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Screening of ß-adrenergic antagonists in human urine by ELISA techniques

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Introduction

The use of ß-adrenergic antagonists is banned in some sports by the International Olympic Committee and International Sports Federations (1). ß-Adrenergic antagonists (or ß-blockers) are forbidden in sports where good psychomotor coordination is required; in these sports, athletes can benefit of the peripheral blockade of several symptoms associated with anxiety. Analytical methods to screen for the presence of these compounds in human urine are applied in routine sports drug testing.

ß-blocker drugs have a general structure of N-alkyl-ß-hydroxy-aryloxypropylamine. Different aromatic rings with different substituents are linked to the oxygen atom, and tert-butyl and isopropyl groups are the most usual substituents of the amine group. There are some exceptions to this general structure, such as sotalol and labetalol that have an α-hydroxy-phenylethylamino structure.

In general, ß-adrenergic antagonists are subjected to an extensive metabolism involving phase I metabolic reactions, consisting of hydroxylations affecting the aromatic ring and its substituents and also N-dealkylations, and phase II metabolic reactions as conjugation with glucuronic acid or sulphate.

Screenings for ß-adrenergic antagonist in most antidoping laboratories are usually performed using gas chromatography coupled to mass spectrometry (GC/MS). A sample preparation step is needed before GC/MS analysis in order to isolate drugs and/or their metabolites from the biological matrix and to form suitable derivatives for GC analysis. Using this methodology, high sensitivity and specificity are achieved although the turnaround time is high (2,3).

Immunological methods are used in some areas of clinical toxicology for screening purposes allowing a substantial reduction in the time spent and the costs of the screening step. One of the main limitations of immunological methods is their high dependence on commercial developments and, in fact, its application in doping control is limited by the lack of tests with adequate specificity to detect the groups of forbidden substances. In the case of ß-adrenergic antagonists, it has been observed in positive samples to ß-blocker drugs a significant cross-reactivity with ELISA tests designed to detect $ß_2$-agonists. This empirical observation is in accordance with the similarity between the chemical structures of ß-adrenergic antagonists and $ß_2$-agonists (specially at the amino group). For this reason, the detection of ß-blockers using ELISA tests commercially available designed to detect ß-agonist drugs has been considered.

The objective of this work was the systematic study of the cross-reactivity of ß-blocker drugs and their metabolites with an ELISA test designed for the detection of $ß_2$-agonists, in order to evaluate its potential applicability in routine sports drug testing.

Experimental

Chemicals and reagents

Elisa tests of Generic Bronchodilators and Clenbuterol (Elisa Technologies Division, Neogen Corporation, Lexington KY, USA) were supplied by Labsystems (Barcelona, Spain).

The parent compounds were supplied by the following pharmaceutical manufacturers: acebutolol (Rhône-Poulenc Farma S.A.E., Barcelona, Spain); bisoprolol and carteolol (Laboratorios Lácer S.A., Barcelona, Spain); clenbuterol (Biomedica Foscama, Italy); labetalol and salbutamol (Laboratorios Glaxo S.A., Madrid, Spain); nadolol (Laboratorios Uriach, Barcelona, Spain); propranolol and atenolol (Laboratorios ICI-Farma S.A., Porriño, Pontevedra, Spain); penbutolol (Hoechst Iberica S.A., Barcelona, Spain); sotalol (Bristol-Myers S.A., Madrid, Spain); terbutaline (Laboratorios Astra-Ifesa S.A., Esplugues del LLobregat, Barcelona, Spain); alprenolol (Hässle, Sweden); metoprolol and oxprenolol (Laboratorios Ciba-Geigy, Barcelona, Spain); timolol (Merck Sharp & Dohme de España S.A., Madrid, Spain); orciprenaline (Laboratorios Boehringer Ingelheim S.A., Barcelona,

Spain); practolol (Ici Pharmaceuticals limited, U.K.); levobunolol (Laboratorios Allergan S.A., Madrid, Spain); celiprolol (Rhône Poulenc Rorer S.A., Alcorcón, Madrid, Spain); mepindolol (Schering España S.A., Madrid, Spain); and pindolol (Laboratorios Sandoz S.A.E., Barcelona, Spain).

