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Short communication

Alcohols which have been in contact with any plastics may interfere in radioimmunoassays of progesterone

Rok Ocvirk[a,b], Jennifer M. Bisson[b], Beverley E. Pearson Murphy[a,b,c,*]

[a] Department of Experimental Medicine, McGill University, 1110 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1
[b] Department of Psychiatry, McGill University, 1033 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1
[c] Department of Medicine, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec, Canada

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ABSTRACT

In recent years there has been increasing use of plastic rather than glass containers for many liquids, including wine. However we have found that residue from commercially obtained 'pure' ethanol dispensed in plastic bottles interferes in some biochemical assays. We have observed a volume-dependent decrease in maximally bound ligand in radioimmunoassays of progesterone. The resulting shift in the standard curve leads to an underestimation of the analyte concentrations and to altered estimation of cross reactivity by competing ligands. These effects became apparent in assays with high sensitivity (500 pg or less). All sources of ethanol obtainable in Quebec contained impurities. A similar effect was also produced by 'pure' methanol. The reduction in maximally bound ligand was amplified when the alcohol was aliquoted using plastic pipette tips. We conclude that alcohols which have had any contact with plastics are not safe to use in immunoassays of progesterone (or its metabolites as estimated according to cross-reactivity after HPLC) and may affect other assays. If the use of alcohol and plastic tips cannot be avoided, the amount of alcohol used should be reduced to 1% or less. This can be accomplished by preparing steroid standards in assay buffers containing albumin or gelatin, which enhance the solubility of steroids in aqueous media.

1. Introduction

Steroids such as progesterone and its metabolites such as pregnanolone and allopregnanolone are highly potent neuroactive compounds [1,2] which have been shown to be implicated in pathophysiological conditions such as depression [3–7] and premenstrual dysphoric disorder [8–9]. The concentrations of neuroactive steroids found in brain tissue and blood are relatively low, in the order of pmol/L [10–12]; data in the literature vary considerably [7,13], and further work is required to elucidate their physiological relevance.

The methods most commonly used to assay neuroactive steroids are gas chromatography–mass spectrometry (GC–MS), and radioimmunoassay (RIA). GC–MS tends to have poorer sensitivity and requires that the analytes be derivatized. While RIA techniques are more sensitive, the measurement is indirect, the identity of the analyte cannot be positively confirmed, and the measured values may be overestimated due to the presence of cross-reacting substances [14].

We have used a method employing high-performance liquid chromatography coupled with RIA to measure progesterone and five of its metabolites, with a limit of detection for progesterone of 20 pg [6,7,11]. A tritiated steroid is typically added to the sample to track recovery through extraction and chromatographic separation. A standard curve is prepared for quantitation of the HPLC fractions in each assay. Due to relatively poor solubility of steroids, these are usually dissolved in ethanol or methanol, serially diluted and aliquoted in glass assay tubes. The solvent is then evaporated and the standards are treated in the same way as the HPLC fractions [7,11,13]. With increasing sensitivity of competitive binding assays, we have encountered peculiarities pertaining to the choice of solvent, and the amount of solvent used in preparation of steroid standards. Methanol and ethanol are often the solvents of choice, with ethanol being preferred due to its safety.

Traditionally, to ensure purity, freshly redistilled ethanol stored in glass containers was used to dissolve steroids. It has been known for over 50 years, however, that most plastic materials dissolve in alcohols and that, in order to keep alcohols pure, any contact with plastics should be avoided [15]. In the 1980s, laboratory stills became illegal in Canada, and high grade ethanol was only

* Corresponding author at: Montreal General Hospital, Room C6-260, 1650 Cedar Avenue, Montreal, Quebec, Canada H3G 1A4. Tel.: +1 514 726 9434; fax: +1 514 934 8361.
E-mail address: bev.murphy@mcgill.ca (B.E.P. Murphy).

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available from laboratories with special permits, in that case we took our own glass bottles to be refilled.

In recent years there has been the increasing use of plastic rather than glass containers for many liquids. Plastic bottles weigh only about 15% as much as glass, greatly reducing transportation costs. At present, ethanol is only available in Quebec from one source and it is illegal to ship it from elsewhere. In the manufacturing process it is briefly exposed to plastic tubing and is then dispensed mainly in 4 L plastic containers with a one-year expiry date, but can also be purchased in 500 mL glass bottles which do not have an expiry date.

