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ISOLATION, DETECTION AND IDENTIFICATION OF SOME ALKALOIDS OR ALKALOID-LIKE SUBSTANCES IN BIOLOGICAL SPECIMENS FROM HORSES WITH SPECIAL REFERENCE TO DOPING

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SUMMARY

The isolation, detection and identification of twelve alkaloids or alkaloid-like substances from aqueous solutions are described. The best extraction was obtained with chloroform, and the detection and identification was performed by thin-layer chromatography.

These extraction and chromatography procedures were applied to the isolation and detection of the same alkaloids from biological materials after their injection and passage through the bodies of horses. Samples of saliva, plasma and urine were examined at different times after the injection. Urine seemed to be the most reliable biological fluid for the detection of these substances. The method fully satisfies the requirements for the rapid determination of these substances in doped race horses and even in human athletes.

INTRODUCTION

Although in human sport such as cycling and swimming doping has only created a furore in recent years, in horse racing it has always been a problem[6,8,20,23,27]. The most usual concept of doping is the administration of a substance with the power to stimulate the animal, thus enabling it to realize a performance superior to its normal capacity. This means "doping to win". But the aim of doping can be the reverse, *viz.* to depress the organism and diminish the racing capacity so that it performs badly and loses the race. This is called "doping to lose".

Hence the substances used for doping can be classified into two groups: the stimulants such as the weckamines or the stimulating alkaloids, and the depressants such as the barbiturates or the tranquillizers.

At present the stimulating doping agents are most commonly used. As established from our own routine examinations for the Belgian and Dutch Horse Racing Associations the weckamines, amphetamine and methylamphetamine, are at present the substances most frequently administered. But nevertheless stimulating alkaloids

such as caffeine, strychine, lobeline and even quinine or alkaloid-like substances such as niketh amide are still used for this purpose.

Because of the small doses used, the "doping" agents only appear as traces in the biological fluids. Hence the methods of isolation, detection and identification must be very sensitive[8,23]. But since the winning owners are often not paid until a negative result has been received from the laboratory, the method must not be time consuming. Because of this we have developed an extraction method to isolate, and a thin layer chromatographic method to detect and identify simultaneously as many alkaloids as possible in the biological fluids taken from race horses.

Although numerous methods have been described for identification of alkaloids[11], most of them relate to the extraction from pure aqueous solutions or from pharmaceutical preparations and only a limited number to the extraction from biological materials such as urine and blood. Most methods are based on paper chromatography[7,13–17,23,26,29,32,33,35,37]. In the last ten years however some papers have been published on thin layer chromatographic detection techniques for one or more alkaloids with especial reference to doping[3,4,10,19,24,27,34].

ISOLATION, DETECTION AND IDENTIFICATION OF THE ALKALOIDS FROM AQUEOUS SOLUTIONS

Materials

Twelve different alkaloids or alkaloid-like substances with a stimulating action on horses were examined (see Table II). The free base form of each alkaloid was prepared by extracting it with chloroform from an aqueous alkaline solution of its respective salt. The crystals or liquids obtained were examined for their purity by means of melting point determination and U.V. spectrophotometry. For each substance tested, a standard curve was prepared, with a solution of the free base in 0.1 *N* sulphuric acid, and the extinction values at the wavelengths of the absorption maxima determined for their respective U.V. spectra. As sparteine did not show an absorption maximum it was impossible to prepare a standard curve for this alkaloid.

Extraction

The purpose of this work was to develop an extraction method for the isolation of some alkaloids after passage through the body from biological materials, such as urine. From our own experience we had established that the extracts of some urines after direct alkaline extraction showed many impurities on the chromatogram and that this disadvantage could be diminished by a preliminary acid extraction followed by the alkaline one. However it was possible that in this preliminary acid extraction some of the alkaloids could be extracted.