Standard solutions

Stock standard solutions were prepared by dissolving the compounds in methanol (1 mg/mL). Working standard solutions were prepared by 1:10 and 1:100 dilution of the stock standard solutions with methanol (100 and 10 μg/mL, respectively). Each concentration was checked by UV spectrophotometry using the solution of 10 μg/mL. All solutions were stored at -20°C.

Urine samples

Spiked urine samples

Standard urines for each compound were prepared at the following concentration levels: 0.3, 1, 3, 10, 30 and 100 ng/mL, for terbutaline; 1, 3, 10, 30, 100 and 300 ng/mL, for other $ß_2$-agonists; and 10, 30, 100, 300, 1000 and 5000 ng/mL, for ß-blocker compounds and orciprenaline.

Urine samples from excretion studies

Urines obtained after administration of different ß-blockers and ß-agonists to healthy male volunteers were analyzed. These urines were obtained according to the clinical trial approved by the Hospital del Mar Ethical Committee (Barcelona, Spain) and the Spanish Ministry of Health (ref. 88/135). Single doses recommended by IOC to obtain reference urines were administered by the oral route. The following excretion studies of ß-blocker compounds were performed: atenolol (50 mg), alprenolol (50 mg), acebutolol (200 mg), bisoprolol (10 mg), carteolol (5 mg), celiprolol (200 mg), labetalol (200 mg), metoprolol (50 mg), nadolol (60 mg), oxprenolol (40 mg), pindolol (5 and 10 mg), propranolol (20 mg), penbutolol (40 mg),

sotalol (80 mg), and timolol (10 mg). The following β_2-agonists were administered: clenbuterol (0.04 mg), orciprenaline (10 mg), salbutamol (2 mg), and terbutaline (2.5 mg).

Urine samples were collected at predose (blank sample) and up to 24 hours after administration at different collection periods. A total of 16 blank samples and 54 samples collected at various intervals after ingestion of the compounds were analyzed in this study.

Samples were maintained at -20°C until the day of analysis.

Urine samples from target sports

Urine samples (147 samples) obtained after competition in sports where ß-blockers are recommended to be tested were analyzed. These sports included: archery (26 samples), bobsleigh (2 samples), diving (10 samples), modern pentathlon (11 samples), shooting (92 samples) and synchronized swimming (6 samples).

ELISA test

The ELISA test Generic Bronchodilators was performed according to the manufacturer's specifications for equine urine except that urine samples were not diluted prior to testing.

ELISA is a competitive immunoassay. The antibody against the target drug is immobilized in a solid support. The sample is added together with a solution of the drug coupled to an enzyme (horse radish peroxidase, HRP). There is a competition between the drug contained in the sample and the conjugate drug-enzyme for the binding sites of the antibodies. The presence of drug in the sample prevents the binding of drug conjugated with the enzyme. After incubation, the conjugate drug-enzyme not linked to the antibodies is removed. In the last step, an enzyme substrate is added and the bound enzyme hydrolyses this substrate to produce a coloured compound at the reaction site. The concentration of drug in the sample is inversely related to the optical density.

Sample aliquots of 20 μl urine were added to each microplate well, along with 180 μL of the diluted terbutaline-HRP conjugate solution. The wells were incubated by shaking (Heidolph

mixer, Labsystems, Spain) for one hour at room temperature. The wells were washed three times with 200 μL of diluted washing buffer (Autowash I, Labsystems, Spain). After washing, 150 μL of peroxidase substrate solution (K-Blue, proprietary composition) were added to each well and incubation for 30 min by shaking was performed for colour development. The optical density for each test well was determined at 650 nm with an automated microplate reader (Miniskan, Labsystems, Spain).

