliquots of the aqueous samples in order to establish possible recovery losses in comparison with the vortexed samples.

The tubes were centrifuged for 10 min at 5000 g and the water phase was discarded; 4.5 ml of the DCE phase was transferred into another Sovirel tube and 1.0 ml of 2×10^{-6}M tetrapentylammonium-hydroxide (TPA) in buffer was added. The mixture was vortexed for 30 s and centrifuged for 10 min at 5000 g. Two 0.25-ml aliquots of the aqueous TPA-extract were transferred to 1.5 ml polypropylene reaction vials and the RRA was carried out.

Calibration graphs were constructed from the results of experiments that involved incubation in the presence of increasing concentrations of drug (10^{-10}–10^{-7}M) which were added to blank plasma, urine or buffer and handled in the same way as the samples to be analysed. The amount of free labelled ligand was expressed in terms of dps-values (disintegrations per s) which were obtained by converting the cps-values; the counting efficiency of these samples was always near 35%.

The amount of drug in standards was plotted against the dps-values. The recovery of the method was determined by comparing the values of the standard curve with those of aqueous TPA solutions containing fixed amounts of ipratropium. The precision of the method was determined by triplicate determination of a standard curve and by repetitive determination of some spiked samples in random order.

Results and Discussion

The optimum tissue concentration for the inhibition curves was estimated by measuring the differences in the concentration of free labelled ligand, in the absence and presence of a fixed concentration of Ipbr which gave approximately 50% inhibition of receptor-bound ^{3}H-NMS, for various tissue concentrations. These data are plotted in Fig. 1. It can be concluded that the optimum tissue concentration at the end of the linear part of the curves is about 6 mg ml^{-1}. Then the amount of displaced radiolabelled ligand is maximal whereas the free fraction labelled ligand is rather small as compared with the corresponding bound fraction.

In Fig. 2 a representative calibration curve for Ipbr is given. The concentration of Ipbr which causes 10% inhibition of receptor-bound ^{3}H-NMS is considered as the lower limit of determination for the method. The determination limit is 1×10^{-9}M which corresponds to 220 pg of Ipbr in the assay.

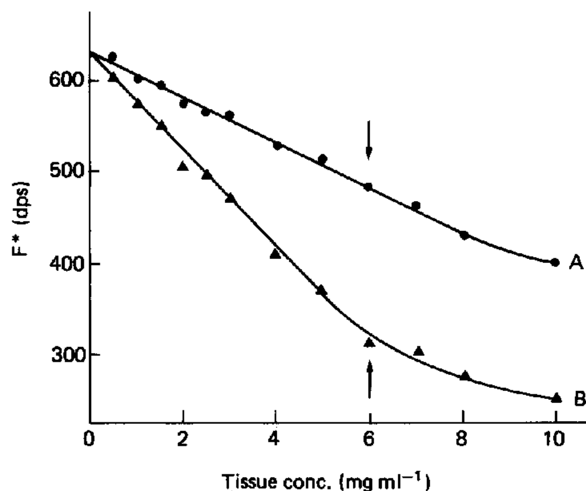

Figure 1
Influence of the amount lyophylised receptor preparation (tissue concentration) on the concentration free labelled ligand (F*) in the presence (A) or absence (B) of 1×10^{-8} M ipratropium bromide. The arrow indicates the optimum tissue concentration.

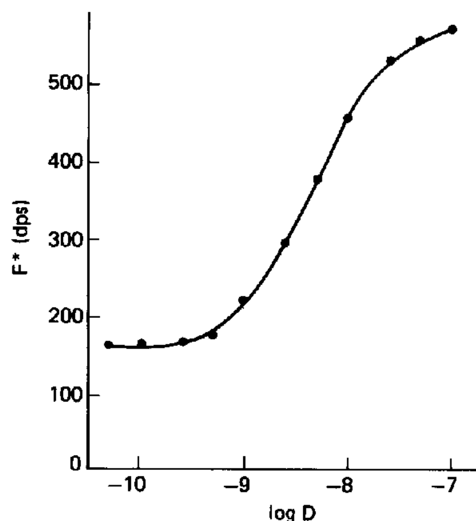

Figure 2
Experimental inhibition curve for ipratropium bromide. F* indicates the concentration free labelled ligand. D indicates added drug concentration in mol l^{-1}.

