THE GAS-LIQUID CHROMATOGRAPH AND THE ELECTRON CAPTURE DETECTION IN EQUINE DRUG TESTING

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ABSTRACT

Three gas-liquid chromatographic (G.L.C.) procedures discussed have been designed around the four "esses" of detection tests – speed, sensitivity, simplicity, and specificity. These techniques are admirably applicable to the very low plasma drug levels encountered in blood testing under pre-race conditions. The methods are equally applicable to post-race testing procedures, where both blood and urine samples are tested.

Drugs can only rarely be detected by the electron capture detector (E.C.D.) without a prior derivatization step, which conveys to the drug(s) high electron affinity. Because of broad applicability, two derivatizing agents, heptafluorobutyric (HFBA) and pentafluoropropionic (PFPA) anhydrides are employed.

The three techniques, allowing broad coverage of various drug classes are:

1) direct derivatization of drugs to form strongly electron capturing amides and esters.

2) reductive fragmentation of drugs with lithium aluminum hydride to form alcohols, with conversion to ester derivatives.

3) oxidative fragmentation of drugs with potassium dichromate to form derivatizable groups, followed by direct derivatization.

INTRODUCTION

Although the methods to be discussed in this paper will be referred to the broader concepts of post-race testing, they were developed principally for rapid blood analyses under pre-race testing conditions. Presently, in the Commonwealth of Kentucky, there is no pre-race drug testing. However, both blood and urine are secured from each horse to be tested.

The analytical laboratory is called upon to detect drugs whose therapeutic blood levels may range from a few picograms per millilitre to about a milligram per millilitre – a variation of one-billionfold* in concentration levels. Obviously, the method adequate for reserpine detection might not be the method of choice for dipyrone or salicylic acid. Also, it would be highly desirable and forensically sound to develop "screening" methods for the parent drug as administered, as opposed to biotransformation or metabolic products.

The great majority of drugs sought by the equine drug analyst lie in a concentration range of one nanogram per millilitre to one hundred micrograms per millilitre in blood or urine, with some notable exceptions. It is to find drugs in this concentration that most modern detection techniques are admirably suited.

Our laboratory employs a "modular" detection scheme for analyses of prohibited drugs. Samples, both blood and urine, are processed in screw-cap culture tubes, 125 mm or 150 mm in length. Biological sample volumes vary from 3 ml to 17 ml. In these tubes, depending upon the procedure(s), samples are hydrolyzed, liquid-liquid extractions are performed, derivatizations are accomplished and final wash steps, if necessary, are completed. Detection procedures employ derivatizations for both thin layer and gas-liquid chromatography; thin layer chromatography, gas-liquid chromatography, and ultra-violet spectrophotometry. Using this "modular" testing concept, the laboratory at the University of Kentucky has, in the first seven months of operation, confirmed one prohibited drug per 145 samples tested. Of the prohibited drugs found, 50% were detected by one or more of the gas-chromatographic procedures to be discussed in this paper.

**In USA 1 billion = 1 thousand million, i.e. 10^9*

A technique which we use in our laboratory, and which has been widely adopted for pre-race blood analyses employs gas-liquid chromatography (G.L.C.) with the electron capture detector (E.C.D.). As a gas chromatographic detector, the electron capture detector is very sensitive, perhaps 1,000 times more so than the commonly used flame ionization detector. The electron capture detector also detects only one-fifth as many substances as the flame ionization detector, and hence is a more specific detector.

The loss of standing current in the electron capture detector is a measure of the amount and electron affinity of the components in the carrier gas. This detector is extremely sensitive to certain molecules, such as organic and inorganic halogen-containing compounds.

Drugs can only rarely be detected by the electron capture detector (E.C.D.) without a prior derivatization step, which conveys to the drugs high electron affinities. Following derivatization, many drugs can be detected in biological fluids at very low concentrations. For simplicity, two derivatizing agents will be discussed, heptafluorobutyric and pentafluoropropionic anhydrides (HFBA and PFPA).

Three derivatization techniques will be discussed:

(1) *direct derivatization* of drugs containing primary or secondary amine or phenolic or alcoholic groups form strongly electron capturing amide and ester derivatives.
(2) *reductive fragmentation* followed by derivatization can be accomplished for some drugs which have groups which may be reduced with lithium aluminium hydride to form alcohols.
(3) *oxidative fragmentation* followed by derivatization is valuable for drugs which can be oxidized to form derivatizable groups.

In Tables I and II are noted a partial list of the many drugs amenable to these procedures. Numerous other drugs which have caused detection problems could be included. The derivatization methods are applicable particularly to those drugs found in low nanogram per millilitre concentrations in biological fluids. The limits of this method are the lability of a compound, the availability of derivatizable sites on a molecule, to some extent molecular weight and extraction and/or derivatization conditions.

The acylation procedures described are used primarily, but not always, with basic drugs. These drugs constitute the bulk of prohibited drugs.