In each strip of eight wells, a blank urine and a control sample were included.

Validation by reference assay procedures

Samples from excretion studies and from target sports were also analyzed by reference assay procedures to confirm the presence or absence of ß-blockers and $ß_2$-agonists and their metabolites.

ß-blockers and most of ß-agonists were tested using a procedure already described (2). Urine samples were subjected to an enzymatic hydrolysis (ß-glucuronidase arylsulfatase) and extracted by means of Bond-Elut Certify™ columns. The residues were then selectively derivatized with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and N-methyl-bis-trifluoroacetamide (MBTFA) to form O-trimethylsilyl-N-trifluoroacetil derivatives. A GC/MS system working in scan acquisition mode was used for instrumental analysis.

The detection of clenbuterol was performed using an specific ELISA test designed for equine urine. The ELISA test was applied according to the manufacturers instructions except that urine samples were not diluted prior to testing. For confirmation purposes, clenbuterol and salbutamol were analyzed by GC/MS (3). Samples were hydrolysed (ß-glucuronidase arylsulfatase) and subjected to a solid-phase extraction using Bond-Elut Certify™ columns. The dry residues were treated with a trimethylboroxine solution to form cyclic methyl boronate derivatives. GC/MS analyses were performed in scan mode.

Calculations

The percentage of activity compared with blank samples (% control activity) was calculated

for all samples as follows: $(B/B_0)x100$, where B is the optical density in the sample and B_0 is the optical density in the blank sample.

The performance characteristics of the ELISA test (sensitivity and specificity) for the cut-off values were calculated by comparing the ELISA results of samples from excretion studies and samples from target sports with those of the reference assay procedures (4). A 2x2 table was created: positive samples in both assays were considered true positives (TP); negative samples in both assays were considered true negatives (TN); and discrepant results were classified as either false positive (FP) or false negative (FN) in comparison with results of the reference assays.

The sensitivity of the assay, defined as the ability to detect a TP result, was calculated according to the following equation: [TP / (TP+FN)] x 100. The specificity, defined as the ability to detect a TN result, was calculated according to the equation: [TN / (TN+FP)] x 100.

The percentages of positive and negative results, FP and FN, and predictive values for positive and negative results (PV+, and PV-, respectively) in routine doping control were calculated taking into account the performance characteristics of the test and the prevalence of positive results in sample population (4). The predictive value of a positive result (PV+) is the percentage of TP results of the total number of positive results obtained by the ELISA test. The predictive value of a negative result (PV-) is the percentage of TN results of the total number of negative results obtained by the ELISA test.

Results and discussion

The ELISA test Generic Bronchodilators was designed to detect terbutaline and other ß-agonists drugs. Concentration-response curves for ß-agonist drugs similar to those reported by the manufacturers were obtained (Figure 1).

Concentration-response curves for different ß-blockers compared to terbutaline are presented in Figure 2. Most of the ß-blockers tested presented cross-reactivity with the test, although

lower than terbutaline. From a practical point of view, this is not a limiting factor because concentrations of ß-blockers in urine after therapeutic doses are, in general, higher than those of ß-agonists.

The highest cross-reactivity was obtained for some compounds with a tert-butylamino substituent (Figure 2.A). This higher cross-reactivity can be explained taking into account the specificity of the antibody that probably recognizes this part of the chemical structure of terbutaline. The lowest cross-reactivity was obtained for labetalol (Figure 2.B) which has a 1-methyl-3-phenylpropylamino substituent.

ß-blockers are subjected to an extensive metabolism involving phase I and phase II reactions, and the analysis of urines obtained after intake of the compounds, where metabolites are present, is of maximum importance to evaluate the usefulness of the ELISA test for doping purposes.

