ELISA ASSAY FOR METHOCARBAMOL

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ABSTRACT

Methocarbamol (1,2-propanediol, 3-(2-methoxy-phenoxy)-,1 carbamate) is classified as a centrally acting muscle relaxant, in that it does not exert its effect directly on the muscles, but is believed to depress the central nervous system. The drug is used frequently in horses. It is normally prescribed for acute muscle spasms associated with trauma, myositis, anxiety or strains/sprains. Due to its potential to depress the central nervous system, it is classified as a Class 4 Drug in the February 1995 RCI *Uniform Classification Guidelines for Foreign Substances*.

Methocarbamol-specific antisera was developed by immunising rabbits with a drug:protein conjugate. A methocarbamol hapten was linked to horseradish peroxidase to form a drug:enzyme conjugate. The binding of the antisera onto microtitre wells was optimised. The conjugate concentration was then altered to yield an optimum standard curve. Standard curves were established in equine urine, equine plasma, equine serum and canine urine.

A background study was performed with a large number of negative equine racing urine samples. Some interference was seen with unknown endogenous urine components. This effect was abolished by a 1:10 dilution of the sample in assay buffer. Little background interference was noted with negative plasma samples. Cross-reactivity studies were performed with a variety of illegal drugs, therapeutic drugs, potential masking agents and drug vehicles. Significant cross-reactivity was seen with guaifenesin (11% cross-reactivity), mephenesin (0.8%) and salicylic acid (0.1%).

The I_{50} of the assay (a measure of sensitivity) with equine plasma is 4.5 ng/ml (parts per million). The I_{50} of the assay with diluted equine urine is 45 ng/ml. The limit of detection of this assay is approximately 2.4 ng/ml. Recognising that methocarbamol is a legitimate therapeutic drug, and that sensitivity of 45 ng/ml may not be desired by some racing jurisdictions, a sample dilution study was performed. A sample dilution of 1:100 produced an I_{50} of approximately 350 ng/ml, whereas a 1:500 dilution increased the I_{50} to approximately 1,000 ng/ml.

Post administration samples were collected and assayed using an enzyme linked immunosorbent assay. Methocarbamol was cleared rapidly. In urine, large amounts were detected at 10 h but could not be detected at 24 h post administration. In plasma, methocarbamol could not be detected after 8 h.

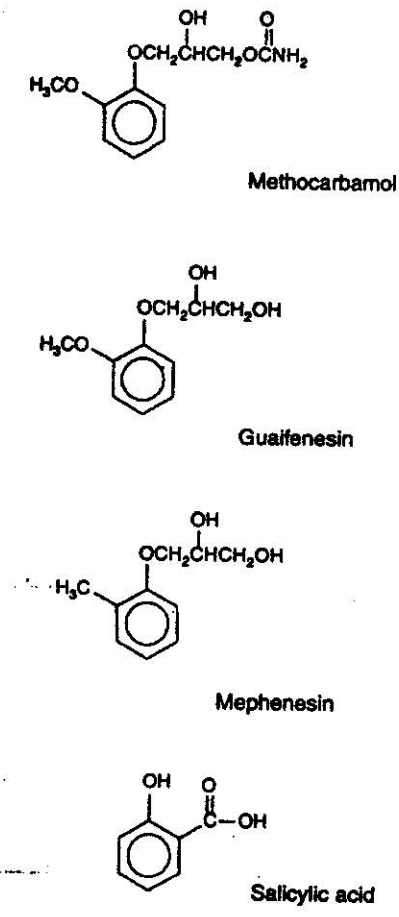

Fig 1: Methocarbamol and cross-reactant structures.