We report here the effects of ethanol and methanol residues on the RIA of progesterone, and of the effects of aliquoting alcoholic steroid standards using plastic pipette tips.

2. Materials and methods

2.1. Materials

All sources of ethanol originated from Commercial Alcohols Inc. (Brampton, Ontario, Canada). They were purchased from McGill University Biostores (anhydrous and 95%, in plastic containers) and from the Société des Alcools du Québec (SAQ) (95% in glass bottles). Methanol (HPLC grade) was obtained from Fisher Scientific, Canada in glass bottles. We were assured that the anhydrous ethyl alcohol "conforms with all U.S. (USP), British (BP), European (EP), and Japanese (JP) Pharmacopoeias and Food Chemicals Codex (F.C.C.) standards". Disposable autoclavable, polypropylene pipette tips used were 1 mL (blue, non-sterilized) and 100 μL (yellow, sterilized and non-sterilized) from Fisher Scientific, Canada. Glass syringes (Hamilton, 10 and 50 μL) were purchased from Fisher Scientific, Canada.

Progesterone was purchased from Steraloids (NH, USA). Tritiated progesterone (*P; [1,2,6,7-3H](N), 90–115 Ci/mmol) was purchased from PerkinElmer (MA, USA). Antiserum to progesterone was raised in rabbits using 4-pregnene-3,20-dione-3-*o*-carboxymethyloxime:bovine serum albumin (Steraloids, NH, USA) as antigen, along with Freund's adjuvant, as described previously [11].

Dextran-coated charcoal solution (DCC) was prepared by dissolving 0.125 mg dextran T-70 (JT Baker, NJ, USA), 1.25 mg Norit A charcoal (Fisher Scientific, Canada) and 0.01 g of sodium azide (Fisher Scientific, Canada) in 1 L of 10% phosphate buffer (0.1 M) in distilled and deionized water.

The equipment used in these studies which came into contact with solutions containing any alcohol, was glass, steel or teflon unless otherwise stated.

2.2. Radioimmunoassays

RIAs were performed in 13 mm × 100 mm glass test tubes (Fisher Scientific, Canada). Empty assay tubes, ethanol, and progesterone standards in ethanol were dried under a gentle stream of air or nitrogen (no difference was noted). The protein-tracer solution (PTS) was prepared by adding antiserum and tracer to phosphate buffer (0.1 M, pH 7.0) containing gelatin (0.5%, w/v). The final concentration of progesterone antiserum was 1/5000 (except in Experiment 1, where titres used were 1/5000, 1/500 and 1/50). PTS was vortexed and briefly incubated at room temperature for equilibration, then aliquoted to assay tubes for a final reaction volume of 100 μL. In each assay, total counts (TC), B_0 (no analyte) and non-specific binding (NSB) controls were included. The NSB controls contained 1000 times more progesterone than the highest amount in the standard curve. In assays containing progesterone standards, additional controls were used, containing solvent only, without any steroid (B_s). Assay tubes were shaken for 1 min, warmed to 36 °C for 10 min, shaken again and incubated at 4 °C for approximately one hour.

The reaction was terminated by adding 1 mL of cold (4 °C) DCC solution to each assay tube (except TC which received cold distilled and deionized water). After 4 min, the tubes were shaken for 1 min and then transferred to a centrifuge. After a 5-min centrifugation (850 × *g* at 4 °C), 0.5 mL of the supernatant was removed, transferred to scintillation vials, and 2 mL of scintillation solution (ScintiSafe 30%, Fisher Scientific, Canada) was added for counting. Although not detailed here, each of the experiments was carried out independently by two different technical personnel with similar results.

2.3. The 'hook' effect in the standard curve

We were not at first aware that our ethanol source had changed since our own glass bottles were refilled at the McGill Biostores from large steel tanks (later deemed to be too large to be safe). After the tanks were replaced by 4 L plastic bottles, we were puzzled by noting 'hooks' in our standard curves, done in duplicate, which usually consisted of aliquots from two solutions: 20, 40 and 80 μL of 1 ng/mL, and 16, 32, 64 and 128 μL of 10 ng/mL progesterone to give a curve ranging from 20 to 1280 pg.

