DOPING CONTROL IN JAPAN

An Automated Extraction Procedure for the Doping Test

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ABSTRACT

Horse racing in Japan consists of two systems, the National (10 racecourses) and the Regional public racing (32 racecourses) having about 2,500 racing meetings in total per year.

Urine or saliva samples for dope testing are collected by the officials from the winner, second and third, and transported to the laboratory in a frozen state. In 1975, 76,117 samples were analyzed by this laboratory.

The laboratory provides the following four methods of analysis, which are variously combined by request.

- (1) Method for detection of drugs extracted by chloroform from alkalinized sample.
- (2) Method for detection of camphor and its derivatives.
- (3) Method for detection of barbiturates.
- (4) Method for detection of ethanol.

These methods consist of screening, mainly by thin layer chromatography and confirmatory tests using ultra violet spectrophotometry, gas chromatography and mass spectrometry combined with gas chromatography.

In the screening test of doping drugs, alkalinized samples are extracted with chloroform. In order to automate the extraction procedure, the authors contrived a new automatic extractor. They also devised a means of pH adjustment of horse urine by using buffer solution and an efficient mechanism of evaporation of organic solvent. Analytical data obtained by the automatic extractor are presented in this paper.

In 1972, we started research work to automate the extraction procedure in method (1) above, and the Automatic Extractor has been in use in routine work since last July. One hundred and twenty samples per hour are extracted automatically by three automatic extractors. The analytical data using this apparatus is presented below.

Introduction

The authors' laboratory is the sole organization in Japan for the testing of doping drugs in race horses. It deals with as many as 75,000 samples a year. In order to test such a large number of samples more efficiently, and to solve the environmental drawback of the testing room where a harmful solvent is used, an automatic extractor was developed. It permits the extraction of 40 samples per hour automatically when combined with a closed recovering system of evaporated organic solvent.

Frozen specimens were used because of transport difficulties and delays, and to prevent decomposition with the large numbers of samples to be handled.

Outline of the Screening Test

A screening test of doping drugs was carried out on alkalinized samples extracted with chloroform. It is summarized in Fig. 1.

Fig. 1. Scheme of the screening test

In an attempt to automate this procedure, each step of the method was reexamined to ascertain whether it

was applicable to an automatic procedure or not. Care was taken not to change the procedure markedly, so that the high detection sensitivity of the method might be maintained.

In the course of the reexamination, a means of pH adjustment of the horse urine sample by buffer solution was devised. Moreover, suitable methods of extraction and evaporation were investigated.

Basic Data on the Automatic Extractor

Adjustment of pH with Buffer Solution

As shown in Fig. 1, formerly the pH of urine was adjusted to 9 with ammonium hydroxide and/or ammonium chloride by checking with hydron paper. Instead of this method, a new one was proposed. In it, the urinary pH was adjusted to around 9 by adding a definite amount of buffer solution.

The sodium sulphite is used to prevent oxidation of drugs in the urine.

At first, about 10,000 urine samples of race horses which had been received by the laboratory in a frozen state were examined by the pH meter at room temperature. No preservatives had been added to any sample. The results obtained are shown in Fig. 2. It was made clear that the urinary pH of race horses was distributed over an extremely wide range of pH.

Fig. 2. Histogram of urinary pH of race horses

In order to adjust these various kinds of urinary samples to around pH 9, several alkaline buffer solutions were examined. Finally, a modified Gomori's buffer solution was selected as the most suitable one.

The titration curve of this buffer solution is shown in Fig. 3. It was decided to use this for further experiments. The solution consisted of 2-amino-2-methyl-1,3-propanediol (4.6 M) adjusted to pH 9.3 with concentrated hydrochloric acid.

The optimum amount of buffer solution to be added to 30 ml of urine was examined. As a result, 99% of 400 urinary samples collected at random were within a range of pH from 8.5 to 9.5 when 2.0 ml of this buffer solution was added.

Fig. 3. Titration curve of modified Gomori's buffer solution

To 2,700 urine specimens chosen at random was added 2.0 ml of this buffer solution to measure the pH of each sample. The results obtained are shown in Fig. 4.

Fig. 4. Histogram of urinary pH after addition of 2 ml of buffer solution

From these results, it was decided to use 2 ml of 4.6 M, 2-amino-2-methyl-1,3-propanediol (pH 9.3) for the pH adjustment of urine samples in the automated procedure.