When drug-free urine is added in small amounts to the incubation medium of the RRA, ^{3}H-NMS binding will be reduced. However, this inhibition is constant and is kept at an acceptable level (approximately 10%) when the urine is diluted about ten-fold with buffer as is the case in the assay (3). Therefore, because of the relatively high drug concentrations in urine samples after therapeutic dosing, it was decided to assay these samples directly instead of applying a laborious sample clean-up procedure followed by dilution, in order to keep the Ipbr concentrations within the range of the calibration curve.

For plasma a sample clean-up and concentration step is unavoidable. On the one hand, plasma proteins have a detrimental effect on the sensitivity and accuracy of the assay because of non-specific binding of ^{3}H-NMS and Ipbr. On the other hand, the drug concentrations in plasma are close to the determination limit of the assay so that dilution of the samples, as with urine, will result in undetectable levels of drug.

Therefore, it was decided that ion-pair extraction and re-extraction procedure were to be used, which is very selective and generally offers high recoveries even in the subnanogram range as was observed for the quaternary anticholinergic, oxyphenonium [4, 5].

Picrate offered the best opportunities for a quantitative extraction of Ipbr; experiments in buffer showed that a picrate concentration of 3×10^{-4}M allows recoveries of more than 99.9% for ipratropium in the organic layer. The re-extraction was carried out with 1 ml of 2×10^{-6}M TPA; TPA has a 10,000-fold higher extraction constant with picrate than ipratropium. So, when the amount of TPA in the aqueous layer is slightly larger than the amount of ipratropium in the organic layer, TPA will displace all ipratropium in the ion-pair with picrate. Concentrations of TPA higher than 10^{-6}M cannot improve the recovery since TPA will start to inhibit the binding of the labelled ligand to the receptor [5].

In Table 1 precision data are given for a number of ipratropium bromide concentrations in buffer, plasma (1 and 2 ml samples) and urine. The following

Table 1
Evaluation of the RRA for Ipbr

Amount of drug in RRA (pmol)	within experiment RSD (%, $N = 6$)	day to day RSD (%, $N = 3$)	day to day RSD (%)
Buffer			
0.25 (100 pg)	14.3	17.6	7.1 ($N = 10$)
1.25 (500 pg)	5.6	9.7	5.0
2.50 (1 ng)	7.5	1.0	1.1
12.50 (5 ng)	5.4	2.4	2.7
Urine			
0.25	12.4	22.1	18.3 ($N = 30$)
1.25	4.9	1.6	3.9
2.50	5.7	4.1	4.3
12.50	14.6	5.5	3.3
Extraction from 1 ml buffer (vortexed)*			
0.25	13.2	16.2	
1.25	16.5	11.2	
2.50	4.4	4.9	
12.50	27.5	18.4	
Extraction from 1 ml plasma (vortexed)*			
0.25	21.8	25.5	14.4 ($N = 10$)
1.25	5.6	8.2	4.9
2.50	6.3	8.4	2.4
12.50	12.8	4.5	5.0
Extraction from 1 ml plasma (rolled)*			
0.25	49.8	41.1	
1.25	4.0	9.6	
2.50	9.1	6.0	
12.50	17.5	10.7	
Extraction from 2 ml plasma (rolled)*			
0.25	31.2	4.8	
1.25	5.2	3.8	
2.50	10.2	6.7	
12.50	6.5	7.1	

*The amount of drug without correction for the recovery of the ion-pair extraction and re-extraction.

recoveries were obtained at the 2.5 pmol level in the RRA (which corresponds to 1 ng of Ipbr) for 1 ml of buffer, 1 and 2 ml of plasma respectively: 104, 91 and 81%. For the rolled samples a comparable recovery was calculated as for the vortexed samples.

As can be concluded from Table 1, the precision of the RRA is dependent on the amount of drug. The precision is optimal in the steepest part of the calibration curve and is worse at the beginning and end of the curve. This is illustrated by the data for 0.25 pmol Ipbr which is approximately half the amount of drug which was arbitrarily assigned as the determination limit of the method.