The distribution profile of control activities for urines obtained in excretion studies is presented in Figure 3. The control activity was in all positive cases lower than 65%, and in most cases lower than 45%. Only one urine collected from 8 to 24h after labetalol intake and some urines obtained after clenbuterol administration presented activities between 45 and 65%. In the case of labetalol, the low signal can be due to the low cross-reactivity of this compound with the test. The low response obtained for urines from clenbuterol excretion studies is probably related to the low concentration of this compound in the urines.

In reference to the blank samples obtained in excretion studies (Figure 3) nearly all samples showed activities higher than 75%.

In order to study the behaviour of the ELISA test when dealing with actual routine samples, urines collected after competition in sports were ß-blockers are recommended to be tested were analyzed. All these samples were previously validated by reference assay procedures: salbutamol and atenolol were detected in two samples; the rest of the samples were found to be negatives to ß-blocker and ß-agonist drugs. The distribution of percentages of control activities obtained after analysis by ELISA of these urines is shown in Figure 4. An activity in the range of 10 to 15% was detected in the two positive samples. A percentage of control activity higher than 60% was obtained for most negative samples, and nearly all samples showed an activity higher than 45%.

Our results show a difference in the distribution profiles of control activities for positive and negative samples. Thus, it seems possible to use the ELISA test Generic Bronchodilators to screen for ß-blockers and, obviously, for ß-agonist compounds. A cut-off value of percentage of control activity must be established for screening purposes.

Taking into account the data obtained after analysis of urines from excretion studies and from target sports (Figures 3 and 4), cut-off values of 45% or 60% of control activity seem to be adequate. These cut-off values correspond to approximate concentrations of 2 ng/mL and 1 ng/mL of terbutaline, respectively. The performance characteristics of the test calculated for each cut-off are listed in Table 1. Sensitivities and specificities higher than 90% were obtained for both cut-off values. As it can be observed, the more sensitive the assay is, the less specific it becomes.

According to the IOC statistics of 1994, the prevalence of positive results of ß-blockers and $ß_2$-agonists in sports where ß-blockers are recommended to be tested is low, accounting for a 0.3% (5). The parameters describing the behaviour of the ELISA test when analyzing routine doping samples predicted for each cut-off value are listed in Table 1.

Due to the low prevalence, the percentage of total positive results will be low for both cut-off values. Logically, the percentage of FP results will be higher when using the cut-off of 60%. The low prevalence determines low predictive values of positive results. In practical terms, it means that only a few of the potential positive results will be confirmed as a true positives (TP).

The number of total negative results will be higher than 90% in both cases. The percentage of FN results will be lower using the cut-off level of 60%. Predictive values for negative results is near to 100% in both cases. Thus, nearly all ELISA negative results will be correctly classified as negatives.

One of the most important datum to evaluate the usefulness of the test for routine screening purposes, in terms of time and costs saved, is the percentage of positive results that will need to be confirmed. Using the ELISA test as screening method, only 5.28 or 1.48% of the samples (depending on the cut-off) will have to be analyzed using GC/MS techniques.

In summary, our results show that the ELISA test Generic Bronchodilators is a powerful tool

to screen for ß-adrenergic antagonists in human urine.

Acknowledgements

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5. International Olympic Committee Statistics, 1994.

TABLE 1. Performance characteristics of the ELISA test and behaviuor estimated in routine doping control for each cut-off value.

	CUT-OFF 45% control activity	CUT-OFF 60% control activity
Terbutaline (ng/mL)	2	1
Sensitivity (%)	92.8	98.2
Specificity (%)	98.8	95.0
	Prevalence 0.3%	
Positive Results (%)	1.48	5.28
False Positives (%)	1.20	4.98
PV (+) (%)	18.86	5.58
Negative Results (%)	98.52	94.72
False Negatives (%)	0.02	0.005
PV - (%)	99.97	99.99

PV (+): predictive value of a positive result
PV (-): predictive value of a negative result

FIGURE LEGENDS:

Figure 1. Cross-reactivity of the Generic Bronchodilators ELISA test for different ß-agonist drugs.