In order to examine this possibility each extraction was made twice, namely directly from an acid solution and directly from an alkaline one. With regard to the extracting solvent, we looked for an organic solvent with a boiling point as low as possible in order to facilitate evaporation, and an extraction rate which was as high as possible. Three solvents were tested, *viz.* ether, chloroform and a mixture of chloroform–isopropanol (4:1). The latter was tested because many authors claim that it is one of the best for the extraction of morphine. The acid as well as the alkaline extractions were made with these three solvents in duplicate. The different aqueous

TABLE I

PERCENTAGE EXTRACTION RATES

Alkaloid	*% alkaline extraction*			*% acid extraction*		
	Chloroform	*Ether*	*Chloroform–isopropanol*	*Chloroform*	*Ether*	*Chloroform–isopropanol*
Brucine, 300 mμ	86	24	83	49	10	69
Caffeine, 273 mμ	78	20	50	85	11	90
Codeine, 284 mμ	92	42	79	2	20	0
Heroin, 280 mμ	38	0	29	0	8	0
Morphine, 285 mμ	0	0	25	0	20	9
Papaverine, 308 mμ	98	98	95	96	10	100
Quinine, 317 mμ	92	95	85	10	4	7
Sparteine, polarography	51	—	50	30	—	—
Strychnine, 255 mμ	85	47	89	41	0	39

solutions with a concentration of 2 μg/ml were extracted thrice with 5 ml of each solvent for 20 minutes. The respective solvent phases were collected, dried over anhydrous sodium sulphate and evaporated under vacuum in a rotary film evaporator.

Simultaneously a blank extraction without the addition of the alkaloid was made.

Determination of the extraction rates

The evaporated residues of the alkaloids as well as of the blanks were redissolved in 4 ml 0.1 *N* sulphuric acid and their optical densities determined by U.V. spectrophotometry against 0.1 *N* sulphuric acid. From the standard curve and the densities obtained it was possible to derive the percentage extraction rates. These are listed in Table I. These percentages are the average of three extraction experiments or six readings for the alkaloid solutions deducted from the average of the six readings for the blanks.

As sparteine did not show any absorption maximum the percentages for this alkaloid were calculated by means of polarography and only for chloroform. The extinction values for nikethamide, lobeline and cocaine, in the concentration used (2 μg per ml) were so low that it was impossible to determine the extraction rates for these substances with accuracy.

These results show that chloroform gives the highest extraction rates for the total group of alkaloids tested.

Detection

The detection of the different substances, as already mentioned, was carried out by means of thin-layer chromatography.

Details of the methods used

We used 20 × 20 cm GF_{254} (Merck) chromatoplates with a layer thickness of about 0.250 mm. We preferred Silica Gel GF because all the alkaloids tested, except sparteine, could be detected at 254 mμ without prior treatment as absorbing spots in quantities ranging from 2 to 5 μg with the exception of cocaine and lobeline which could only be detected from 20 μg. After trying different solvent systems the most satisfactory separation for the twelve alkaloids was obtained with a mixture of hexane–acetone–diethylamine (6:3:1). The chromatoplates were developed in this mixture to give a solvent front rise of 15 cm. This took about 30–40 min. Then the plates were dried with cold air and examined under U.V. light at 254 mμ.

Finally the sheets were consecutively sprayed with:

(*a*) *Dragendorff's reagent modified by Meunier and Macheboeuf.* Solution A: 0.85 g basic bismuth nitrate; 10 ml acetic acid 96%; and 40 ml distilled water. Solution B: 20 g KI dissolved in 50 ml water.

Both solutions were mixed and kept in a dark brown bottle. 10 ml acetic acid and 35 ml water were added to 5 ml of this mixture just before spraying. The chromatograms were sprayed till the orange-yellow spots of the alkaloids were just visible.

(*b*) *A saturated solution of silver sulphate in 10% sulphuric acid.* The chromatograms were sprayed until they were uniformly grey.

(*c*) *Dragendorff's reagent.* The plates were sprayed again with this reagent until they were homogeneously light brown coloured.

As a result of these three successive sprays, the spots of the different alkaloids became still sharper and more pronounced as compared with the colour gradation after the first spray with Dragendorff's reagent. Moreover, in this way it was possible to make caffeine visible as a brick-red spot as mentioned previously by HAUCK[13], and nikethamide as an orange spot with a grey border, whereas these two substances were not detectable after the first spray with Dragendorff's reagent.

By this thin layer chromatographic method it was not only possible to detect the twelve alkaloids mentioned but also some of the weckamines such as methamphetamine, mephentermine and phenmetrazine.

