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Distribution and metabolism of topically applied progesterone in a rat model

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Abstract

This study investigated the transdermal uptake and subsequent tissue distribution of [^{3}H]progesterone applied in a commercially available progesterone cream in a rat model. Concentrations of lipid- and water-soluble metabolites of [^{3}H]progesterone were also measured in plasma, urine and selected tissues (uterus, liver, kidney, salivary gland) 3 h after its topical application. Female rats were ovariectomized and adrenalectomized to remove all endogenous progesterone, and 4 weeks later were anaesthetized and 150 mg Pro-Feme® cream (containing progesterone 3.2% w/w and 200 μCi [^{3}H]progesterone) was applied to the abdominal skin. Six arterial blood samples were then obtained from a carotid cannula over the following 3 h, and urine and selected tissue samples were collected after the final blood sample. Plasma progesterone increased progressively until 90 min, then remained relatively stable. Plasma levels of [^{3}H]progesterone were high by the 15-min sample and increased only slightly thereafter. Water-soluble metabolites were detectable in plasma at 15 min, whereas lipid-soluble metabolites became apparent only by 60 min then increased progressively to 180 min. The tissue:plasma concentration ratio for [^{3}H]progesterone exceeded 1 in all tissues, most notably in uterus (8.4) and lung (9.6), whereas urinary [^{3}H]progesterone levels were only half those in plasma. Concentrations of lipid- and water-soluble progesterone metabolites were most prevalent in liver and kidney, and both reached very high concentrations in urine. These results demonstrate that topically applied progesterone is rapidly absorbed transdermally and that its patterns of distribution and metabolism are comparable to those previously reported for intravascularly administered progesterone.

Keywords: Progesterone; Hormone replacement therapy; Topical cream; Transdermal uptake; Steroid metabolism

1. Introduction

The use of combined synthetic estrogens and progestins is common to the majority of current hormone replacement therapy (HRT) regimens [1,2]. Estrogens can relieve menopausal symptoms related to vasomotor instability, urogenital atrophy and psychosomatic complaints [3,4], prevent bone loss and reduce the risk of coronary heart disease [4]. The addition of progestin therapy opposes estrogen action to protect against endometrial and other forms of gynecological cancers, although the risk of breast cancer remains slightly increased [4]. Progestins may also be beneficial by promoting bone formation either directly or by buffering the osteoporotic effects of glucocorticoids [5,6]. On the other hand, synthetic progestins can be mildly androgenic and exhibit unpleasant side effects [6,7], and so the use of 'natural' progesterone has been promoted as an alternative component of HRT [8,9]. However, the very low uptake and rapid clearance of orally administered progesterone has precluded its general use in favor of synthetic progestins [10,11]. Alternative administration regimens for progesterone include intramuscular injection, vaginal suppositories and as a topical cream [12]. Several studies indicate that although topically applied progesterone is absorbed transdermally [13,14] and appears to exert biological effects on the endometrium [15] and vasomotor activity [16], there remains considerable uncertainty as to the resultant progesterone levels in target tissues [17,18]. For example, although salivary progesterone levels were elevated in both pre- and post-menopausal women following application of topical progesterone, no change in plasma progesterone levels was detected [19]. Subsequently, Carey et al. [20] reported significant increases in plasma progesterone and urinary pregnanediol-3-glucuronide following topical application of progesterone cream, but these changes were relatively small and appeared quite variable. Thus, although topically applied progesterone appears to exert biological activity, there remains uncertainty about its pattern of uptake, metabolism and subsequent tissue distribution. In the present study, therefore, we used an

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experimental rat model to follow the uptake and metabolism of topically applied progesterone. Rats were first adrenalectomized and ovariectomized to remove endogenous progesterone and estrogen, and cream containing both authentic progesterone and [^{3}H]progesterone was applied to the skin. Concentrations of progesterone, [^{3}H]progesterone and its [^{3}H]metabolites were determined in sequential blood samples obtained over the next 3 h, and in selected tissues and urine collected after the final blood sample.

