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Regulatory significance of procaine residues in plasma and urine samples: preliminary communication

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Summary

Plasma and urinary concentrations of procaine and the duration of response to procaine after its administration as a local anaesthetic to horses were studied. Following injection of a clinical dose of procaine HCl (80 mg), the concentration of procaine in plasma was less than the lower limit of quantitation and unsuitable for threshold determination. Therefore, the urinary concentration of procaine was determined after injection of a dose of 5 mg procaine HCl, the highest no-effect dose (HNED) of this agent. Free unconjugated procaine in equine urine reached a peak concentration of 23.7 ng/ml, while total (unconjugated plus conjugated) procaine peaked at 37.9 ng/ml (mean urine pH of 8.5). Because a basic drug may concentrate substantially in acidic urine, a threshold concentration of 25 ng/ml of unconjugated procaine is a reasonable and conservative threshold for procaine at this time.

Horses were administered abaxial sesamoid blocks containing 2% procaine HCl (40, 80, 160 and 320 mg) and 2% procaine HCl (40 and 320 mg) with epinephrine (1:100,000) in local anaesthetic experiments. There was a significant local anaesthetic (LA) effect for all doses of procaine HCl with the duration of effect ranging from 30 min (40 mg) to 60 min (320 mg). The addition of epinephrine significantly increased the duration of local anaesthesia to 180 min for a 40 mg dose and 420 min for a 320 mg dose. Because epinephrine may extend the duration of local anaesthesia beyond a reasonable period of confinement for horses before the starting time of a race, the increased LA effect following the addition of epinephrine to procaine has regulatory significance.

Introduction

Procaine is one of the most commonly detected drugs in post race urine samples (Tobin and Blake 1977), with 73 positive samples in the United States during a 3.5 year period (January 1990 to July 1993) (R. Gowen, personal communication). Many of these positives result from administration of procaine as procaine penicillin. Because procaine penicillin is a legitimate and highly beneficial therapeutic agent, used widely by veterinarians, and the urinary excretion of procaine is prolonged after its administration, inadvertent procaine 'positives' are a major problem for equine veterinarians, horsemen and regulatory officials. Less probable sources of procaine in equine urine are administrations of this drug as nerve blocks, which usages are of regulatory concern (Tobin *et al.* 1977a,b; Tobin 1981; Kellon and Tobin 1995).

There are two proposed solutions to the problem of procaine detection in post race urine samples. One is to determine the minimum effective dose of procaine to cause a significant pharmacological effect and set a plasma and/or urinary threshold based on this dose. A second approach is to sequestre procaine-treated horses for a period prior to a race. Since the pharmacological effects of procaine are thought to be short-lived, a reasonable period of sequestration should effectively prevent the improper use of procaine for its local anaesthetic (LA) effects. Here, we report the plasma and urinary concentrations of procaine associated with no-effect threshold (NET) doses of this agent in horses and the duration of the local anaesthetic effects of procaine when administered with and without epinephrine.

Materials and methods

Horses

Six mature Thoroughbred mares, weighing 413–602 kg, were used. All horses were acclimatised to their stalls 24 h prior to the protocol. Because of the critical role of superficial skin temperature in these experiments, no LA quantification experiments were performed when the ambient temperature was less than 10°C. At least 7 days elapsed between individual LA dose response curve experiments.

Drug administration

Plasma procaine concentration experiment: A standard clinical dose (80 mg) of 2% Procaine HCl[1] was administered subcutaneously into the posterior fetlock of the left front leg in 2 concurrent doses, 40 mg over the lateral and 40 mg over the medial sesamoid bones. Blood samples were collected at 0, 10, 20, 30, 45, 60, 120, 180, 240 and 360 min after injection, separated by centrifugation, and stored at -20°C until analysed. Blood samples were collected via venepuncture into tubes containing sodium fluoride at a final concentration of 5 mg/ml blood, sufficient to prevent hydrolysis of procaine by plasma esterases (Baselt *et al.* 1985).