Figure 2. Cross-reactivity of the Generic Bronchodilators ELISA test for different ß-blocker drugs compared to terbutaline.

Figure 3. Distribution profiles of percentages of control activities for urines obtained in excretion studies of ß-blockers and ß-agonists.

Figure 4. Distribution profiles of percentages of control activities for urines obtained in sports where ß-blockers should be tested.

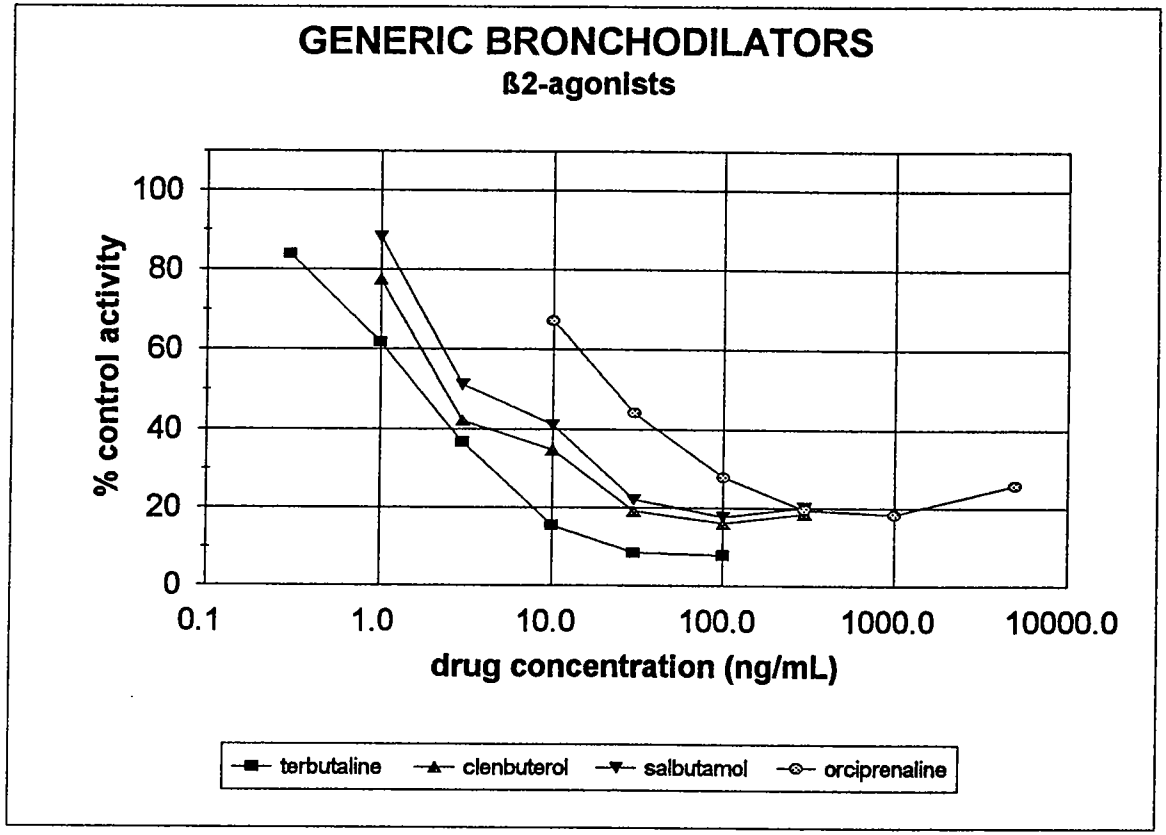
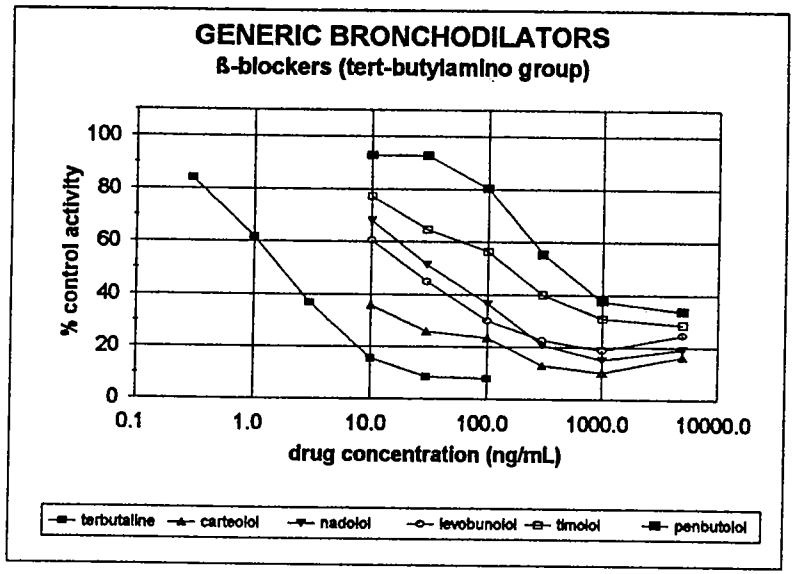
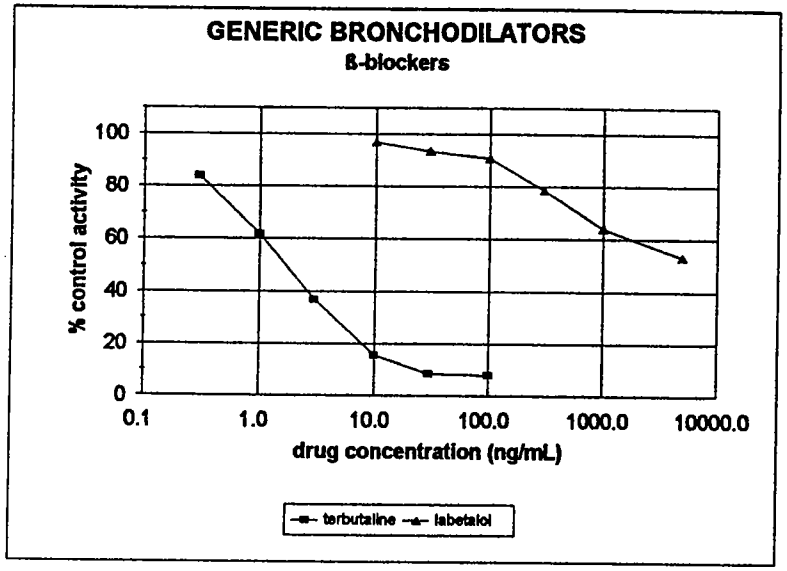
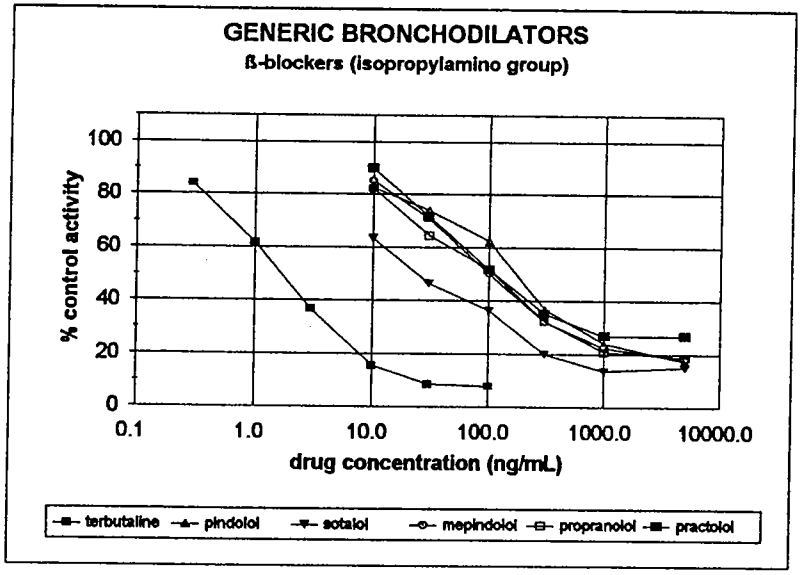