2. Materials and methods

2.1. Animals and chemicals

Nulliparous albino Wistar rats, 3–5 months old and weighing 242 ± 14 (mean ± S.E.) on the day of experimentation, were obtained from the Animal Resources Centre (Murdoch, WA, Australia). Housing management and conditions were as previously described [21], with a 14:10 h light:dark cycle. Four weeks prior to experimentation each rat was adrenalectomized and ovariectomized via two dorso–lateral incisions under halothane:nitrous oxide anesthesia. Thereafter, rats were provided with normal saline (0.9% NaCl) for drinking, and 1 week prior to the experiment this was supplemented with dexamethasone acetate (0.25 μg/ml) to replace glucocorticoid. Topical progesterone cream (Pro-Feme® cream containing 3.2% w/w progesterone) was obtained from Lawley Pharmaceuticals (Mt. Lawley, Australia). Progesterone and 20α-hydroxypregn-4-en-3-one (20α-OHP) were obtained from Sigma (St. Louis, MO, USA). [1,2,6,7-^{3}H]progesterone (specific activity 81 Ci/mmol) and [4-^{14}C]progesterone (specific activity 55 mCi/mmol) were obtained from Amersham Australia (Sydney, NSW, Australia). The [^{3}H]progesterone was re-purified immediately prior to experimental use by thin-layer chromatography (TLC) (see below). TLC plates precoated with silica gel 60 F_{254} were supplied by Merck (Darmstadt, Germany).

2.2. Application of progesterone cream and collection of blood and tissue samples

Rats were anesthetized with pentobarbitone sodium (40 mg/kg i.p. maintained with hourly doses of 10 mg/kg s.c.) and the entire ventral skin surface exposed by carefully clipping all fur. The rat was placed on a warming blanket to maintain body temperature and a polyethylene cannula (Dural Plastics, Dural, NSW, Australia; 0.5 mm i.d., 0.8 mm o.d.), filled with 20 IU/ml heparinized saline, inserted into the left carotid artery as previously described [22]. After a brief stabilization period (15–20 min), 150 mg Pro-Feme® cream containing approximately 200 μCi [^{3}H]progesterone was applied evenly to the entire ventral abdominal area. The [^{3}H]progesterone had been added to the cream in 20 μl saline and mixed thoroughly immediately prior to application. Arterial blood samples were collected via the cannula into heparinized tubes at 15, 30, 60, 90, 120 and 180 min after cream application. Samples were immediately centrifuged at 13,000 × *g* for 5 min, and plasma stored at −20 °C until analysis. To maintain blood volume, heparinized whole blood from a male rat, equivalent in volume to that removed for sampling, was administered via the cannula after every second sample. After collection of the final blood sample the abdomen and thorax were opened and samples of urine, uterus, liver, kidney, lung and submaxillary salivary gland were collected, snap frozen on liquid nitrogen, and stored at −20 °C until analysis.

2.3. Analysis of blood and tissue samples

Concentrations of progesterone were determined by radioimmunoassay in all plasma samples using a Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA). All samples were measured in a single assay and the intra-assay coefficient of variation was 3.6%. Blood and tissue concentrations of total tritium, lipid-soluble tritium, [^{3}H]progesterone and [^{3}H]20α-OHP were determined as previously described [23]. Briefly, samples of plasma (0.25 ml) or urine (0.5 ml) were extracted with 3.0 ml chloroform:methanol (1:2) and sufficient water to yield a solvent ratio of 1:2:0.8 for chloroform:methanol:water. Procedural losses were monitored by the addition of approximately 500 cpm [^{14}C]progesterone. Total tritium content (i.e. [^{3}H]progesterone and its lipid- and water-soluble metabolites) of each sample was estimated by counting 0.5 ml of this initial mixture. The lipid-soluble phase was separated by the addition of 0.8 ml chloroform and lipid-soluble tritium estimated from an aliquot of this phase. The remainder of the lipid-soluble phase was applied to a TLC plate with 5 μg each of authentic progesterone and 20α-OHP, and samples chromatographed using dichloromethane:diethyl ether (5:2) as solvent system to isolate progesterone and 20α-OHP fractions. We have previously demonstrated that this single TLC system is sufficient to achieve radioisotope homogeneity of both [^{3}H]progesterone [24] and [^{3}H]20α-OHP [25] in arterial blood of pregnant rats. The ^{3}H and ^{14}C contents in all aliquots and eluates were determined by liquid scintillation spectrometry, with sufficient counts accumulated to ensure a counting error of less than 2%. Tissue samples (approximately 100 mg) were homogenized using an Ultra-Turrax homogenizer (Janke and Kunkel, Germany) in 3.0 ml chloroform:methanol (1:2) and sufficient water to yield a final solvent ratio of 1:2:0.8 for chloroform:methanol:water. The resultant extract was then processed as described for plasma and urine samples.