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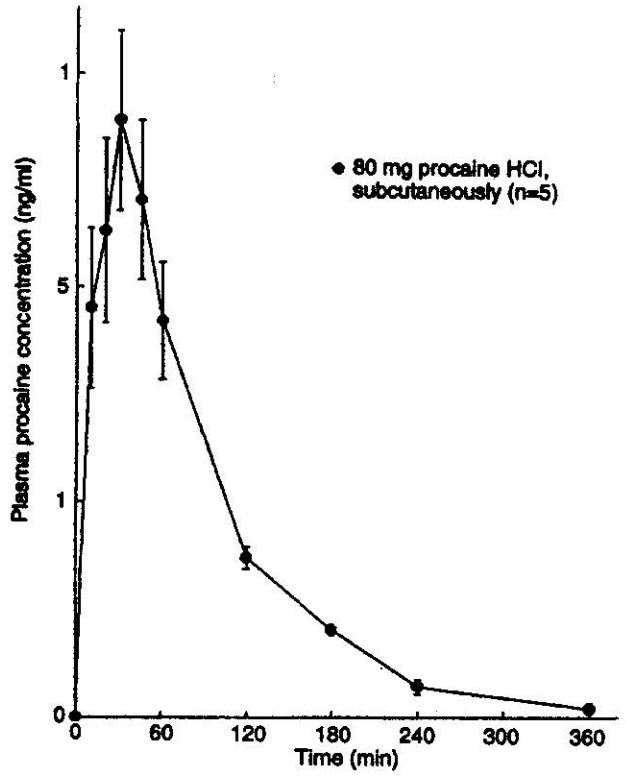

Fig 1: Plasma concentration of procaine following subcutaneous injection of 80 mg procaine HCl.

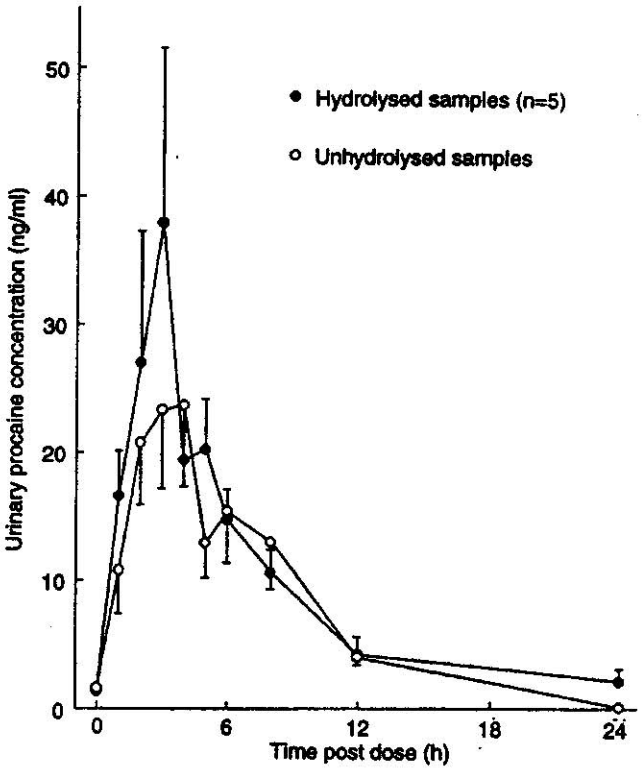

Fig 2: Urinary procaine concentrations of hydrolysed and unhydrolysed samples following subcutaneous injection of 5 mg procaine HCl.

Local anaesthetic quantification experiment: Randomly selected doses (40, 80, 160, 320 mg) of 2% Procaine HCl and doses (40 and 320 mg) of procaine HCl with epinephrine (1:100,000) were injected subcutaneously. The site of injection was into the area of the lateral volar nerve where it passes lateral (abaxial) to the lateral sesamoid bone. In clinical practice, this block is known as an abaxial sesamoid block. To control for possible effects of pressure or volume, a similar volume of normal saline was injected into the contralateral leg, which was tested in parallel with the LA-treated leg.

Urinary procaine concentration experiment: The highest no-effect dose of Procaine HCl (5 mg; Harkins *et al.* 1995) was injected subcutaneously into the posterior area over the lateral sesamoid bone of the left front leg. Urine samples were collected at 0, 1, 2, 3, 4, 5, 6, 8, 12 and 24 h after injection and stored at -20°C until analysed.

Site preparation

Before each LA quantification experiment, the hair on the front and lateral side of the foreleg pasterns was clipped and the pastern was blackened with stamp pad ink[2] to ensure equal and consistent heat absorption independent of skin and hair colour. Contralateral legs were also clipped, blackened and tested to assess any systemic effect of the LAs.