TABLE II

DETAILS OF THE THIN-LAYER CHROMATOGRAPHIC DETECTION METHOD

Alkaloid	*Sensitivity (in μg) under U.V. light at 254 mμ*	*Sensitivity (in μg) after spraying*	R_F *values*	*Colour reactions*
Brucine	2	2	0.16	yellow-brown
Caffeine	5	5	0.44	brick-red
Cocaine	20	5	0.86	brown
Codeine	5	2	0.45	orange-brown
Heroin	5	5	0.42	orange-yellow
Lobeline	10	5	0.65	yellow-brown
Morphine	5	5	0.12	orange-yellow
Nikethamide	5	20	0.56	orange
Papaverine	5	5	0.50	yellow
Quinine	5	2	0.37	orange-yellow
Strychnine	2	5	0.36	yellow
Sparteine	—	2	0.96	orange

Identification

Identification was possible at first by means of the R_F values under U.V. light before and after spraying. As shown in Table II the R_F values for some of these alkaloids do not differ much from each other so that it is often difficult to differentiate them by the R_F values alone. This is especially true for strychnine, quinine, heroin, caffeine and codeine.

A second identification method however is offered by the coloration of the spots after spraying. Caffeine can be identified by its specific brick-red colour; strychnine is yellow, codeine is orange-brown and heroin gives an orange-yellow colour. Quinine is the only alkaloid which gives a clear-blue fluorescent spot under U.V. light at 366 mμ after the first Dragendorff's spray and even after the silver sulphate spray.

A third possibility of identifying the different alkaloids is offered by two-dimensional thin-layer chromatography. Reference substances are run on the same plate in the following way. 5 cm from the right hand edge a line is scored up the whole length of the plate. A similar line is scored 5 cm from the upper edge. These 5 cm tracks are used to spot the reference substances. The reference spots are positioned in each track so that they move alongside the mixture of drugs in the first and second runs, respectively. The reference tracks may themselves be scored further to give three 1.5 cm tracks, or two 2.5 cm tracks depending upon whether three or two reference spots are used. Thus, the mixture is spotted in the lower left hand corner, 1.5 cm from the lower and left edge, and the reference compounds are spotted separately 1.5 cm from the lower edge in the right hand track, and 1.5 cm from the left hand edge in the upper track. First, the chromatograms are developed with a mixture of chloroform–methanol–diethylamine (95:5:0.05) till the front reaches the 15 cm line. Thereafter the plates are dried and placed in the second solvent composed of a mixture hexane–acetone–diethylamine (6:3:1). The chromatograms are again developed in the second direction until the liquid reaches the other 15 cm line. A colour reaction, after spraying, identical with the reference spots and with an equal R_F value in both directions confirms the nature of the alkaloid examined.

A final identification method is based on the U.V. spectrum. After the second spray with Dragendorff's reagent the spots are discoloured and eluted. This discolouration is performed by the consecutive treatment of the spot with a drop of aqueous sodium sulphite 10 %, barium chloride 10 % and ammonium hydroxide 80 %[9]. Thereafter the silica gel in the position of the spot is scraped off and the alkaloid is eluted from the silica gel with chloroform. The chloroform layer is centrifuged, evaporated, and the residue redissolved in 0.1 *N* sulphuric acid for spectrophotometric examination.

ISOLATION, DETECTION AND IDENTIFICATION OF THE ALKALOIDS FROM THE BIOLOGICAL FLUIDS OF HORSES AFTER PASSAGE THROUGH THE BODY

Materials

Ten different female horses with a body-weight of 400–500 kg were injected I.M. with therapeutic doses of the twelve alkaloids, separately or in combination (see Table III).

Just before the injection the animals were placed in the experimental box and their mouth thoroughly rinsed with tepid water. Thereafter control samples of urine,

saliva and blood were taken. The collection of urine was performed by catheterization and complete emptying of the bladder; the blood was collected by puncture of the jugular vein and collected in a receptacle containing a few drops of concentrated sodium citrate solution. Saliva was collected by rubbing the lining of the mouth and the teeth with swabs of gauze held in a long forceps. After these samples had been taken the alkaloid under investigation was injected. Samples of blood, urine and saliva were taken again in the same manner 1.5 h, 3 h and 6 h after the injection in order to study the excretion. For the more slowly absorbed or excreted substances, even 12 and 24-h samples of saliva and urine were taken.