TABLE 1: Cross-reactivity study

Cross-reactant	% cross-reactivity	Cross-reactant	% cross-reactivity
Guaifenesin	10.95	Mephenesin	0.79
Salicylic acid	0.11	6-α methylprednisolone	< 0.01
Aminophylline	< 0.01	Ascorbic acid	< 0.01
Baclofen	< 0.01	Carisprodol	< 0.01
Chlorzoxazone	< 0.01	Clenbuterol	< 0.01
Codeine	< 0.01	Curare	< 0.01
Cyclobenzaprine	< 0.01	Dantrolene	< 0.01
Decamethonium bromide	< 0.01	Dezocine	< 0.01
Diazepam	< 0.01	Diclofenac	< 0.01
Dimethylsulfoxide	< 0.01	Dipyrone	< 0.01
ε-amino caproic acid	< 0.01	Ethyl *p*-aminobenzoate	< 0.01
Flunixin	< 0.01	Flurazepam	< 0.01
Furosemide	< 0.01	Gallamine triethiodide	< 0.01
Glycopyrrolate	< 0.01	Hordenine	< 0.01
Hydrocortisone	< 0.01	Ibuprofen	< 0.01
Levallorphan	< 0.01	Mebeverine	< 0.01
Metaproterenol	< 0.01	Methylene blue	< 0.01
Naproxen	< 0.01	Niacinamide	< 0.01
Orphenadrine	< 0.01	Oxymorphone	< 0.01
Oxyphenbutazone	< 0.01	Pancuronium bromide	< 0.01
Papaverine	< 0.01	Pentoxifylline	< 0.01
Phenothiazine	< 0.01	Phenylbutazone	< 0.01
Polyethylene glycol	< 0.01	Prednisolone	< 0.01
Procaine	< 0.01	Procyclidine	< 0.01
Pyrantel	< 0.01	Quinine	< 0.01
Ritodrine	< 0.01	Salbutamol	< 0.01
Salicylamide	< 0.01	Succinylcholine	< 0.01
Thiamine	< 0.01	Tolperisone	< 0.01
Tubocurarine	< 0.01	Xylazine	< 0.01

INTRODUCTION

Methocarbamol (Fig 1) is classified as a muscle relaxant. The drug is marketed by A.H. Robins (Virginia, USA) as Robaxin. Methocarbamol does not exert its effect directly on the muscles, but is believed to depress the central nervous system. Therefore, it is known as a centrally acting muscle relaxant. Methocarbamol is an effective therapeutic agent in equine medicine (Anon 1991). However as a potential central nervous system depressant, abuse in the racing industry can occur.

Enzyme linked immunosorbent assays (ELISA) have great value as screening tools for drugs in race samples (Tobin *et al.* 1988). The development of such a test for methocarbamol is described.

A competitive ELISA format was chosen for this test. In this format, antibodies specific to methocarbamol are immobilised onto microtitre plate wells. The sample is added along with a methocarbamol:enzyme conjugate. Methocarbamol in the sample or control competes with the conjugate for antibody binding sites during an incubation phase. Following this incubation, unbound drug and conjugate are removed by washing. An enzyme substrate and chromophore are then added. Bound enzyme conjugate activity produces a blue colour; the intensity of which is inversely proportional to the concentration of methocarbamol in the sample.

REAGENT DEVELOPMENT

Methocarbamol was derivatised by introducing a carboxylic acid group into the molecule. An immunogen was made from this derivative by linking the hapten to bovine serum albumin through NHS (N-hydroxysuccinimide; Sigma Chemical Co, Missouri, USA) and EDC (1-ethyl-3-(diaminopropyl)carbodiimide, Sigma) active ester chemistry (Grabarek and Gergely 1990). New Zealand White rabbits were inoculated with the immunogen in Freund's Adjuvant. The rabbits were boosted monthly and bled at least once a month. The blood was allowed to clot and the serum was isolated by centrifugation. Rabbit antisera were stored at <-20°C.

The same hapten was used to make drug:enzyme conjugates. Active ester chemistry was used to link the hapten to horseradish peroxidase (Boehringer Mannheim Corporation; Indiana, USA). Conjugates were purified by liquid chromatography (Kwik Columns; Pierce, Illinois, USA).

