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Simple Radioligand Binding Assay for the Determination of Urinary Scopolamine

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Received November 2, 1982, from the **Human Pharmacology Institute, Ciba-Geigy, Tübingen, Federal Republic of Germany* and the ‡*Research Department of the Pharmaceutical Division, Ciba-Geigy, Basel, Switzerland.* Accepted for publication March 29, 1983.

Abstract □ A sensitive radioligand binding assay is described for the determination of scopolamine in human urine. As a measure for the drug concentration, the quantitative displacement of scopolamine of tritiated quinuclidinyl benzylate from rat brain receptors was used. The assay is sensitive to concentrations as low as 1.2 ng/mL, surpassed only by GC–MS techniques. It can be performed easily and quickly and does not include extraction procedures. Scopoline and scopine, possible metabolites of scopolamine, do not interfere with the assay. After transdermal administration of scopolamine, 34% of the drug is found in the urine. Of the total scopolamine excreted, 79% is conjugated to glucuronic and/or sulfuric acid and 21% is excreted in the unbound form.

Keyphrases □ Scopolamine—urinary determination, radioligand binding assay, □ Radioligand binding—simple assay, determination of urinary scopolamine □ Excretion, urinary—scopolamine, determination by radioligand binding assay

The lack of suitable methods for measuring low concentrations of scopolamine and other strongly acting parasympatholytic substances in biological fluids after therapeutic dosages in humans makes it difficult to obtain exact pharmacokinetic data on these drugs. Several methods exist for the determination of scopolamine, but these do not allow a rapid and sensitive measurement of low drug concentrations. GC (1) or acid–dye techniques (2) are not sensitive enough, while a GC–MS method (3) is not specific and involves difficult extraction procedures.

The development of radioligand binding techniques for studies of drug interactions with cholinergic receptors, using tritiated quinuclidinyl benzylate (I) as the radioligand (4), allows (analogous to RIA) the measurement of drugs having strong affinity to muscarinic receptors. Using tritiated I we developed a radioligand binding assay for the determination of the very low urinary concentrations of scopolamine that are found after transdermal administration of the drug.

EXPERIMENTAL

Preparation of Rat brain Homogenate—Male rats (Tif RAI f SPF) of 150–250-g body weight were used. After being sacrificed by decapitation, the brains were removed and the cerebral cortices dissected on ice and homogenized[1] for 30 s, at position 5, in 20 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.3). The homogenate was centrifuged at 26,000×*g* for 10 min, and the pellet was resuspended in the same volume of fresh buffer; this step was repeated three times. All procedures were performed at 4°C. The final pellet was suspended in 20 volumes of Tris-HCl and divided into 1-mL aliquots, which were rapidly frozen in liquid propane (~−160°C) and stored at −30°C. (After storage at −30°C for up to 3 months no loss of binding capacity was observed.) For the radioreceptor assay, the 1-mL aliquots were thawed for 5 min in a water bath (37°C) and diluted 17-fold by addition of ice-cold Tris-HCl. Membrane suspensions were not used in the assay for longer than 12 h after thawing.

Radioligand Binding Assay—The radioreceptor assay was performed at room temperature in 5-mL polystyrene tubes in a total volume of 2 mL of Tris-HCl containing brain membranes [~100 μg of protein as determined according to Lowry *et al.* (5)], 0.2 nM [^{3}H]-I[2] (33 Ci/mmol), and a 50-μL urine sample.

After 30 min, the incubation was terminated by filtration through a glass-fiber filter (2.4 cm diameter[3]) under reduced pressure. The filters were rinsed twice with 5-mL ice-cold Tris-HCl. After incubation of the filters for 30 min with 1-mL of tissue solubilizer[4] (diluted 1:1 with propanol), 10 mL of acidified scintillation fluid[5] was added and the radioactivity was determined in a liquid scintillation counter[6]. Nonspecific binding was defined by 10^{-7} M unlabeled I[7]. Scopolamine hydrobromide trihydrate[8] was used as a standard.

For the calibration curve, different concentrations of scopolamine were added to blank urine and tested as described above. The displacement (in percent) of ^{3}H-QNB from the muscarinic receptors in the brain homogenate was plotted on logit–log scale against the scopolamine concentration. Unknown urinary scopolamine concentrations were calculated from the calibration curve. To determine the total scopolamine (conjugated and free drug), urine samples were incubated at 37°C for 3 h with an equal volume of 1 M acetate buffer (pH 4.75) and 7.5 μL of β-glucuronidase–sulfatase[9]/mL of the mixture. The pH was adjusted to 7.3 with NaOH before the binding assay was performed.