Determination of local anaesthetic effect

For the LA quantification experiments, dose and time response relationships for procaine and procaine with epinephrine were determined with a heat projection lamp described previously (Harkins *et al.* 1995). Briefly, focused radiant light/heat was used as a noxious stimulus and was directed onto the pastern of a horse to elicit the classic flexion-withdrawal reflex. Hoof withdrawal reflex latency (HWRL) was defined as the time between lamp illumination and withdrawal of the hoof. These times could be adjusted by varying the intensity of the heat output with a rheostat. In general, the intensity of the light beam was adjusted so that HWRL period was about 3 or 4 s, with the actual HWRL recorded on an electronic timer built into the lamp. In the anaesthetised leg, the duration of light exposure was limited to 10 s to prevent damage to the skin. A secondary unfocused light beam (sham light) was used to confound the horse, reducing the possibility that the flexion-withdrawal reflex was to visual, rather than thermal, perception of the focused light beam.

Dose and time response relationships

For the LA quantification experiments, HWRL was measured at -30 and -15 min and immediately before injection of the LA. These 3 HWRL times (-30, -15 and 0 min) were used to establish a control value for HWRL in each horse. The HWRL was also measured at 7.5, 15, 30, 45, 60, 75, 90 and 120 min after administration of procaine HCl. For doses containing epinephrine, measurements of HWRL were continued every 30 min until cutaneous sensation returned. The HWRL is expressed as a per cent of control values.

Analysis of plasma and urinary procaine concentration

To an aliquot of each urine sample, calibrator and negative control was added 4 ml of 50% ammonium hydroxide solution, 5 ml of dichloromethane (DCM) and 100 ng of procainamide[3] (20 µl of 5 µg/ml methanol solution) as internal standard. To each plasma sample, calibrator, and negative control was added 5 ml of a saturated solution of sodium tetraborate in water, 5 ml of DCM

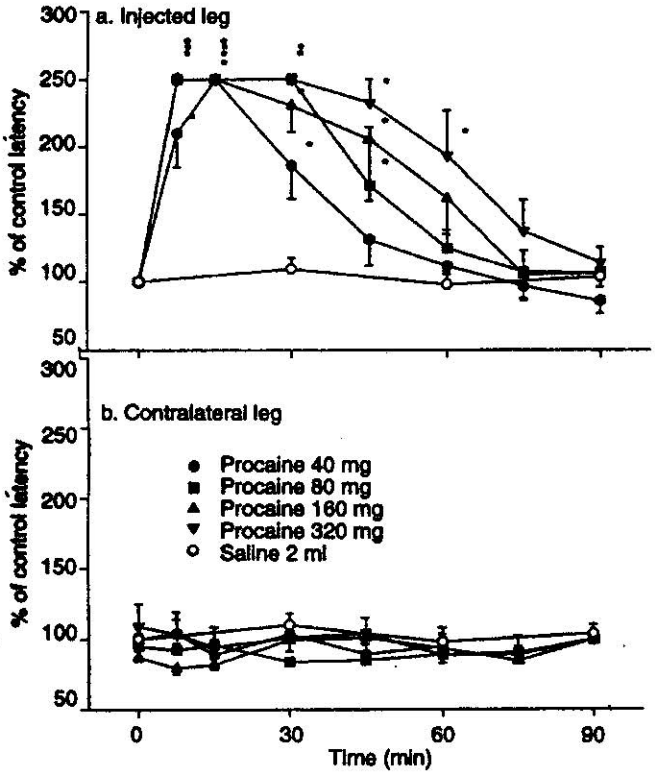

*Fig 3: a) Per cent increase in HWRL following injection of procaine doses without epinephrine; b) Per cent change in contralateral leg. *significantly (P<0.05) different from control (saline) values.*