2.4. Change with increasing sensitivity

Three sets of progesterone standard curves were prepared (A, 3.2–100 ng; B, 320 pg–10 ng; C, 32 pg–1 ng). Each set consisted of two standard curves in duplicate, one prepared using plastic pipette tips, and the other using glass Hamilton syringes. Progesterone was dissolved in anhydrous ethanol (from plastic containers) and solutions containing the desired amount of steroid per 50 μL ethanol were prepared by serial dilution. The standards were then aliquoted into the glass assay tubes using disposable 100 μL plastic pipette tips or 50 μL Hamilton syringes. The different standard curves were assayed by RIA with proportional amounts of reagents. Standard curve A was assayed with an antiserum titer of 1/50 and amount of *P equivalent to approximately 3×10^6 dpm/assay tube. Curves B and C were assayed with reagent concentrations 1/10 and 1/100 those of assay A, respectively. Each assay included solvent-only controls (B_s; 50 μL ethanol).

2.5. Effects of different batches of ethanol

Three sources of ethanol were tested for solvent effect in RIA: Anhydrous and 95% (Commercial Alcohols, Canada) and 95% (SAQ). The same volumes of methanol (Fisher Scientific, Canada) were also assayed. Containers were opened immediately prior to the experiment. Aliquots from each source – 100 and 400 μL – were pipetted in quadruplicate into assay tubes, using glass volumetric pipettes. Four empty assay tubes were included as B_0 controls. Samples were evaporated and RIA was performed.

2.6. Effects of pipetting using disposable plastic tips

Anhydrous ethanol 50 μL was pipetted in quadruplicate into tubes using sterilized and non-sterilized 100 μL pipette tips or a 50 μL glass syringe. Empty assay tubes were included as B_0 controls.

2.7. Different ways of preparing standard curves

Standard curves (each in duplicate) were prepared by pipetting known amounts of progesterone dissolved in RIA buffer [16] containing only 1% ethanol, or ethanol alone. Standards prepared in RIA buffer were aliquoted in 50 μL volume. Those in ethanol were prepared by aliquoting the same amounts of progesterone in 10 and 50 μL anhydrous ethanol.

2.8. Data analysis

The mean NSB in each assay was subtracted from raw counts per minute (cpm), which were then expressed as % bound in the absence of any analyte or solvent (%B/B_0). Where different treatments were employed, the results were analysed by analysis of variance (ANOVA).

3. Results and discussion

3.1. The hook effect

An example of the distortion of the standard curve which first drew our attention to the effects of plastics residues in alcohol is shown in Fig. 1. In addition to the displacement of the tracer by progesterone, the effects of the impurities in the alcohol are superimposed on the standard curve. This results in a hook which becomes apparent between points of the curve where different stock solutions are used, and the volume of ethanol used changes dramatically between adjacent points. After observing such hooks repeatedly, we tested varying amounts of ethanol alone and observed that a curve with increasing volumes of ethanol is produced in the absence of any steroid, as shown in Fig. 2. The batch tested in Fig. 2 had minimal effects due to 25 μL of ethanol but the effect of increasing volumes mimicked a standard curve.

Neurosteroids measured with the same antiserum, which cross-react less than 100% compared with progesterone are overestimated to an even greater degree since the contribution by the impurities is inflated when the amount measured is adjusted for cross-reactivity. Such an error occurred in our determinations of cross-reactivities for various neurosteroids published previously [11]. Overestimated cross-reactivities led to underestimations of the concentrations of these compounds. Although statistical significance found between groups is not affected by linear transformations and the findings remain relevant so far as we can tell, the absolute levels of the compounds as reported were underestimated [6,7].