2.4. Statistical analyses

Changes in arterial plasma concentrations of authentic progesterone, [^{3}H]progesterone and [^{3}H]lipid- and water-soluble metabolites over time were assessed by repeated measures analysis of variance (ANOVA) and least

significant difference (LSD) tests. Among tissue comparisons for [^{3}H]progesterone and [^{3}H]lipid- and water-soluble metabolite concentrations were made by one-way ANOVAs and LSD tests.

3. Results

3.1. Plasma concentrations of progesterone

Plasma progesterone concentrations prior to application of topical progesterone cream were extremely low (2.0 ± 0.8 nmol/l), consistent with the absence of ovaries and adrenals in these animals. Plasma progesterone then increased over time following cream application ($P < 0.001$, ANOVA), although a significant increase from time zero was not observed until 60 min (10.5 ± 1.6 nmol/l; $P < 0.01$, LSD test). Plasma progesterone then increased progressively to a maximum of 19.2 ± 3.4 nmol/l by the final sample (see Fig. 1).

3.2. Plasma concentrations of ^{3}H-progesterone

[^{3}H]progesterone was readily detectable in plasma obtained 15 min after application of progesterone cream and thereafter, although concentrations varied significantly ($P < 0.05$, ANOVA), these changes were relatively minor (see Fig. 2). Due to more rapid uptake of [^{3}H]progesterone relative to authentic progesterone, the specific activity of [^{3}H]progesterone in plasma peaked at the 15-min sample (175 ± 47 cpm/nmol), declined to 50 ± 10 cpm/nmol at 90 min ($P < 0.01$, ANOVA) and stabilized thereafter (40 ± 7 cpm/nmol at 180 min). In contrast, the total tritium concentration in plasma, which includes [^{3}H]progesterone and its tritiated metabolites, increased progressively ($P < 0.01$) after the 30 min blood sample (see Fig. 2). Thus, while [^{3}H]progesterone accounted for more than 75% of the total tritium radioactivity detectable in plasma at 15 min, this proportion fell to only 42% by 180 min, indicative of accumulation of progesterone metabolites in plasma.

3.3. Plasma concentrations of lipid- and water-soluble progesterone metabolites

Lipid-soluble metabolites of [^{3}H]progesterone in plasma were not detectable (i.e. did not differ significantly from zero, Paired *t*-test) until the 30-min sample time. Thereafter, lipid-soluble metabolites increased progressively ($P < 0.01$) to peak at the final sample (180 min). Water-soluble metabolites, however, were readily detectable at 15 min, then remained relatively stable before increasing by the final sample (see Fig. 3).