Scopolamine Medication and Collection of Urine—Scopolamine was administered transdermally to nine healthy volunteers as a transdermal therapeutic system with scopolamine[10]. The drug system was applied on the skin surface behind the ear. Each system contains 1.5 mg of scopolamine base and can deliver ~0.5 mg of drug over 72 h. The system is programmed to release 0.15 mg as a loading dose during the first few hours; afterward, ~5 μg of scopolamine/h pass through the skin into the blood circulation. The drug system was applied for 64 h, delivering ~0.45 mg of scopolamine. Urine was collected in plastic bottles before the medication, in 12-h periods during the application, and for 8 × 6 h after removal of the drug. Aliquots were stored at −20°C in plastic tubes until assayed. For each subject, an individual urine blank for the calibration of the assay was collected before the medication.

[1] Polytron; Kinematika, Luzern, Switzerland.

[2] New England Nuclear, Boston, Mass.
[3] GF/B; Whatman.
[4] Soluene; Packard.
[5] Aquasol; Packard.
[6] Tri-Carb 460C; Packard.
[7] Synthesized by Dr. A. Storni, Ciba-Geigy, Basel, Switzerland.
[8] EGA Chemie, Steinheim, West Germany.
[9] Calbiochem.
[10] Scopoderm TTS; Ciba-Geigy.

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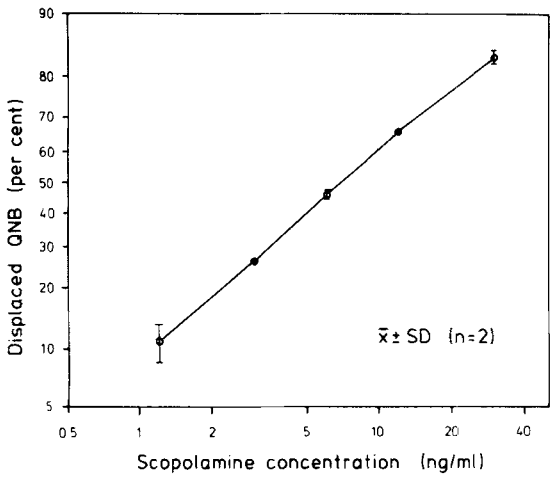

Figure 1—*Displacement of tritiated quinuclidinyl benzylate (QNB) from rat brain membranes by scopolamine (logit-log scale). Different concentrations of scopolamine were added to blank urine. The displacement of the labeled I from rat brain membranes was determined under the assay conditions described in* Experimental. *The values are means* ±SD *of two measurements.*

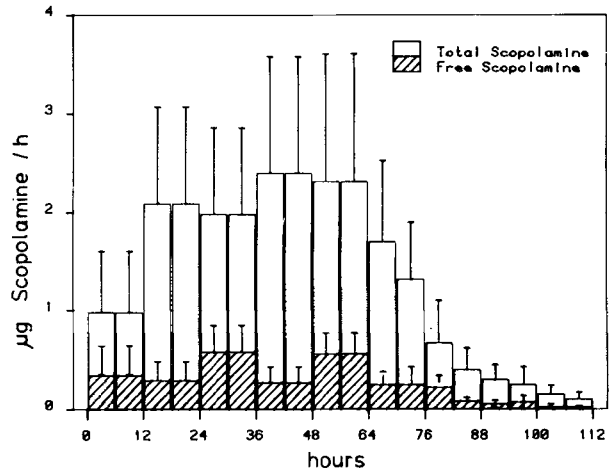

Figure 2—*Urinary excretion of scopolamine after transdermal administration. Application time was 64 h. Urine was collected as described under* Experimental. *The values are means* ±SD *from nine volunteers.*

RESULTS AND DISCUSSION

Under the test conditions selected, there was a linear correlation on logit-log scale between inhibition of [^{3}H]-I binding and scopolamine concentration in the range of 10^{-10}–2.5×10^{-9} M. This corresponds to a 1.2–30-ng/mL scopolamine concentration in 0.05-mL urine samples (Fig. 1). The IC_{50} value was 4.7×10^{-10} M.

To test the intra- and interassay reproducibility of the binding assay, blank urine samples containing 2, 10, and 25 ng/mL of scopolamine, respectively, were assayed repeatedly on a single day or different days. The results of these measurements are shown in Table I.

To exclude interference of the radioligand binding assay by scopoline and scopine, possible degradation products of scopolamine, these two substances were added to the urine blank in different concentrations (10^{-10}, 10^{-9}, and 10^{-8} M in the incubation mixture). Under these conditions, no displacement of [^{3}H]-I from brain membranes was observed.