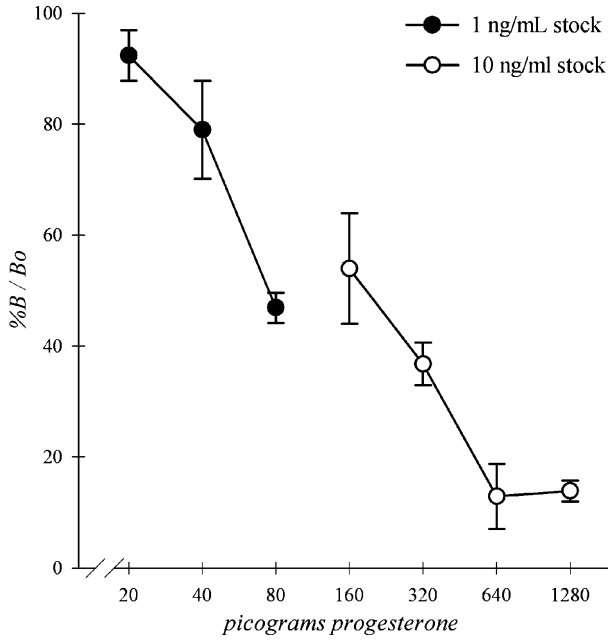

Fig. 1. A progesterone standard curve produced by aliquoting varying volumes of progesterone from two stock solutions in ethanol. The 20, 40 and 80 pg points were made with 20, 40 and 80 μL of 1 ng/mL stock; the subsequent points were made with 16, 32, 64 and 128 μL of 10 ng/mL stock solution). The 80 pg point (80 μL ethanol) appears stronger than the 160 pg point (16 μL of ethanol), giving a 'hook' appearance.

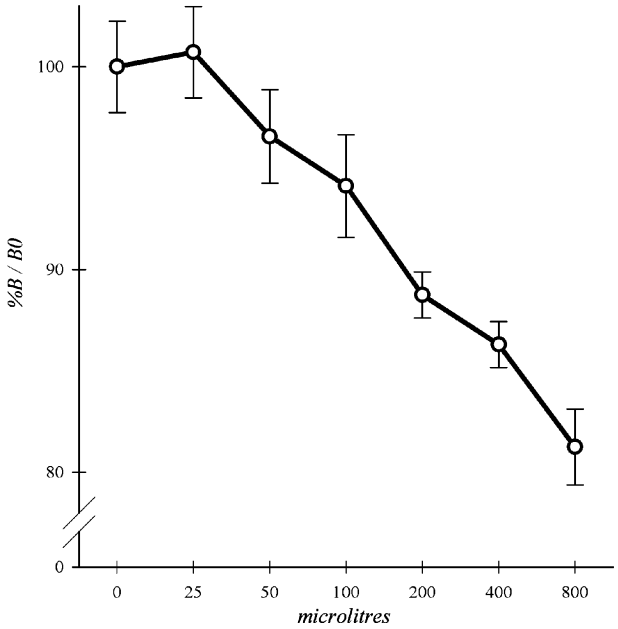

Fig. 2. Residue from evaporating increasing amounts of ethanol alone produces volume-dependent displacement of bound radio-labelled tracer.

Change with increasing sensitivity (Fig. 3). In the least sensitive assay (Fig. 3A), ethanol residue alone did not produce a significant decrease in the amount of radioligand bound, and the progesterone standard curves prepared with plastic tips and glass syringes did not differ. In the assay with medium sensitivity (Fig. 3B), some effect of solvent was observed, but did not reach significance. However, in the assay with highest sensitivity (Fig. 3C), ethanol produced a significant ($p \leq 0.05$) decrease in the amount of radioligand bound (approximately 25% apparent reduction in bound fraction compared with B_0). This resulted in a significant flattening of the curve. This flattening was exaggerated when plastic tips were used to pipette the standards.

3.2. Effects of different batches of ethanol (Fig. 4)

When three sources of ethanol were pipetted using new and carefully cleaned glass syringes, evaporated and then assayed without any steroid added (B_s), the smaller volume of 100 μL decreased the binding from slight for the batches of methanol and anhydrous ethanol, to about 35% for the 95% ethanol obtained from the liquor commission (SAQ), while the 400 μL volume decreased it much further in every case. Special care was taken such that all sources of ethanol were opened immediately prior to the experiment so that no contamination with progesterone could have occurred. Even from the same source we found that different solvent batches varied widely—in some, volumes even smaller than 50 μL produced substantial decreases in B_s. The effect was present whether or not the alcohol arrived in glass or plastic bottles.