3.4. Tissue concentrations of ^{3}H-progesterone and its metabolites

The tissue concentration of [^{3}H]progesterone 3 h after cream application exceeded its plasma concentration for all tissues analyzed, and this was most notable in uterus, lung and kidney (see Table 1 and Fig. 4). In contrast, the concentration of [^{3}H]progesterone in urine was only half that in plasma. Total tritium radioactivity was also higher ($P <$

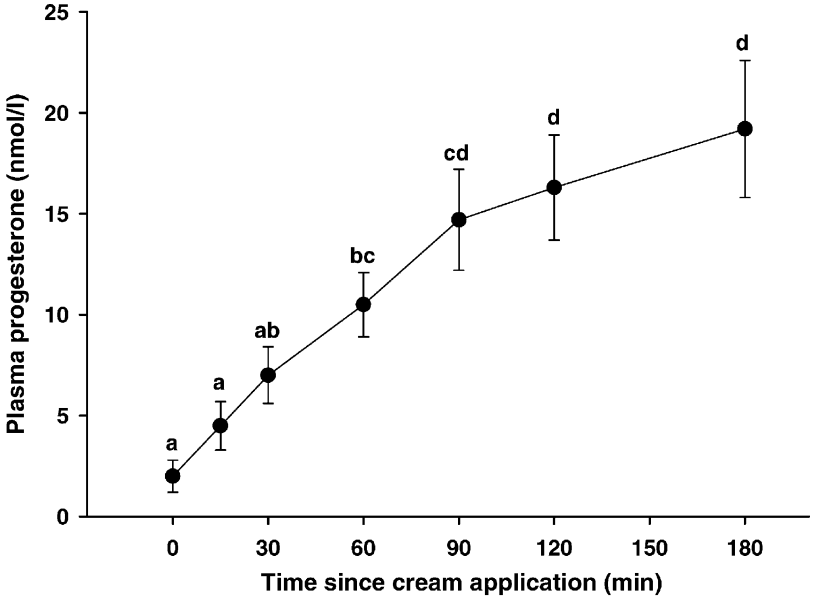

Fig. 1. Plasma concentrations of progesterone during the 3-h period following application of topical progesterone cream containing [^{3}H]progesterone. Values are the mean ± S.E. ($n = 5$). Cream was applied to anesthetized rats and arterial blood samples obtained sequentially via a carotid cannula. Progesterone concentrations were measured by RIA. Plasma progesterone levels varied significantly with time ($P < 0.001$, ANOVA) and data points without shared notations (a, b, c or d) differ significantly ($P < 0.05$, LSD test).

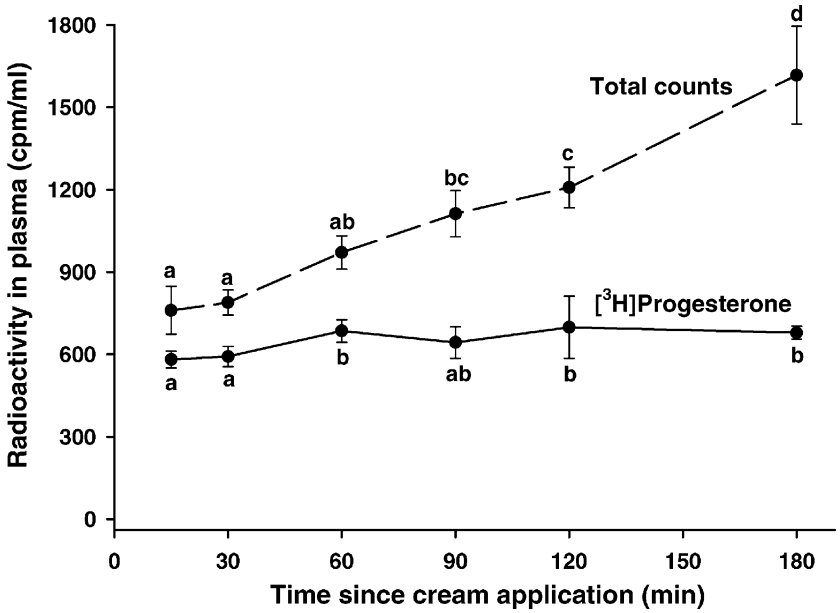

Fig. 2. Plasma concentrations of [^{3}H]progesterone during the 3-h period following application of topical progesterone cream containing [^{3}H]progesterone. Values are the mean ± S.E. ($n = 5$). Cream was applied to anesthetized rats and arterial blood samples obtained sequentially via a carotid cannula. [^{3}H]progesterone was quantitated following isolation from extracted plasma by TLC. There was significant variation with time for both total tritium ($P < 0.01$, ANOVA) and [^{3}H]progesterone ($P < 0.05$), and for each profile those data points without shared notations (a, b, c or d) differ significantly ($P < 0.05$, LSD test).