Extraction

Saliva. Immediately after their collection in the extraction tubes, the swabs of gauze were drenched in 25 to 30 ml 1 *N* hydrochloric acid. After shaking the tubes for 30 min the liquid was poured off and the swabs wrung out. After centrifuging the supernatant was made alkaline to pH 9–10 with 50 % NaOH and extracted three times with 5 ml chloroform for 20 min by rotating the tubes. The organic phases were collected, dried over sodium sulphate and evaporated.

Blood. The blood was centrifuged and 25 ml plasma were deproteinized with 5 ml trichloroacetic acid, 20 %. After centrifuging the supernatant solution was made alkaline and extracted with chloroform in the same way as described for the saliva.

Urine. As can be seen from the extraction data, some alkaloids could be as satisfactorily extracted at an alkaline pH as at an acid pH. For that reason, the extraction of the urine was made at both alkaline and acid pH, with ether and chloroform as extracting solvents.

From each urine sample, 25 ml were pipetted into 4 glass-stoppered 40 ml extracting tubes. Two of them were made alkaline to pH 9–10 with 50 % NaOH for the alkaline extraction and extracted three times with 5 ml chloroform or ether for 20 minutes. The other two were acidified to pH 2 with concentrated HCl and extracted in the same way. Because of the formation of an emulsion in some extracts, especially the alkaline ones, it was necessary to centrifuge the tubes for 10 minutes at 2000 r.p.m. in order to obtain a sharp separation of the two phases. The chloroform extracts were collected, dried over anhydrous sodium sulphate and evaporated.

Detection and identification

The evaporated residues were dissolved in 0.1 ml chloroform and spotted on the chromatoplates. Since the R_F values varied from plate to plate and were altered by salts and other contaminants of the biological extracts it was necessary for identification to co-chromatograph on the same plate reference spots of the alkaloid to be examined and extracts of the control sample with and without the addition of the same alkaloid. The chromatograms were developed by ascending chromatography till the liquid reached 15 cm above the starting point, examined under U.V. light at 254 mμ and sprayed as described above.

RESULTS

The results of the different experiments are summarized in Table III.

TABLE III

RESULTS AFTER THE INJECTION AND PASSAGE THROUGH THE HORSE'S BODY

Alkaloid	*Dose (g)*	*Urine*									
		Acid extraction									
		Ether					*Chloroform*				
		*1.5**	*3*	*6*	*12*	*24*	*1.5*	*3*	*6*	*12*	*24*
Brucine	0.100	—	—	—	—	—	—	—	—	—	—
	0.150	—	—	—	—	—	—	—	—	—	—
	0.175										
Caffeine	2.000	—	—				+	+			
Cocaine	0.400	—	—	—			—	—	—		
	0.500	—	—	—			—	—	—		
	0.500				—	—				—	—
	0.700	—	—	—	—	—	—	—	—	—	—
Codeine	0.650	—	—	—			—	—	—		
	0.750	—	—	—			—	—	—		
Heroin	0.150	—	—	—			—	—	—		
Lobeline	0.030	—	—	—			—	—	—		
	0.060	—	—	—			—	—	—		
	0.080	—	—	—			—	—	—		
Morphine	0.250	—	—	—			—	—	—		
	0.275	—	—	—			—	—	—		
	0.300	—	—	—	—	—	—	—	—	—	—
	0.350	—	—	—	—	—	—	—	—	—	—
Niketharnide	3.750	—	—				+	+			
Papaverine	0.350	—	—	—			—	—	—		
	0.500	—	—	—	—	—	—	—	—	—	—
	0.600	—	—	—	—	—	—	—	—	—	—
Quinine	0.750	—	—	—			—	—	—		
	0.750	—	—	—			—	—	—		
Sparteine	0.250	—	—	—			—	—	—		
	0.400	—	—	—			—	—	—		
Strychnine	0.050	—	—	—			—	—	—		
	0.070	—	—	—			—	—	—		
	0.075	—	—	—			—	—	—		
Combination											
Niketharnide Caffeine	1.000 1.000										
Lobeline Caffeine	0.080 1.000										
Niketharnide Sparteine Strychnine	1.000 0.400 0.075										

* Time in hours after injection.