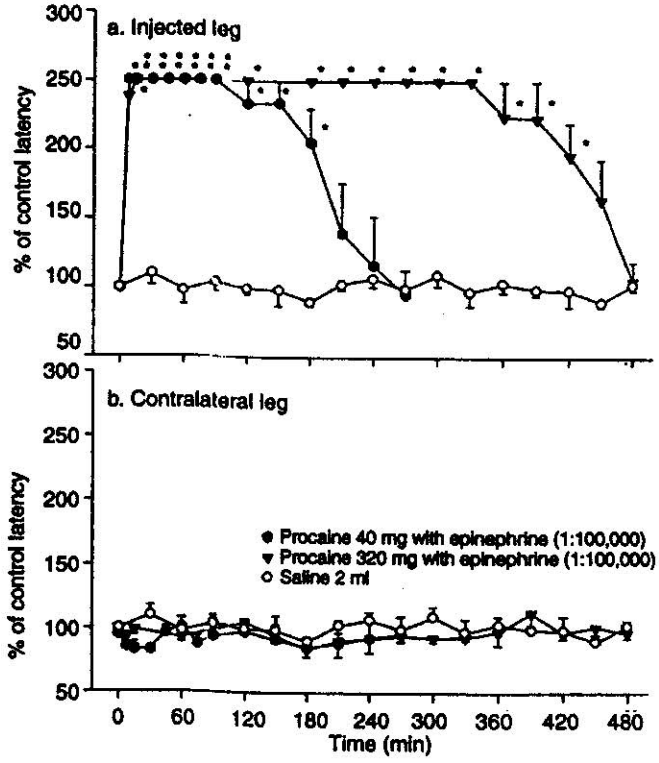

*Fig 4: a) Per cent increase in HWRL following injection of procaine doses with epinephrine; b) Per cent change in contralateral leg. *significantly (P<0.05) different from control (saline) values.*

and 100 ng of tetracaine[3] (5 µl of 10 µg/ml methanol solution) as internal standard. The samples were mixed by rotation for 20 min and then centrifuged 30 min to reduce the emulsions. The upper (aqueous) layer was discarded by aspiration and the DCM phase was evaporated to dryness under a stream of N2.

To samples that were to be hydrolysed was added 2 ml sodium acetate buffer (pH 5) and 1 ml of β-glucuronidase (5000 u/ml). The tubes were vortexed and then incubated at 63°C for 3 h.

The instrument employed was a Beckman System Gold High Pressure Liquid Chromatography (HPLC) System[4] with 2 Model 110B Solvent Delivery Pumps and a Model 167 Scanning Detector. The column was a Regis Workhorse Octadecyl, 4.6 mm x 300 mm with 10 µ particle size[5]. The mobile phase consisted of acetonitrile: 0.0165 M triethylamine pH 3.0 (86:14) at a flow rate of 1 ml/min. The UV detector wavelength was set at 288 nm. All injections were made onto a 20 µl loop.

Each sample extract was redissolved in 200 µl of methanol. An aliquot of each sample (20 µl) was injected on the HPLC. The height of the peaks corresponding to procaine and internal standard were measured. The internal standard peak heights were used to normalise the procaine peak heights. The procaine standard curve was used to calculate the procaine concentration for each sample. The analytical method was modified from that of Stanley (1994). The limits of quantitation of this method were 10 ng/ml and 2 ng/ml for plasma and urine, respectively.

This analytical method detected unconjugated procaine. When the enzyme hydrolysis step was applied prior to extraction of the procaine, the concentration of procaine measured included both unconjugated and conjugated procaine.

Statistical analysis

Analysis of variance with repeated measures was used to compare control and treatment HWRL values for the different doses of procaine with and without epinephrine. Significance was set at P<0.05.

Results

Plasma procaine concentration

Following subcutaneous injection of 80 mg procaine HCl, procaine was detected in plasma at the first sampling point (10 min). Plasma procaine reached a peak concentration (13.88 ng/ml) at 30 min and remained detectable through 360 min after injection (Fig 1).

Urinary procaine concentration

Following subcutaneous injection of 5 mg procaine HCl, procaine was detected in the urine at the first sampling point (1 h) for the hydrolysed and unhydrolysed samples. Total procaine concentration reached a peak (37.9 ng/ml) 3 h after injection and unconjugated procaine concentration reached a peak (23.7 ng/ml) 4 h after injection. Urine unconjugated procaine concentrations were less than the limit of quantitation 24 h after injection (Fig 2).

Dose and time response curves

The LA effect of procaine HCl is illustrated in Figure 3a. Following all injections, there was a significant LA effect at the first measurement (7.5 min). For the 40 mg dose, the significant LA effect persisted through 30 min; for the 80 and 160 mg doses, the significant LA effects persisted through 45 min; and for the 320 mg dose, the LA effect was extended through 60 min. Therefore, increasing the procaine dose 8-fold (40 mg vs. 320 mg) lengthened the duration of local anaesthesia by only 2-fold (30 min

and 60 min, respectively). There was no LA effect in the contralateral leg (Fig 3b).