0.01, Unpaired *t*-tests) in all tissues compared with plasma, but there were considerable differences in the distribution of this accumulated radioactivity (see Fig. 4). In the uterus, the clear majority of tritium was present as progesterone (93 ± 7%), with no water-soluble metabolites detectable and only relatively low levels of lipid-soluble metabolites. Most tritium was also attributable to progesterone in both the lung (83 ± 5%) and salivary gland (60 ± 8%), whereas [^{3}H]progesterone accounted for only 5.1 ± 0.5% of total tritium in the urine. Most of the urinary tritium was due to comparable amounts of lipid- and water-soluble metabolites. High concentrations of [^{3}H]progesterone were evident in the

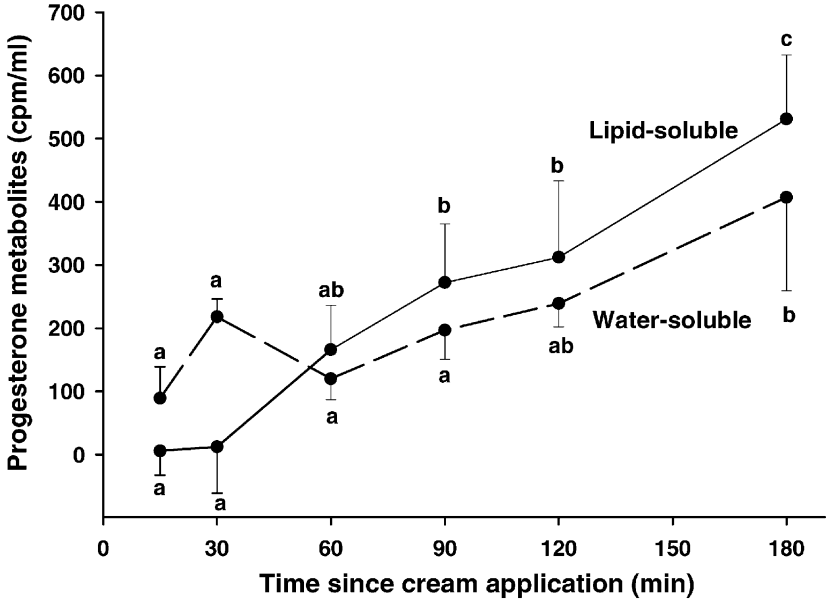

Fig. 3. Plasma concentrations of lipid- and water-soluble metabolites of [^{3}H]progesterone during the 3-h period following application of topical progesterone cream containing [^{3}H]progesterone. Values are the mean ± S.E. ($n = 5$). Lipid- and water-soluble metabolites were derived from the total tritium, lipid-soluble tritium and [^{3}H]progesterone concentrations. There was significant variation with time for both lipid- and water-soluble metabolite concentrations ($P < 0.01$, ANOVA), and for each profile those data points without shared notations (a or b) differ significantly ($P < 0.05$, LSD test).