Extraction

A centrifugal cup extraction mode was devised as a suitable mechanism for automatic extraction. It is a characteristic of this mode of extraction that the drug can be extracted with the same amount of solvent as the urine sample, without emulsification.

The extraction cup is shown in Fig. 5. This cup is 150 mm in height and 62 mm in diameter. The inner side of the cup is coated with Teflon.

Fig. 5. Mode of cup extraction

The cup spins continuously. Therefore, the sample, solvent, and reagents, having entered the cup, are spread as thin films on the wall of the cup, as shown on the left side of the figure. The spinning film of the immiscible solvent is ideally suited for extraction by another film of the sample sliding over its surface. Fig. 6 shows the results of recovery by this mode of extraction with 30 ml of caffeine solution and the same amount of chloroform.

Fig. 6. Relationship between recovery and rotating time

From this experiment, it was found that the amount of a drug recovered did not increase with the prolongation of rotating time, contrary to expectation. To increase the rate of recovery, the cup was spun (2500 r.p.m. at maximum) for 7 seconds until the film of the two solutions reached the top of the cup. The spinning was stopped within 13 seconds, so that the two solutions might go down slowly to the bottom of the cup and come to a standstill. Thirteen seconds was a minimum time for this. The standstill condition was indispensable to prevent urine and solvent from being emulsified. The effect of repeated spinning and stopping (ON-OFF) of the cup is shown in Fig. 7.

Fig. 7. Effect of repeated spinning and stopping of the cup on drug recovery

Recovery of caffeine was improved to some extent by repeated spinning and stopping of the cup. Table I shows recovery of some drugs with chloroform by repeating 11 times.

TABLE I
Recovery of some drugs with chloroform

Drugs	Concn. (µg/ml)	Recovery (%)
Caffeine	5.0	70.5
Theophylline	30.0	7.0
Aminopyrine	10.0	70.9
Ephedrine	50.0	49.9

When the results of drug recovery were compared, the cup extraction method was inferior to the shaking method with 1 part of the sample and about 4 or 5 parts of solvent for extraction.

From the results of these experiments, it was decided to use 30 ml of urine (twice as much as urine used in the shaking method) to maintain the detectable limit of the method.

Extraction Solvent

To improve poor recovery of such a drug as theophylline with chloroform, as shown in Table I, addition of some alcohols was investigated. The addition of n-butanol exhibited a remarkable effect, as is clear from Table II.

The addition of 5% butanol to chloroform was effective not only for theophylline, but also for some other drugs, such as theobromine, lidocaine, and ephedrine. For this reason, it was decided to adopt this solvent in the automatic procedure.

TABLE II
Effect of addition of alcohols on recovery of theophylline

i) Alcohol in Chloroform

Drug \ Solvent	Recovery (%)				
	A	B	C	D	E
Theophylline	7.0	9.0	9.7	14.5	24.9

- Solvent A : Chloroform
B : 5% Methanol in Chloroform
C : 5% Ethanol in Chloroform
D : 5% Isopropanol in Chloroform
E : 5% n-Butanol in Chloroform

ii) n-Butanol in Chloroform

Drug \ n-Butanol %	Recovery (%)				
	1	2	3	4	5
Theophylline	6.1	9.7	12.9	17.4	22.2

Evaporation of Solvent

One of the troublesome problems on the rapid evaporation of the solvent is to prevent the solvent from bumping under reduced pressure. To solve it, the vapor of the solvent is introduced into the cooling units by the aid of a vacuum pump to supply almost the same amount of dried air as the vapour simultaneously, as shown in Fig. 8.

Fig. 8. Method of evaporation

To prevent the loss of the drug contained in the evaporating vessel during the heating of the solvent, two heating baths are adopted. One of the baths is regulated

at 85°C to evaporate about 95% of 30 ml of the solvent within 15 minutes, and the other at 45°C to evaporate the solvent completely.

Design of Automatic Extractor

On the basis of the above-mentioned results, a screening procedure was established as summarized in Fig. 9. An automated procedure was designed, as shown in Fig. 10.

Fig. 9. Scheme of a new screening test

Fig. 10. Schematic diagram of automatic extractor

As shown in Fig. 10, 30 ml of sample in a beaker is sucked into a coiled tube by the aid of the pump P₁ through the valve V₃ and transferred to the extraction cup (Extractor) with its pathway changed by the valve V₃. At the same time, 0.8 ml of 12.5% Na₂SO₃ and 2.0 ml of buffer solution are poured into the extractor by the pumps P₂ and P₃, and also 33.5 ml of chloroform containing n-butanol by pumps P₄ and P₅.