The radioligand binding assay was used to measure the urinary excretion of scopolamine after administration of the transdermal system for 64 h. During the time of medication and 48 h thereafter, a total urinary excretion of 156 µg (80–276 µg) of scopolamine was observed (mean of nine volunteers). Steady state was reached after ~12 h of medication, lasting until the removal of the drug system (Fig. 2).

Scopolamine is excreted mainly in its conjugated form (79% *versus* 21% in unbound form), as determined after β-glucuronidase–sulfatase incubation. Assuming a total delivery of 0.45 mg of scopolamine to the circulatory system, an average of 34% (17–60%) of the administered scopolamine can be detected in the urine. Scopolamine, in very small amounts, was recovered from the urine even in the last collection period [42–48 h after the removal of the system (Fig. 2].

The use of radioligand binding techniques was originally established for studies of drug–receptor interactions *in vitro*. Radioreceptor assays were also exploited to measure concentrations of hormones (6), neurotransmitters (7, 8), or drugs (9) in biological fluids (10). Recently, such an assay for the measurement of atropine in plasma was published (11).

Table I—Intra- and Interassay Reproducibility of the Radioligand Binding Assay [a]

n	Conc. Given, ng/mL	Conc. Found, ng/mL	CV, %	Recovery, %
		Within Day		
10	2	2.3	13.7	113
10	5	5.5	5.0	109
10	10	11.7	8.0	117
10	25	27.8	7.9	111
		Day-to-Day		
11	2	2.3	32.1	114
10	5	5.7	11.3	115
20	10	10.3	12.1	103
21	25	25.6	12.6	103

[a] Scopolamine was added to the urine blank in different concentrations (2, 5, 10, and 25 ng/mL). The samples were assayed as described in the text either in one series or on different days.

The method described here was developed to determine the very low amounts of scopolamine excreted in the urine after transdermal administration of the drug. In spite of similar detection limits with this method (1.2 ng/mL) and that of Metcalfe (0.9 ng/mL) (11), scopolamine concentrations in plasma could not be measured after application of a transdermal delivery system. This could be explained by the different amounts of drug administered. While Metcalfe injected 2 mg im of atropine, the transdermal system only delivers 0.5 µg of drug/h to the blood.

The sensitivity of the radioligand binding method is only surpassed by the GC–MS technique (3), which has a detection limit of 50 pg/mL. However, this GC–MS assay has several disadvantages compared with the radioligand binding test. First, deuterated scopolamine has to be synthesized as an internal standard. Second, the method includes a complicated and time-consuming extraction procedure. Third, scopolamine is hydrolyzed to scopoline in this method, and the latter substance would be detected under the same peak. Additionally, the authors state that scopine and apo-scopolamine (possible metabolites of scopolamine) would also interfere with their assay.

The radioligand binding assay is easily and quickly performed; 50 samples can be determined in half a day, because no extraction is needed. The assay also shows good reproducibility (Table I). Measurements of the urinary excretion of scopolamine after administration of the transdermal system indicate that only 21% of scopolamine is excreted in free form, while 79% is conjugated to glucuronic and/or sulfuric acid. This confirms similar observations from animal studies (12), where the 9′-glucuronide was found to be the main metabolite of scopolamine in the mouse.

If one assumes that during a 64-h period 0.45 mg of scopolamine is absorbed through the skin, then an average of 7% of the total drug is excreted in the urine. This agrees with the results of Bayne *et al.* (3) and with those of Shaw *et al.* (13), both using a GC–MS method. Bayne *et al.* (3) recovered 4–5% of an oral dose unchanged in the urine, while Shaw *et al.* (13) measured 10% of the drug as free scopolamine given either intravenously or intramuscularly. Comparable results from this study, therefore, show the suitability of the radioligand binding technique.

Up to 48 h after removal of the drug system, scopolamine can still be detected in urine, despite its short elimination half-life of ~1 h (14). Similar results were obtained by Shaw and Urquhart (15), who assumed that an intradermal reservoir continues to deliver scopolamine to the circulation even after termination of the transdermal medication.

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Determination of 17β-Hydroxy-1α-methyl-17α-propyl-5-α-androstan-3-one in Plasma by Gas Chromatography–Mass Spectrometry with Single-Ion Detection

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Abstract □ The topical anti-androgen 17β-hydroxy-1α-methyl-17α-propyl-5α-androstan-3-one is determined in plasma samples by extracting with ether and subsequent mass fragmentography with single-ion detection at *m/z* 303. 17β-Hydroxy-1α-methyl-17α-pentyl-5α-androstan-3-one, added to the samples before extraction, is used as the internal standard. Reproducibility was calculated to be ±5.9% at the 5-ng/mL level and 0.4% at the 20-ng/mL level. The limit of detection is ~1 ng/mL. Total gas chromatography–mass spectrometry analysis time is ~10 min/sample.