Figure 1 depicts direct acylation and reductive fragmentation followed by acylation using methylphenidate as the drug model. Figure 2 represents oxidative fragmentation followed by acylation, with promazine as the drug model.

TABLE I

A representative listing of drugs which may be directly derivatized with fluorinated acylating agents

Amphetamine	Codeine
Methamphetamine	Phenothiazine
Ephedrine	Procaine
Mephentermine	Benzocaine
Pentazocine	Desipramine
Morphine	Apomorphine
Ketamine	Mephenesin

TABLE II

A representative listing of drugs which may be acylated following (a) reductive fragmentation and (b) oxidative fragmentation

Reductive Fragmentation

Cocaine	Indomethacin
Scopalamine	Anileridine
Meperidine	Atropine

Oxidative Fragmentation

Propiopromazine	Triflupromazine
Promazine	Imipramine
Acetylpromazine	Doxepin

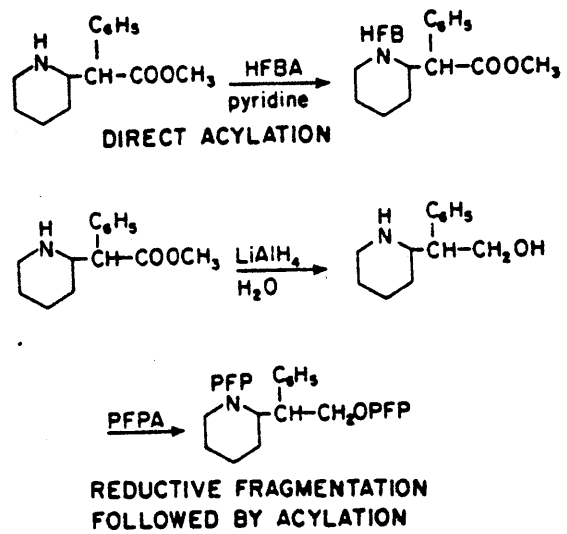

Figure 1

Acylation of methylphenidate by (a) direct derivatization with HFBA and (b) reductive fragmentation followed by direct derivatization with PFPA (Huffman and Blake, 1974).

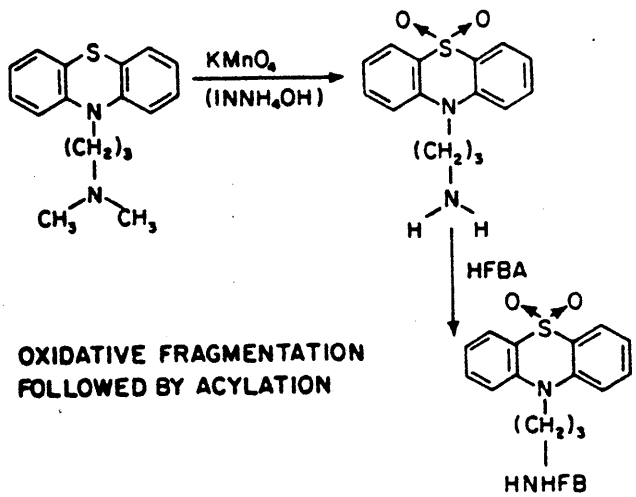

Figure 2

Acylation of promazine by oxidative fragmentation with $KMnO_4$ followed by direct derivatization with HFBA (Blake and Tobin, 1976).

Numerous variations of each procedure can be employed; – solvents such as cyclohexane, benzene, or dichloromethane, catalysts such as pyridine, ethylacetate or heat and washes using aqueous solutions of various pH may be used. The complexity of the acylation procedures may be adjusted to meet the conditions of testing, notably the time frame. Under pre-race conditions, if 80-100 blood samples are to be analyzed in four hours, certain procedures can be used, if five or six hours are available, more complex and more drug encompassing procedures may be used.

To explain the three procedures the "rule(s)" of "three and four" will be used.

Direct derivatization or acylation of a drug involves the extraction of 3 ml of plasma mixed with 3 ml of saturated sodium tetraborate into 3 ml of cyclohexane. To the cyclohexane phase is added 50 microlitres of heptafluorobutyric or pentafluoropropionic anhydrides (HFBA or PFPA) and 50 microlitres of a pyridine/benzene catalyst. Derivatization reaction time is 4 minutes, after which the cyclohexane phase is washed with 4 ml of 0.5N sodium hydroxide or 8 ml of saturated tetraborate solution. The derivatized drug(s) in the cyclohexane phase are now ready for G.L.C. analysis.

Reductive fragmentation followed by direct derivatization employs the extraction of 3 ml of plasma mixed with 3 ml of saturated sodium tetraborate into 3 ml of cyclohexane. To the cyclohexane phase is added 100 microlites of a saturated lithium aluminum hydride etherate to be followed by a 3-minute reduction reaction time. Twenty microlitres of distilled water is then added to the cyclohexane to stop reduction and destroy the excess $LiAlH_4$. From this point on, the procedure is identical to the direct derivatization cited. Pentafluoropropionic anhydride with a tetraborate wash is used where alcoholic sites are to be derivatized.