In contrast, there was a significant LA effect following procaine injection (40 and 320 mg) with epinephrine (1:100,000) through 180 and 420 min, respectively (Fig 4a). The addition of epinephrine (1:100,000) to 40 mg procaine HCl increased the duration of local anaesthesia 6-fold over the duration of local anaesthesia from the same dose without epinephrine. Similarly, the addition of epinephrine to 320 mg procaine HCl increased the LA duration 7-fold over the duration of local anaesthesia from the same dose without epinephrine. There was no LA effect in the contralateral leg (Fig 4b).

Discussion

In our initial plasma procaine concentration experiments, we chose 80 mg procaine HCl as a standard clinical dose based on suggested threshold doses for this agent from the scientific literature (Stevenson *et al.* 1992). In a parallel study (Harkins *et al.* 1995), we determined that the highest no-effect dose (HNED; i.e. 'the highest dose of a drug at which there is no reasonable possibility of a pharmacological effect during a race') of procaine was 5 mg. We also determined in that study (and verified in the current study) that any LA effect from 80 mg procaine disappeared by 60 min after drug administration.

The mean procaine concentration in plasma 60 min after injection was ~9.2 ng/ml (Fig 1). Extrapolating from the plasma procaine concentration curve (Fig 1), plasma concentration of procaine 1 h after a 5 mg injection would be about 0.5 ng/ml, a concentration well below the routine quantification ability of our laboratory. Therefore, we concluded that a plasma threshold approach was not practical for 3 reasons: 1) the very low concentration of procaine in plasma following administration of the HNED for procaine, 2) the problem of hydrolysis of procaine by plasma esterases, which must be inhibited with physostigmine or sodium fluoride added when plasma is collected (Tobin *et al.* 1976) and 3) many racing jurisdictions do not collect blood samples for analysis.

Because measuring plasma procaine concentration was not practical and because procaine is present in urine at higher concentrations and for longer durations than in plasma (Tobin and Blake 1977), we focused our research on measuring procaine concentration in urine. However, the concentration of procaine (and most drugs) found in the urine is influenced by 2 factors: urine pH and the ratio of hydrolysed/unhydrolysed compound in the sample. In this study, peak procaine concentrations (hydrolysed - 37.9 ng/ml, unhydrolysed - 23.7 ng/ml; Fig 2) were measured in urine samples with a mean pH of 8.3 units.

The pH of the urine sample probably influences the concentration of unconjugated procaine in post race urine (Stanley *et al.* 1995). Local anaesthetics, such as procaine, are weak bases, and the concentration of the unconjugated form of these compounds in urine is liable to increase as urinary pH decreases (Houston *et al.* 1985; Wood *et al.* 1990; Gerken *et al.* 1991). Furthermore, strenuous exercise causes acidosis and urinary acidification in horses (Houston *et al.* 1985) and there are good theoretical reasons to believe that urinary concentrations of basic drugs such as procaine may be concentrated up to 1000-fold in acidic urine samples (R. Sams, personal communication).

On this basis, the work reported here suggests a minimum threshold for unconjugated procaine in alkaline urine (pH ~8.3). At this time, we do not know which urinary procaine concentration would result from administration of a no-effect threshold (NET) in acidic urine, other than that it would probably be substantially higher than 25 ng/ml. Therefore, our suggested urinary threshold for procaine is very conservative.

We also studied the relationship between unconjugated and conjugated procaine in urine samples. As shown in Figure 2, hydrolysis increased the concentration of unconjugated procaine in the samples by about 60%. This is important because the urinary concentration of unconjugated procaine is the probable concentration that relates most closely to the pharmacological effect of the drug. The conjugated fraction is pharmacologically inactive and is not likely to relate in any predictable way to the concentration of unconjugated procaine in the horse. Therefore, we believe that, for forensic purposes, the critical concentration is the concentration of unconjugated procaine in the urine. Furthermore, regulatory officials (e.g. equine forensic chemists, Commission Veterinarians and Equine Medical Directors) need to distinguish between unconjugated and conjugated procaine in urine samples when they interpret their analytical data.