Alkaline extraction										*Saliva*					*Plasma*				
Ether					*Chloroform*					*Chloroform*					*Chloroform*				
1.5	*3*	*6*	*12*	*24*	*1.5*	*3*	*6*	*12*	*24*	*1.5*	*3*	*6*	*12*	*24*	*1.5*	*3*	*6*	*12*	*24*
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
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					+	+	+			−	−	−			+	−	−		
					+	+	+			−	−	−			−	−	−		
					+	+	+			−	−	−			−	−	−		

Saliva

Of the twelve alkaloids injected, seven were never detected. These were brucine, heroin, lobeline, morphine, papaverine, sparteine and strychnine.

Quinine was the one and only alkaloid that could be detected in all the saliva samples examined and this in a diminishing concentration from 1.30 h to 6 h after the injection. The identification of quinine was made much easier by the strong blue fluorescence under U.V. light at 366 mμ after the first spray with Dragendorff reagent and even after the silver sulphate spray.

Caffeine also could be identified in all the saliva samples with certainty, but only after the injection of the highest doses. The remaining three alkaloids, cocaine, codeine and niketharnide, also were detected after the highest doses, but only in some of the samples.

Plasma

In the plasma only six of the twelve alkaloids injected were identified. Four of them were the same as for saliva, *viz.* caffeine, codeine, niketharnide and quinine. In addition to these lobeline and sparteine were also detected, whereas cocaine, like brucine, morphine, papaverine and strychnine, in contrast to the saliva, was not detectable at all in plasma.

Here also quinine was the only one alkaloid that was detectable in all the samples examined and again in diminishing concentration from 1.30 h to 6 h.

Urine

Three of the twelve alkaloids administered, heroin, morphine and papaverine could not be detected in either the alkaline or acid extracts.

Moreover acid extraction with ether did not reveal any of the twelve alkaloids. In the acid chloroform extracts only the highest dosed alkaloids such as caffeine and niketharnide could be identified.

In the alkaline ether extracts, in addition to the three alkaloids already mentioned, niketharnide was never detectable. The remaining alkaloids on the contrary could be detected more or less readily. Quinine and sparteine were the only alkaloids that were identified in all the samples examined. Besides the normal fluorescent spot for quinine after the spray with silver sulphate a second fluorescent spot was detectable on each chromatogram just beyond the starting-point. This spot was probably a metabolite of quinine and its concentration, as could be derived from the size and the intensity of the spot, increased with time after the injection. A second spot with an R_F value a little lower than that for sparteine was also seen. On each chromatogram this second spot was more concentrated than the corresponding spot for sparteine and this was especially the case in the extracts of the 3-h samples. After the injection of 70 and 75 mg of strychnine, two spots, with an R_F value just beyond the spot of strychnine, were seen on the chromatograms for all the samples, the concentration increasing from 1.5 h till 6 h.

After alkaline extraction with chloroform all the alkaloids except the three substances already mentioned, were detected in most of the samples investigated. The chromatograms developed after the highest doses of brucine showed, besides the spots of brucine, two other spots with R_F values 0.13 and 0.6, respectively. It was noteworthy that the intensity and the size of the spots for caffeine increased with th

course of time after injection in contrast to the ether extracts which did not show any gradation. The higher the dose of cocaine injected the better and longer was it detectable in the urine samples. The spots of codeine for the chloroform extracts were much more intense than those for the ether extracts, with a progressive fall in intensity and size towards 6 h. After the highest doses a second spot was seen just beyond the starting point. Besides the two fluorescent spots already mentioned for quinine in the ether extracts, a third spot with a weaker fluorescence appeared just above the spot for quinine (R_F value = 0.53). In addition to quinine two metabolites of quinine were probably isolated by extraction with chloroform. After extraction with chloroform the spots for lobeline were more pronounced but, as for quinine, impurities interfered somewhat. Just like in the ether extracts the same spots were detected for sparteine in the chloroform extracts, but they were greater and more intense. In agreement with the alkaline extraction with ether, strychnine could be isolated from the chloroform extracts after the injection of 75 mg, but not after the 50 mg dose. In contrast, after the 70 mg dose it was detectable in the chloroform extracts. The two metabolite spots were also revealed in the chloroform extracts as for the ether extracts, but they were more pronounced. Moreover after the 75 mg dose a fourth spot with an R_F value of 0.73 appeared on the chromatoplates. This spot probably corresponds to a third metabolite.