GENERIC BRONCHODILATORS
ß2-agonists
% control activity
100
80
60
40
20
0
0.1
1.0
10.0
100.0
1000.0
10000.0
drug concentration (ng/mL)
terbutaline
clenbuterol
salbutamol
orciprenaline

FIGURE 1.


GENERIC BRONCHODILATORS
ß-blockers (tert-butylamino group)
% control activity
drug concentration (ng/mL)
terbutaline
carteolol
nadolol
levobunolol
timolol
penbutolol


GENERIC BRONCHODILATORS
ß-blockers
% control activity
drug concentration (ng/mL)
terbutaline
labetalol


GENERIC BRONCHODILATORS
ß-blockers (isopropylamino group)
% control activity
drug concentration (ng/mL)
terbutaline
pindolol
sotalol
mepindolol
propranolol
practolol

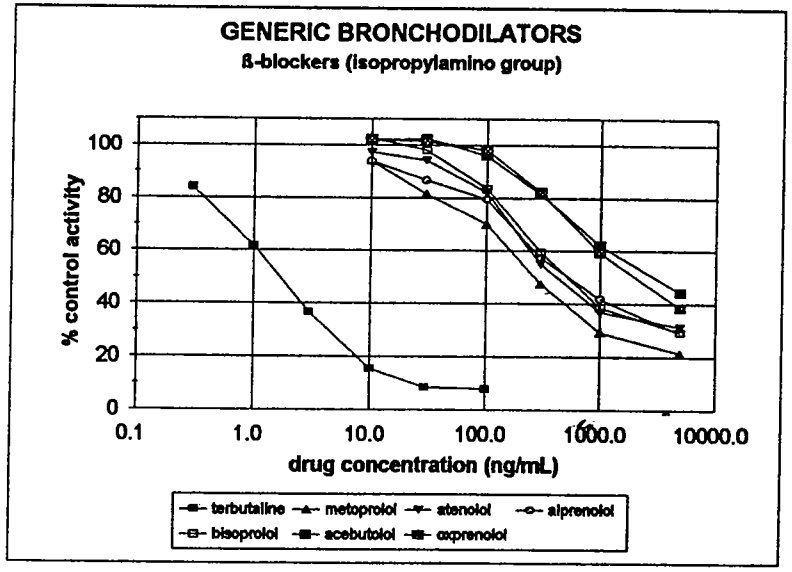

GENERIC BRONCHODILATORS
ß-blockers (isopropylamino group)
% control activity
drug concentration (ng/mL)
terbutaline
metoprolol
atenolol
alprenolol
bisoprolol
acebutolol
oxprenolol

FIGURE 2.