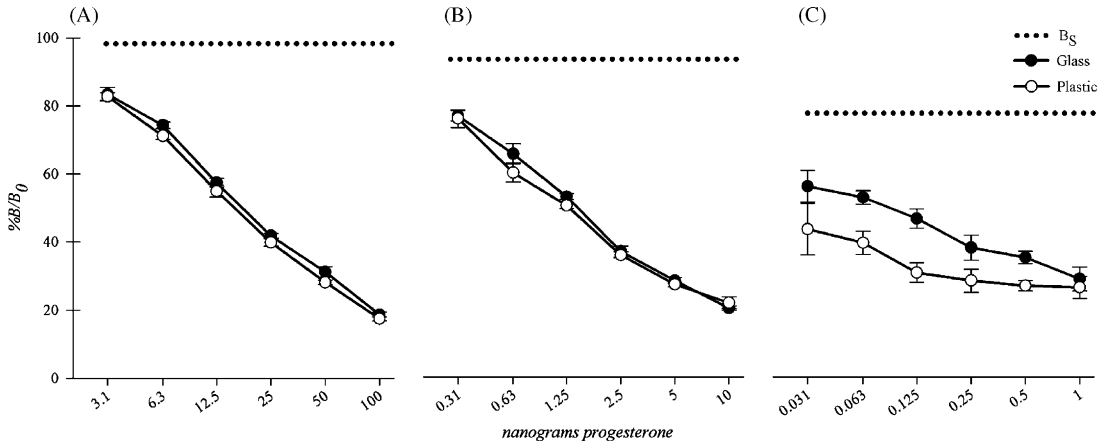

Fig. 3. Progesterone standard curves assayed by RIA with different sensitivities. Standard curves were prepared using either glass Hamilton syringes or disposable plastic pipette tips. The dotted line represents the effect of solvent alone (B_s). (A) Progesterone 3.2–200 ng, antiserum titre 1/50. (B) Progesterone 0.32–20 ng, antiserum titre 21/500. (C) Progesterone 32 pg–1 ng, antiserum titre 1/5000.

3.3. *Effects of aliquoting standards with plastic pipette tips compared to those with glass (Fig. 5)*

Even aliquoting the same ethanol solution was affected by the brief exposure to plastic occurring with the use of plastic tips, and the extent of the decrease varied with the batch of tips, in the case shown here being greater when non-sterilized tips rather than sterilized tips were used. It is therefore essential to test whether plastic tips are safe to use prior to performing RIA's.

3.4. *Different ways of preparing standard curves*

Progesterone standard curves prepared in RIA buffer solutions containing only 1% ethanol were compared with those using standards in 50 and 10 μL ethanol (Fig. 6). Those prepared in buffer and the smaller amount of ethanol (10 μL) were similar. Ethanol 50 μL caused more apparent displacement of *P, producing a shift in the standard curve, which was significant ($p < 0.01$).

These studies lead us to believe that the use of alcohol in contact with any plastics should be assessed to ensure the above effects are avoided. Where alcohol is used, it is crucial to include B_s controls in the assay. Due to the variability between batches of solvent, we suggest that a volume of the solvent several-fold higher than that used for standards be tested and that controls be included routinely.

It is fortunately possible to prepare standards in essentially aqueous media by adding bovine serum albumin or gelatin to assay buffers [16]. These protein compounds enhance solubility of steroids in aqueous media, and may improve performance of immunoassays as shown in Fig. 6. It is not possible to avoid the use of alcohol entirely—the most concentrated stock solutions cannot be made in buffer solutions. However the amount of ethanol can be minimized by preparing the more dilute solutions in this way.

In simple RIAs, in which no chromatographic separation is performed, it is possible to add known amounts of standards to the biological matrix, and these can be processed in the same way as the samples. This may include extraction of steroids from the biological matrix, or addition of the matrix containing the analyte or standard directly to the reaction mixture. This, however, is not possible when multiple steroids from the same sample are measured and chromatographic separation of the analytes is performed prior to measurement by RIA. Such methods include those profiling structurally related steroids, which cross-react with the same antiserum, e.g. pregnane neurosteroids [6,7,11]. When RIA is coupled with HPLC, many chromatographic runs would be required just to compose a standard curve for each analyte. This would be time-

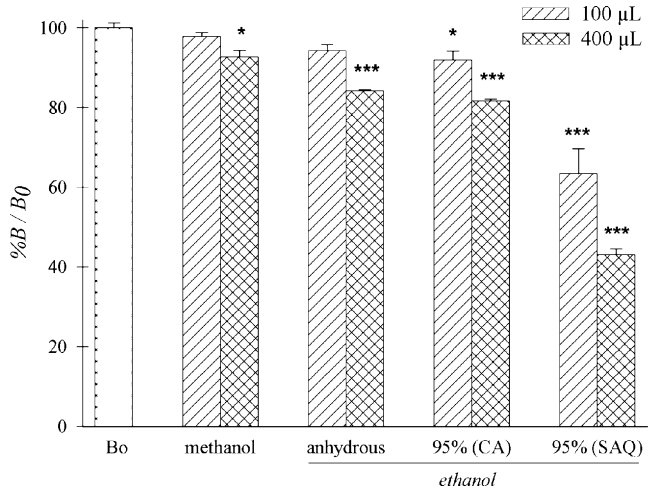

Fig. 4. The effect of methanol and ethanol residues on progesterone RIA. Three different sources of ethanol were tested, each in two volumes (100 and 400 μL, in quadruplicate). * and *** indicate a statistically significant decrease from B_0 ($p \leq 0.05$ and $p \leq 0.001$, respectively.