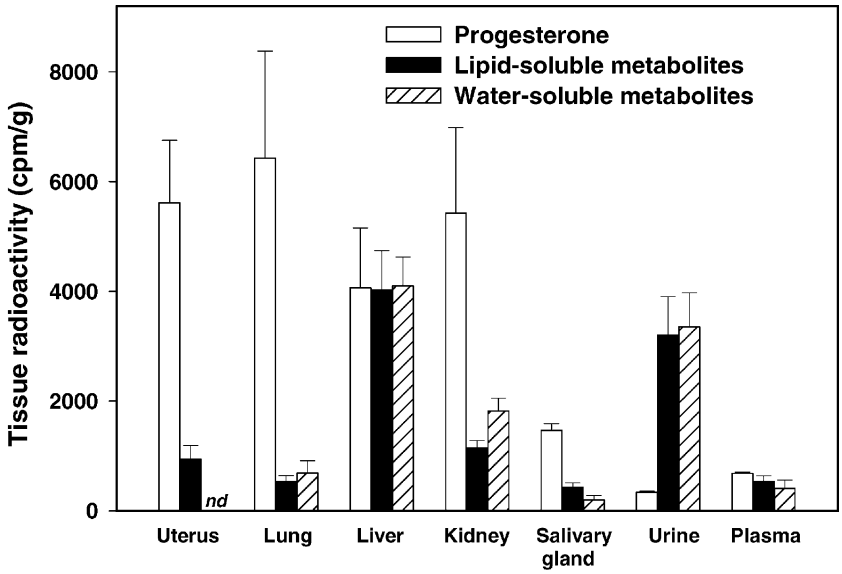

Fig. 4. Concentrations of [^{3}H]progesterone and its lipid- and water-soluble metabolites in various tissues and urine obtained 3 h after application of topical progesterone cream containing [^{3}H]progesterone. Values are the mean ± S.E. ($n = 5$). Tissue samples were obtained immediately after collection of the final blood sample and snap frozen in liquid nitrogen. [^{3}H]progesterone was quantitated following isolation from extracted homogenized tissues by TLC, and lipid- and water-soluble metabolites were derived from the total tritium, lipid-soluble tritium and [^{3}H]progesterone concentrations; nd: not detectable.

Table 1
Tissue:plasma ratio of [^{3}H]progesterone in tissues 3 h after application of topical progesterone cream containing [^{3}H]progesterone

Tissue	[^{3}H]Progesterone tissue:plasma ratio
Uterus	8.36 ± 1.82[a]
Lung	9.63 ± 3.01
Salivary gland	2.18 ± 0.23
Liver	8.02 ± 2.38
Kidney	5.82 ± 1.37
Urine	0.49 ± 0.04

[a] Values are the mean ± S.E. ($n = 5$).

liver and kidney, and these tissues also exhibited high concentrations of lipid- and water-soluble-metabolites, consistent with their known capacity for progesterone metabolism.

4. Discussion

This study demonstrates that in a rat model, topically applied progesterone is absorbed transdermally and then distributed and metabolized in essentially the same manner as progesterone that enters the blood directly. The immediate and progressive increase in plasma progesterone suggests that transdermal uptake begins effectively, immediately upon cream application, and continues over at least the following 3-h period. Moreover, topically applied [^{3}H]progesterone accumulated in various tissues, including progesterone target tissues, at concentrations well in excess of plasma levels, consistent with it being physiologically active. Therefore, our results suggest that topical application of progesterone cream is a potentially viable option for progesterone therapy.

The rapid and progressive increase in plasma progesterone concentrations to a maximum of around 20 nmol/l following application of topical progesterone cream clearly shows that progesterone is absorbed into the blood transdermally. This contrasts with a recent report in which women receiving topical progesterone in the same cream formulation used in this study did not show any significant rise in plasma progesterone. This was despite a clear increase in salivary progesterone 30 min after cream application [19]. This apparent discrepancy likely reflects the differences in relative dose used in the two studies. In addition, removal of effectively all endogenous progesterone by ovariectomy and adrenalectomy prior to experimentation in the rat model clearly facilitated the detection of increased plasma progesterone levels.

The initial rate of [^{3}H]progesterone entry into blood was more rapid than that of authentic progesterone, presumably due to it being entirely in solution within the cream. Indeed, the increase in plasma [^{3}H]progesterone to near maximal levels by 15 min is similar to the rapid appearance and stabilization of exogenous progesterone in rat plasma following subcutaneous injection of progesterone in oil [21]. In contrast, because progesterone is added in micronized form during manufacture of the Pro-Feme® cream, much of it likely remains in crystalline form. If so, the proportion of the applied [^{3}H]progesterone dose absorbed should be greater than that of authentic progesterone, and this does indeed appear to be the case. Thus, based on a steady state [^{3}H]progesterone plasma concentration of 680 cpm/ml and a metabolic clearance rate (MCR) of 27 ml/min for progesterone in anaesthetized rats [24], the uptake rate of [^{3}H]progesterone was 3.3×10^6 cpm over the measurement period, or around 1.6% of the total