The radioreceptor assay has been employed in pharmacokinetic studies in healthy volunteers after oral administration of 2, 5, 10 and 20 mg of Ipbr, after intravenous administration of 2 mg of Ipbr and after slow inhalation of 2 mg of Ipbr. As expected, there were considerable variations in plasma levels and urinary excretion. For this reason the volume of urine added to the assay was 25 or 50 μl for oral and inhalation administration whereas a further 100-fold dilution of the urine samples after intravenous administration was necessary in order to obtain sample concentrations within the range of 10–90% inhibition of the calibration curve. This is illustrated in Fig. 3 with the cumulative excretion curves after administration of 2 mg of Ipbr by three different routes. It can be seen that there are enormous variations in bioavailability, dependent on the route of administration.

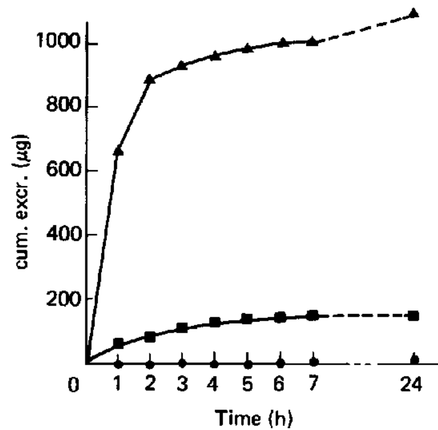

Figure 3
Cumulative excretion curves of ipratropium bromide after intravenous (▲), oral (●) and inhalation (■) administration of 2 mg of Ipbr.

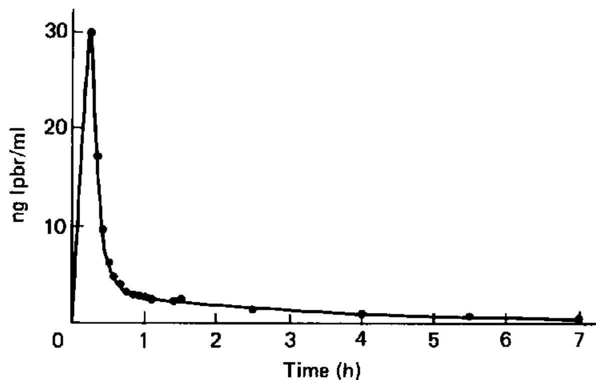

Figure 4
Plasma concentration–time curve after intravenous administration of 2 mg of Ipbr.

For plasma samples comparable variations were expected; however, the actual concentrations were much lower than those for the corresponding urine samples. Therefore, 2 ml samples were assayed in the case of low oral dosages. In Fig. 4 a typical plasma concentration-time curve after intravenous administration of 2 mg of Ipbr is shown.

The determination limit of the assay as such corresponds to a 5 ng ml^{-1} determination limit when 25 μl of the urine sample is used.

For plasma the determination limit is 1 ng ml^{-1} or 500 pg ml^{-1} when 1 or 2 ml sample is extracted, respectively.

It seems that the present assay is sensitive enough for pharmacokinetic studies of Ipbr when therapeutic doses are administered.

Acknowledgements: This study was supported by a research grant from Boehringer Ingelheim (Ingelheim, FRG; Alkmaar, The Netherlands).

References

[1] J. von Adlung, K. D. Höhle, S. Zeren and D. Wahl (1976). Untersuchungen zur Pharmacokinetik und Biotransformation von Ipratropiumbromid an Menschen. *Arzneim. Forschung* **26**, 1005–1010.

[2] K. Ensing, K. G. Feitsma, D. Bloemhof, W. Gin t'Hout and R. A. de Zeeuw (1986). Centrifugation or filtration in quantitative radioreceptor assays. *J. Biochem. Biophys. Meth.* **13**, 85–96.

[3] K. Ensing, F. Kluivingh, T. K. Gerding and R. A. de Zeeuw (1984). Development of a sensitive radioreceptor assay for oxyphenonium in plasma and urine. *J. Pharm. Pharmacol.* **36**, 235–239.

[4] J. E. Greving (1981). Ph.D. thesis: *Bioanalysis and pharmacokinetics of oxyphenonium bromide.* Groningen, The Netherlands.

[5] K. Ensing (1984). Ph.D. thesis: *Bioanalysis of anticholinergics with muscarinic receptors in relation with chronic obstructive lung diseases.* Groningen, The Netherlands.

[Received for review 16 October 1985; revised manuscript received 5 March 1986]