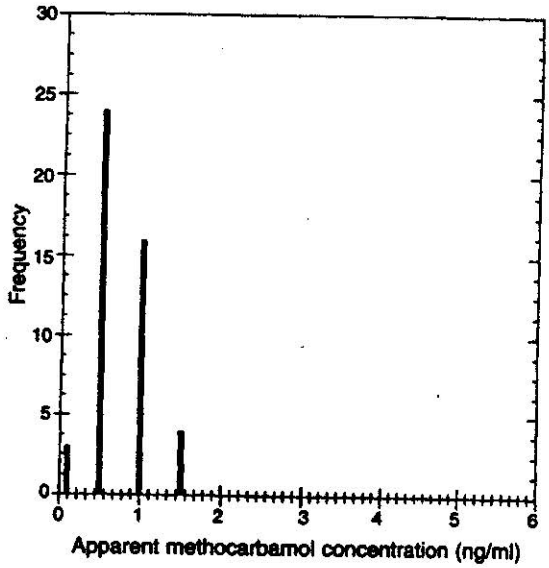

Fig 2: Diluted equine urine matrix effects. Negative track samples were diluted 1:10 in EIA and quantitated in the methocarbamol assay.

Antisera selection

The immobilisation of anti-methocarbamol antisera was optimised. Three different means of binding the antibodies to the plate were examined. The antisera were diluted and allowed to bind:

1) Directly to the wells through hydrophobic interactions;
2) To wells pre-coated with goat anti-rabbit IgG antisera (Zymed, California, USA); or
3) To wells pre-coated with Protein A (Repligen, Massachusetts, USA).

Protein A produced the best results and was used for all assay development activities. Antisera were titred and pooled. Antisera and conjugate levels were adjusted to yield optimum standard curve performance.

Assay format

Anti-methocarbamol assay plates are supplied ready to use. Samples are diluted in enzyme immunoassay (EIA) buffer and 20 µl are added to the wells. To maximise conjugate stability, the methocarbamol:enzyme conjugate is provided as a concentrate. The conjugate is diluted 1:180 in EIA buffer and 180 µl are added to each well. The reaction is incubated for 1 h at room temperature. The wells are washed with buffer (provided with the kit). A one-step peroxidase substrate is added to the wells (150 µl per well). Colour is allowed to develop for 30 min at room temperature. The absorbance of each well is measured on a microtitre plate reader.

Cross-reactivity

Cross-reactivity to a variety of drugs was determined by spiking the drug into EIA buffer and determining the apparent methocarbamol concentration in the spiked samples. Those drugs that demonstrated some competition with the conjugate for antibody binding were assayed again. In the second assay, a standard curve was produced for the cross-reactant. Percent cross-reactivity was determined by dividing the I_{50} concentration of methocarbamol by the I_{50} of the cross-reactant.

Results are shown in Table 1. Significant cross-reactivity was seen with guaifenesin (Fig 1). The I_{50} of the guaifenesin curve was 42 ng/ml compared to 4.9 ng/ml in the methocarbamol curve. Slight cross-reactivity was seen with mephenesin (I_{50} = 594 ng/ml) and salicylic acid (I_{50} = 4,125 ng/ml).

Sample matrix effects

Negative equine track samples were assayed in the methocarbamol assay. No false positives were noted with equine plasma samples. However, significant matrix effects were seen with some equine urine samples. The matrix effects are reversed by dilution in EIA buffer. When the urine samples were diluted 1:10 in EIA, the interference was reduced to an acceptable level (Fig 2). Therefore, 1:10 dilutions of urine samples are recommended to prevent false positive results due to matrix effects.

Urinalysis of a portion of the samples revealed no common thread between those samples with no matrix effect compared to samples with large matrix effects. Pre-incubation of samples with protease substrates (horse serum or bovine serum albumin) or a protease inhibitor (EDTA) failed to relieve the interference. Therefore, urine proteases were eliminated from consideration as the causative agent. Likewise, pre-incubation of unconjugated peroxidase with samples failed to reduce the matrix effects. Therefore, a peroxidase binder or inhibitor does not appear to cause the effects. Finally, 2 normal urine components (in man) with some structural similarities with methocarbamol were tested as cross-reactants. These compounds, hydroxyindole acetic acid (HIAA) and vanillylmandelic acid (VMA), did not cross-react in the assay.