applied dose (based on uptake rate (cpm/min) = MCR (27 ml/min) × plasma concentration (680 cpm/ml) [26], applied dose = 2×10^8 cpm). The uptake rate of authentic progesterone, on the other hand, was 97 nmol over the 180 min measurement period (based on the apparent plateau of 20 nmol/l in Fig. 2), or only 0.6% of the applied dose of 15 μmol. Importantly, this relatively slow but sustained transdermal uptake of authentic progesterone from topical cream may be considered ideal in a clinical setting.

Despite the more rapid uptake of [^{3}H]progesterone, its subsequent distribution and metabolism suggest that progesterone entering the blood via the transdermal route behaves essentially the same as endogenously secreted progesterone. Specifically, in tissues obtained 180 min after application of progesterone cream containing [^{3}H]progesterone, the relative concentrations of [^{3}H]progesterone and its lipid- and water-soluble metabolites were similar to those measured after intravenous constant infusion of [^{3}H]progesterone [23,27]. Constant infusion of labeled steroids is thought to mimic secretion of endogenous steroids, and is thus the conventional approach for measurement of steroid distribution and metabolism [26]. The key features of the distribution of transdermally absorbed [^{3}H]progesterone were high levels of [^{3}H]progesterone relative to its metabolites in uterus, lung and salivary gland. High concentrations of [^{3}H]progesterone (relative to plasma) were also observed in the liver and kidney, but these tissues also had high concentrations of lipid- and water-soluble metabolites, particularly the liver, consistent with their important roles in progesterone metabolism [26,28]. Urine, on the other hand, had low relative concentrations of [^{3}H]progesterone (approximately half that of plasma), but high levels of lipid- and water-soluble metabolites, consistent with their high levels in kidney.

The marked accumulation of [^{3}H]progesterone evident in all tissues was most notable in the uterus and lung where its concentration exceeded that in plasma by more than eight-fold. We have previously reported a similar retention of [^{3}H]progesterone in the lung of pregnant rats during its constant infusion [27]. This retention most likely reflects the high lipid solubility of these tissues as well as the presence of progesterone receptors. High tissue concentrations of [^{3}H]progesterone (relative to plasma) are also consistent with the observation that increases in salivary, but not plasma, progesterone levels were detected after application of progesterone cream in humans [19].

The collection of sequential blood samples following cream application enabled assessment of how quickly lipid- and water-soluble metabolites of [^{3}H]progesterone appeared in plasma. Predictably, concentrations of both metabolite fractions increased to maximal levels at the final sample (180 min). The initial appearance of the two fractions in plasma, however, differed considerably, with water-soluble metabolites readily detectable at 15 min then showing a transient peak at 30 min. Then the subsequent decline in this fraction was associated with the first appearance of lipid-soluble metabolites at 60 min. These patterns suggest that progesterone metabolism initially involves conjugation in the liver, the products of which are subsequently hydrolyzed, most likely in the gut [29], thus leading to the delayed increase in the lipid-soluble fraction in plasma. [^{3}H]progesterone, on the other hand, was close to maximal levels by the 15 min sample, and showed only a slight increase thereafter. This suggests that [^{3}H]progesterone quickly reached a steady-state, with its transdermal uptake and subsequent entry into plasma equivalent to its irreversible exit via metabolism and excretion.

In conclusion, the present study establishes that topically applied progesterone is absorbed effectively immediately into the blood via a transdermal route. Its subsequent pattern of tissue distribution and metabolism appears to be essentially the same as that for progesterone administered intravascularly, and thus endogenously secreted progesterone. The accumulation of [^{3}H]progesterone in several tissues, most notably the uterus, after topical application is consistent with transdermally absorbed progesterone being biologically active.

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