Keyphrases □ 17β- Hydroxy -1α-methyl-17α- propyl -5α- androstan -3-one—determination in plasma, gas chromatography—mass spectrometry, single-ion detection □ Mesterolone—17α-propyl analogue, determination in plasma, gas chromatography–mass spectrometry, single-ion detection

17β-Hydroxy-1α-methyl-17α- propyl -5α-androstan-3-one, the 17α-propyl analogue of mesterolone, is currently under investigation as a topical anti-androgen for the treatment of acne and seborrhea. Pharmacological data of this compound, which may act similarly to the 17α-propyl analogue of testosterone (1), have been reported earlier (2).

Topical anti-androgens act locally in the skin, and the systemic availability of this class of compounds should be as low as possible in order to avoid endocrinological disorders. Therefore, any analytical method applied to the determination of these drugs in plasma must be very sensitive to be able to detect very small portions of absorbed substances. Because the drug molecule may be metabolized during passage through the skin, there are also certain requirements regarding specificity of the assay. In the present report an analytical method for the determination of plasma concentrations of 17β-hydroxy-1α-methyl-17α-propyl-5α-androstan-3-one (I) is described, which is devoid of tedious extraction or derivatization procedures and may, therefore, be useful in clinical tolerance studies of this compound when relatively high doses of drug are administered. Furthermore, it may serve for controlling the specificity of an RIA procedure.

EXPERIMENTAL

Subjects and Medication—Two healthy male volunteers (40 and 31 years of age, and 88 and 70 kg, respectively) were treated topically two times daily with 10 mL of a 3% solution of I in 70% aqueous ethanol. The drug was spread over the total body of the volunteers. During the first week the skin area to which the drug has been applied was kept under occlusion for 22 h/d. Blood was drawn into heparinized syringes before the treatment and in weekly intervals up to 8 weeks after the beginning of the 6-week treatment. The samples were immediately centrifuged, and the plasma stored frozen until analysis.

Chemicals—All solvents (ethanol, *n*-hexane, and ether) were of analytical reagent grade[1] and were used without further purification.

Extraction Procedure—Fifty nanograms of the internal standard (17β-hydroxy-1α-methyl-17α-pentyl-5α-androstan-3-one) dissolved in 50 μL of *n*-hexane was transferred into a 15-mL stoppered test tube and evaporated to dryness under a stream of nitrogen. One milliliter of plasma was added, followed after a short mixing time by 3 mL of ether. After thoroughly mixing on a vortex mixer for 1 min and centrifugation at 1200×*g* for 5 min, the organic phase was transferred to another test tube and evaporated to give a final volume of ~10 μL. One microliter of this solution was analyzed by gas chromatography–mass spectrometry (GC–MS). The recovery of the extraction procedure was determined by spiking 1-mL plasma samples with either 20 or 50 ng of radiolabeled I, with subsequent determination of the radioactivity in the organic phase.

Gas Chromatography–Mass Spectrometry—A GC–MS system[2] with the following components was used: a gas chromatograph[3], a quadrupole mass spectrometer[4], and a data system[5]. The gas chromatograph was equipped with a CP-SiL 5-coated open-tubular glass column (25 m × 0.25 mm) used in the direct-coupling mode or, alternatively, a CP-SiL 5 CB wall-coated open-tubular fused silica column (25 m × 0.23 mm), which was directly introduced into the ion source.

The temperature program applied to the column was 240°C for 1 min, then rising to a final temperature of 260°C at a rate of 10°C/min. Using the fused silica column the program was 200°C for 2 min, then 10°C/min up to 300°C. The injector was kept at 290°C, the direct coupling device at 280°C. Helium was used as carrier gas with a pressure of 14 psi in front of the column. The split ratio was 1:5.

Calibration Curve—The standard curve was constructed with 1-mL blank plasma samples containing 0, 5, 10, 15, 20, and 50 ng of I and 50 ng of the internal standard. These samples were extracted by the method described above. The peak areas of the internal standard and drug were measured, and the calibration curve (peak area ratio of drug/standard *versus* the concentration of I) was constructed. The precision of the assay was calculated from consecutive determinations of the drug within 1 d and by comparison with the results obtained on different days.

[1] E. Merck, Darmstadt, West Germany.
[2] Finnigan 4021.
[3] Finnigan 9610.
[4] Finnigan 4000.
[5] Incos-type 2100.

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