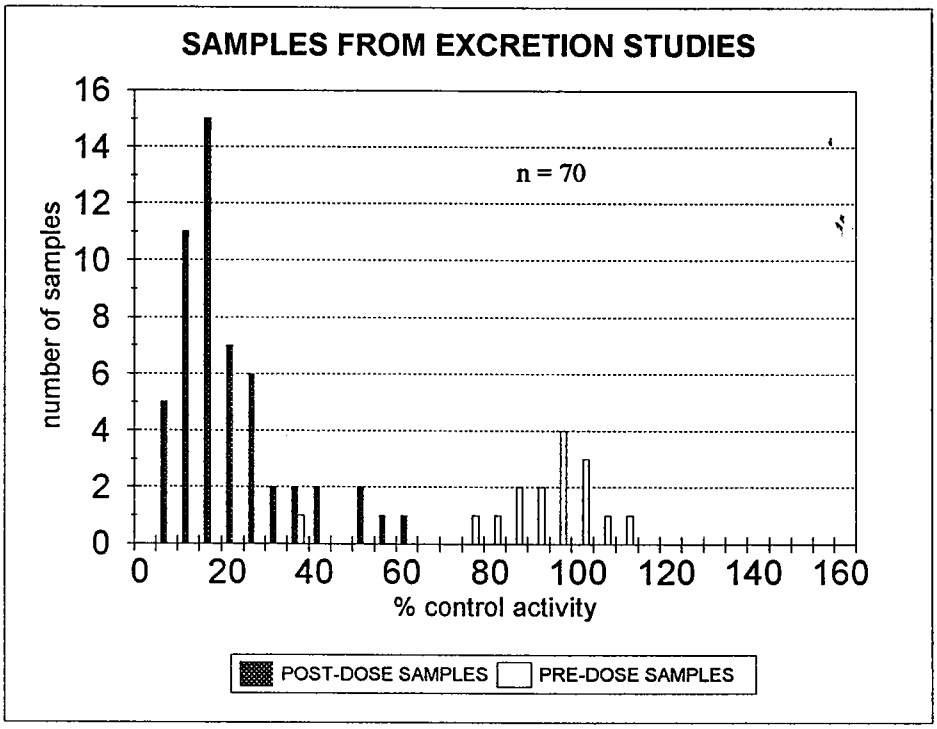


FIGURE 3.

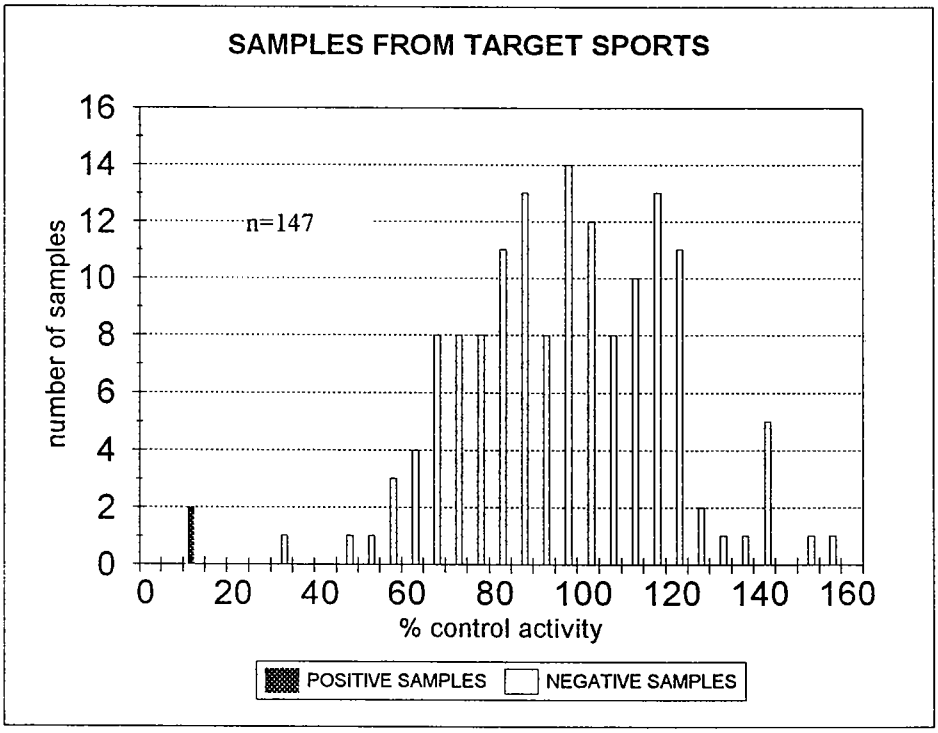


FIGURE 4.