After ON-OFF extraction is performed 11 times, 29 ml of the solvent is sucked by the pump P₅ and introduced into the evaporating vessel by opening the valve

V₇. To this vessel 0.1 ml of 1% tartaric acid solution in alcohol is added before evaporation. The solvent is heated and evaporated in two heating baths. The vapour produced is led to two condensers. By this process about 85% of the solvent can be recovered.

The pathways of the sample and solvent are so designed that the sample may be washed each time to avoid cross contamination.

All these steps are carried out automatically, and 40 samples can be treated in an hour. If any trouble occurs to the automatic extractor, all the operation is suspended. This is made known by a buzzer.

Detection of Doping Drugs by Automatic Extractor

To determine the minimum detectable limit of a drug by the automated procedure a given amount of the drug was added to 30 ml of pooled horse urine, which was extracted by the automatic extractor. The resulting residue was detected by two systems of thin layer chromatography according to the method illustrated in Fig. 1.

In this determination, 4 samples were examined for each dose of the drug. A minimum amount of the drug showing a positive reaction in all the 4 samples was regarded as a minimum detectable limit.

The results obtained are shown in Table III accompanied with the data obtained by shaking method, as shown in Fig. 1, using 15 ml of the same urine sample.

A good detectable sensitivity of the method was maintained by the automatic procedure.

TABLE III
Minimum detectable limits of drugs by automated procedure

Drug μ g added to 30 ml of horse urine	AUTOMATIC		SHAKING	
	TLC-I	TLC-II	TLC-I	TLC-II
4-Aminoantipyrine	30.0	60.0	40.0	60.0
Aminopyrine	10.0	40.0	10.0	40.0
Antipyrine	5.0	10.0	5.0	10.0
Atropine	5.0	5.0	10.0	5.0
Brucine	10.0	10.0	10.0	10.0
Caffeine	2.5	2.5	2.5	5.0
Chlorpromazine	10.0	10.0	10.0	10.0
Chlorpromazinesulfoxide	5.0	5.0	5.0	5.0
Cocaine	5.0	10.0	10.0	20.0
Dibucaine	2.5	2.5	2.5	2.5
Dihydroxypropyltheophylline	100.0	80.0	100.0	80.0
Ephedrine	30.0	5.0	30.0	5.0
Lidocaine	10.0	40.0	10.0	40.0
Methamphetamine	20.0	20.0	60.0	20.0
Methapyrilene	10.0	10.0	2.5	10.0
4-Methylaminoantipyrine	20.0	80.0	20.0	80.0
Methylephedrine	20.0	20.0	20.0	20.0
Methylphenidate	20.0	40.0	20.0	40.0
Morphine	10.0	20.0	10.0	20.0
Nicotine	5.0	20.0	2.5	10.0
Nikethamide	30.0	30.0	30.0	30.0
Oxyethyltheophylline	10.0	—	20.0	—
Oxypropyltheophylline	10.0	40.0	10.0	40.0
Pentetrazol	20.0	20.0	30.0	20.0
Phenylbutazone	50.0	—	50.0	—
Pipradrol	20.0	10.0	20.0	10.0
Phenacetin	5.0	10.0	5.0	10.0
Procaine	5.0	10.0	5.0	10.0
Promazine	5.0	10.0	5.0	10.0
Scopolamine	20.0	20.0	20.0	20.0
Strychnine	20.0	10.0	20.0	10.0
Tetracaine	5.0	20.0	5.0	20.0
Theobromine	5.0	10.0	5.0	10.0
Theophylline	20.0	20.0	20.0	20.0

DISCUSSION

HOUGHTON: What type of molecular separator do you use with your mass spectrometer?

CARTONI: The best thing is to have no molecular separator at all and when we work with a capillary column we send all the column effluent inside the ion source. For packed columns we use the silicone membrane separator where the possibility of absorption of the sample is reduced. We have had very bad results with the Biemann separator, and there is a strong absorption of basic and high-boiling compounds. The silicone membrane separator seems the best, if you have to use a membrane.

HOUGHTON: We have the silicone membrane connected to our organic MS-20 and occasionally we have had problems with amphetamine. Have you noticed any problems of this type at all?

CARTONI: For amphetamine we have had no problems, at 150° or 160° it will pass. Our problems have been with the much higher boiling compounds, tranquillisers and so on.