Assay sensitivity

The I_{50} was determined for methocarbamol standard curves in EIA buffer, equine urine, equine

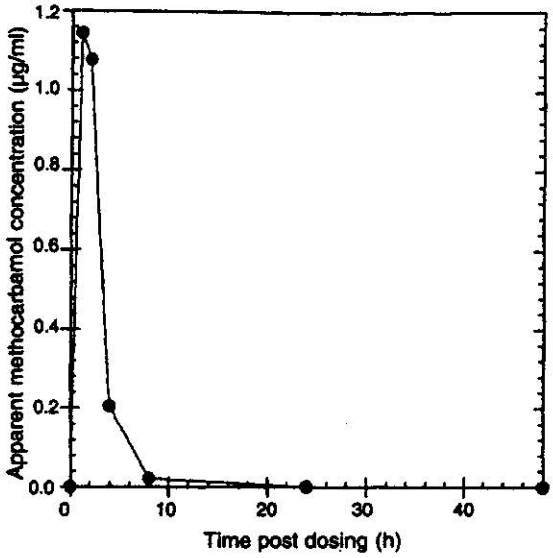

Fig 3: Duration of detection in plasma. Standardbred mare - 10 g iv. Plasma was collected and refrigerated until assayed. All samples were quantitated at the same time with the same methocarbamol standard curve.

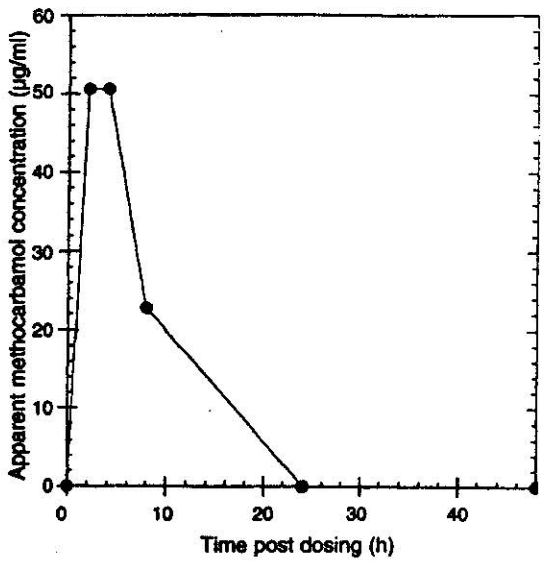

Fig 4: Duration of detection in urine. Standardbred mare - 10 g iv. Urines were collected and refrigerated until assayed. Samples were diluted 1:10 in EIA buffer and quantitated with the same methocarbamol standard curve.

plasma and equine serum. The I_{50}s of the EIA, plasma and serum curves were very similar, ranging from 4.5–5.2 ng/ml. As expected, the 1:10 dilution of the urine increased the I_{50} of this curve to 45 ng/ml.

As methocarbamol is a legitimate therapeutic drug, some racing jurisdictions may not desire a screening assay with a sensitivity of 45 ng/ml. Therefore, a sample dilution study was performed. Methocarbamol standards were made in an equine urine sample. These standards were then diluted in EIA buffer and assayed. The normal 1:10 dilution produced an I_{50} of 36.5 ng/ml. A 1:100 dilution increased the I_{50} to 341.2 ng/ml, whereas a 1:500 dilution gave an I_{50} of 1 µg/ml. Therefore, a laboratory that does not wish to confirm urine samples with less- than 1 µg/ml methocarbamol should dilute samples up to 1:500 before assaying.

Duration of detection

A Standardbred mare was given 10 g of methocarbamol iv. Urine and plasma samples were collected over the next 48 h. The samples were assayed and methocarbamol concentrations were determined from standard curves. Results are shown in Figures 3 and 4. The drug was not detected in plasma after 8 h. Urine methocarbamol levels were very high after 10 h, but not detected at 24 h.

Conclusion

An ELISA assay has been developed for methocarbamol. The assay is specific and sensitive. The assay may be more sensitive than is desired for an approved therapeutic drug. This problem can be circumvented by sample dilution.

Acknowledgements

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