Oxidative fragmentation followed by direct derivatization involves the extraction of 8 ml of whole blood into 3 ml of dichloromethane. Prior to extraction, 3 ml of a 1N ammonium hydroxide solution saturated with potassium permanganate is added to the whole blood, well mixed, allowing for an oxidation period of 4 minutes. Following extraction the procedure follows that of direct derivatization, except that after a sodium hydroxide wash, the dichloromethane must be carefully evaporated to dryness, and the residue picked up in benzene prior to chromatography.

Gas-liquid chromatographic conditions employ short, three or four feet, glass columns packed with a non-polar phase such as 3% OV-1, 3% OV-101, or 3% SE-30. In the laboratory, the Varian machines with scandium-tritide foils have proved to be excellent units for routine analyses, although other chromatographs would suffice.

In summary, the detection of drugs in the biological fluids of racehorses has taken a new trend because of the development and availability in the last 10 years of excellent sensitive, analytical instrumentation. General derivatizing procedures for drugs have provided specific and sensitive gas chromatographic tests. Procedures have been and will further evolve toward smaller sample volumes using simpler, more specific, more instrumented and probably more automated techniques.

REFERENCES

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DISCUSSION

SCHOTMAN: I am surprised at the number of positives you have now found.

BLAKE: This is in a post-race situation where we do have the availability of hydrolysing our samples. About 16% of our positive drugs, particularly the phenothiazine tranquillisers, synthetic narcotics such as pentazocine do require a hydrolysis procedure. About 16% of these drugs are found as hydrolysed products, the others can be determined equally well in either blood or urine by direct extraction.

SCHOTMAN: Last year in Oslo there was an international congress of Jockey Clubs. On pre-race testing, someone reported that in the United States they had done about 20,000 pre-race samples and only one was positive, but it was a preventative measure. There was a great difference at the various racetracks, one is post-race testing, another is pre-race. You have had many more positive results.

BLAKE: Again, it depends on the training of the technicians, the analyst running the particular laboratory, the maintenance of equipment, and optimum conditions and time available. In one State they only have a very minimal amount of time, approximately 30 minutes, in which to run 10 blood samples for pre-race testing. Whereas in the other two racing States they have anywhere from 1½ to 2 hours per 10 samples and hence could certainly do a more thorough job. It is true, I think, as a rule of thumb there are fewer positives on a pre-race test but this need not necessarily be so if the technicians are well trained.

DELBECKE: We have had a scandium detector for 3 years and found it very difficult to clean the detector. Now Varian has a new model which is easier to clean but we have seen that the standard current drops very rapidly in a few moments from 80% to 20%. We think for qualitative work it is better to work with a nickel-63 detector instead of a scandium detector.

BLAKE: I would agree, depending on the person running the laboratory, nickel-63 might be a better choice with the new systems, giving extended linear dynamic range which is very important. The one reason that the scandium detector works so well in our laboratory is the fact that we use 3 and 4ft columns, hence we run our drugs at temperatures probably 30° to 40° under what most analysts would run their drugs. For instance, Tobin in his laboratory has the same machine using a 6ft column of OV-101 and indeed they are running into the same problems that you are experiencing, whereas we are running at 30° lower hence we rarely exceed a detector temperature of 260 to 270°. Most detectors are very easy to clean just by putting them into a scintillation vial with a little methanolic sodium or potassium hydroxide and heating them for one hour, rinsing them very carefully, wearing rubber gloves and taking necessary precautions. I found in the first 7 months of operation we had to change our columns only twice and we run approximately 100 samples per day through these detectors six days a week and our standing current has dropped about 25%, but it is true that your experience is the experience of most people using scandium detectors and the only difference I can see is the temperature we usually run our samples.

DELBECKE: Are you assured that after reduction with lithium aluminium hydride and after the washings that you obtain a pfp derivative of the amino and the hydroxol groups because we think that after reduction with lithium aluminium hydride and the washings, the acid fraction is not so stable and that we have obtained only a pfp of the amino group.

BLAKE: It depends on the pH of the wash. When you wash with an aqueous solvent of pH 9.5 or less I think you find by mass spectrometry that you do indeed get derivatization of both sites. The key is to wash with the lowest pH buffer you can to remove whatever undesirable components are present. It is true when you derivitize alcohol or phenol groups if the pH of your aqueous wash is too high you will indeed rapidly derivitize. In fact some of the original literature on procaine, where it was put into sodium hydroxide, they actually cleaved the ester function. So it depends on the aqueous wash. We have shown by mass spectrometry that we do have the derivatives of both the alcoholic and amine site on the piperidine ring. This is true of cocaine, we do get pfp derivatization in both contiguous hydroxyl sites.