Distinguishing between unconjugated and conjugated procaine is also important because conjugated procaine probably represents the major portion of the procaine in a urine sample. As shown in Figure 2, the glucuronide metabolite at peak urinary concentration represented about 40% of total procaine present in the urine sample from our horses. More importantly, field reports (R.A.S. and S.D.S.) suggest that the fraction of total procaine present as the glucuronide may be much higher under certain circumstances. For example, Sams (unpublished data) reports that in track samples 80 to 90% of the procaine recovered is in the form of the glucuronide fraction, which is consistent with our experience with morphine.

In our work with morphine, Combie *et al.* (1982) measured the concentration of morphine in urine samples with and without enzymatic hydrolysis for 6 days after a single i.v. dose of this agent. Throughout that period, an average of only 12% of the measured morphine was excreted in the unconjugated form and 88% was excreted in the conjugated form. Beyond this, the ratio of unconjugated morphine to its glucuronide changed throughout the excretion period, although the glucuronide conjugate was always the dominant form.

Consistent with these findings, the 24 h time point in Figure 2 shows negligible unconjugated procaine but measurable total procaine. These results suggest that, under approximately steady state conditions, such as occurs after administration of penicillin-G procaine, we may expect a large (90%) proportion of the procaine in urine to be in the glucuronide form.

During analysis of urine samples for procaine, the laboratories of most racing jurisdictions routinely subject them to enzymatic hydrolysis, which effectively converts the glucuronide complex to unconjugated procaine. Since the predominant form of procaine in the urine is procaine glucuronide, this procedure would be expected substantially to increase the urinary concentration of unconjugated procaine. Therefore, we do not recommend the taking of regulatory action on the results obtained from hydrolysed samples. Rather, we suggest a threshold for unconjugated procaine measured in unhydrolysed urine of 25 ng/ml.

It is interesting to note that the California Horse Racing Board has just introduced a urinary threshold for procaine of 10 ng/ml, a very conservative threshold. Further research to determine unconjugated procaine urinary concentrations in acidic urine characteristic of post race samples (pH=4.5–9; Houston *et al.* 1985) will more accurately establish procaine thresholds for urine at different pH values.

Since the LA effect of a large dose of procaine HCl (320 mg) returned to control values 60–90 min after injection, sequestration of horses treated with procaine HCl or procaine penicillin for a period of 2 h before start of a race would effectively ensure that procaine had no effect on the performance of the horse during the race, assuming that the only effect possible is local anaesthesia. However, from the LA quantification experiments, the addition of epinephrine (1:100,000) significantly increased the duration of local anaesthesia produced by procaine HCl. Presently, there are no commercial preparations of procaine HCl containing

epinephrine; however, epinephrine could be added to procaine HCl to subvert this restriction, rendering the sequestration strategy useless.

In this regard, it is worth noting that the Canadian experience with their regulatory protocols for procaine suggests virtually no tendency for horsemen to misuse this agent or use it to cover misuse of procaine (Weber 1995). Bearing this experience in mind, it could be worthwhile to develop the sequestration approach, if only on a pilot basis.

In summary, these reports suggest that, due to the very low concentrations of plasma procaine associated with its local anaesthetic effects, establishment of a plasma threshold for procaine is not a useful regulatory approach. On the other hand, administration of 5 mg of procaine subcutaneously, the highest no-effect dose (HNED), is associated with peak concentrations of unconjugated procaine in unhydrolysed urine of about 25 ng/ml. Because this value was obtained in horses at pasture, it represents a very conservative 'no-effect threshold' in equine urine for this important therapeutic agent.

We also evaluated the approach of sequestering horses during the possible period of action of procaine as a local anaesthetic. Procaine HCl had a very brief duration of pharmacological action, and, for this agent, a 2 h sequestration would be adequate. However, addition of small quantities of epinephrine to procaine greatly extended the duration of its pharmacological effect; nevertheless, the experience of Canadian regulators suggests that sequestration should at least be tried on a pilot basis.

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Manufacturers addresses

[1]Abbott Laboratories, North Chicago, Illinois, USA.
[2]Dennison Manufacturing Co, Framingham, Massachusetts, USA.
[3]Sigma Chemicals, St. Louis, Missouri, USA.
[4]Beckman Instruments, Fullerton, California, USA.
[5]Regis Technologies, Morton Grove, Illinois, USA.

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