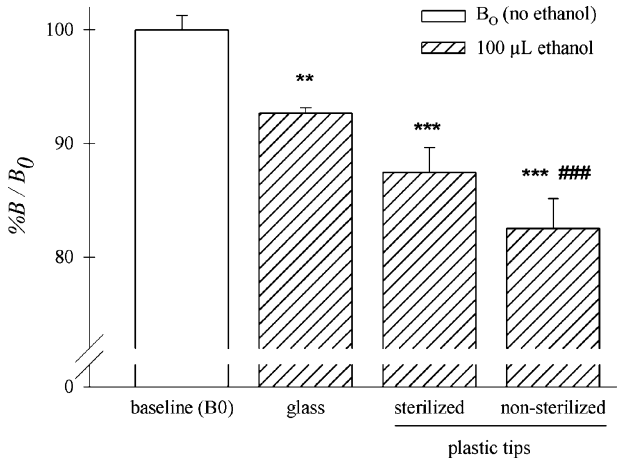

Fig. 5. Change in baseline signal (B_0) due to ethanol aliquoted with glass syringes and using non-sterilized or sterilized plastic pipette tips. ** and *** indicate a significant difference from B_0 ($p \leq 0.01$ and $p \leq 0.001$, respectively). ### indicates a significant change from ethanol pipetted with a glass syringe ($p \leq 0.001$).

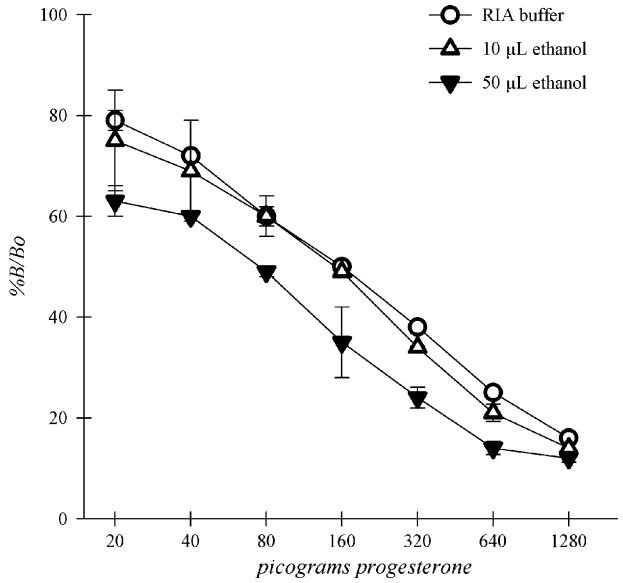

Fig. 6. Progesterone standard curves prepared in three different ways: known amounts of progesterone were serially diluted and aliquoted in 50 μL RIA buffer, 50 or 10 μL anhydrous ethanol. 50 μL ethanol produced a leftward shift in the standard curve (significant at $p \leq 0.01$).

consuming and would not allow standard curves to be measured in the same assay as the analytes. Instead, an internal standard (radiolabelled steroid) is used to track recovery through the extraction from tissue samples and the separation by HPLC, and the HPLC fractions are dried and assayed alongside known amounts of a single standard aliquoted in alcohol [10,11]. The results are then calculated according to the cross-reactivities of the various ligands.

4. Conclusions

We have shown that impurities are present whenever alcohol that has been in contact with any plastic is involved. While it is not clear in what way the impurities interact with the progesterone RIA, it seems likely that similar effects may occur in competitive binding assays for other steroids, particularly as the sensitivity of such assays is steadily increasing. The effects seen here occurred despite the claims of purity meeting international standards by the manufacturer.

Furthermore we are concerned about the potential health risks of this phenomenon. Recently consumable alcohol such as wines (alcohol content 11–13%) is increasingly being packaged, stored (often for years), and sold in plastic containers, and such containers do not have an expiry date. Since wine is drunk by many people in substantial quantities (e.g. 200 mL, – 10,000 times the 20 μL volume used here) on a daily basis, we believe the safety of such practices should be investigated.

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