DISCUSSION

As can be deduced from our results after the injection of the different substances into horses, it can be seen that the thin-layer chromatographic detection method used, fully satisfies the requirements for the investigation of the possible presence of these substances in biological materials from doped race horses, and even from human athletes.

In the first place it is very simple and not time consuming. Further it has a very broad spectrum because not only could the twelve alkaloids injected by us be identified, but also the alkaloids pilocarpine, atropine and eserine and even the weckamines methamphetamine, mephentermine and phenmetrazine. The examination of the chromatoplates under U.V. light at 254 mμ immediately after development, the different specific colour reactions after the combined spraying with Dragendorff's reagent and silver sulphate, and the different R_F values all help to identify and differentiate with certainty most of the substances examined. Eventual doubts can definitely be removed by using the two-dimensional chromatography technique described as well as the elution method followed by spectrophotometry.

Finally the detection method used is very sensitive. Except for nikethamide which could be detected only in quantities of 15–20 μg all the other alkaloids were readily detectable in quantities of 5 μg and even 2 μg.

In addition to the detection method, the extraction method is also of great importance in this work. It must have such a high yield that the extracts contain sufficient quantities for them to be detected chromatographically. From the mean extraction values it is seen that the best results were obtained after alkaline extraction with chloroform for nearly all the alkaloids tested, excepting morphine where the mixture chloroform–isopropanol gave the best results. The comparative extraction rates with ether at an alkaline pH were mostly remarkably smaller. It is well known

that alkaloids, in view of their basic character, are very readily extracted from aqueous alkaline solutions by means of organic solvents. This was again proved by the high rates for the alkaline extractions and confirmed by the results of the extractions from biological fluids after passage through the body of the horse. Nevertheless from our results it appears that some alkaloids are also very readily extracted from aqueous acid solutions, especially with chloroform. Besides the well-known example of caffeine, papaverine and to a lesser extent strychnine and brucine could be extracted in the same way. These facts were confirmed by our results on the urine extracts for caffeine and even nikethamide. Hence preliminary extraction of urine with chloroform at acid pH, in order to obtain a more suitable and pure alkaline extract, can result in a loss of a great part of the substances. A preliminary ether extraction from acid solution on the other hand seems to be less dangerous.

The results obtained by application of the method described to the detection of doping agents in biological materials after passage through the body seemed to be very satisfactory and very similar to those obtained for the extractions from aqueous solutions. Many methods are described for the extraction from pure aqueous solutions and sometimes from biological fluids after the addition of the substances examined[11]. This does not mean however that these methods are always applicable and sensitive enough for the detection of the same substances after injection and passage through the body.

Except for heroin, all the doses injected were lower than the maximum allowed therapeutic doses. Heroin alone in the dose administered, provoked some signs of excitation and raised irritability, whereas for the other alkaloids administered no symptom of excitement or nervousness was observed, so that we can conclude that the dose necessary to dope a horse with success must at least be as high as our maximal doses.

Furthermore, it seems from our results that urine is the most reliable biological fluid for the detection and control of doping agents in race horses, since nine of the twelve alkaloids tested could be identified in the urine after alkaline extraction with chloroform. The higher the doses administered, the better and the longer after the injection could the drugs be detected. With the exception of nikethamide the other alkaloids were detectable in the ether as well as in the chloroform extracts but not always for so long after the injection, as for example brucine and cocaine, or only after the highest doses as for strychnine. This means that chloroform would be a better extracting solvent than ether. This coincides fully with our determinations of the extraction rates. On the other hand the chloroform extracts now and then revealed on the chromatoplates a greater quantity of impurities than the ether extracts, so that for some drugs as *e.g.* quinine and lobeline, the spots were not so well defined and their identification was not so easy. However we believe that this advantage of ether over chloroform does not counterbalance the higher extraction rates for chloroform, since detection of doping agents is a question of micrograms. We must also take into account that the urine samples from our experimental horses were always very concentrated and slimy because of the strongly alkaline pH, hence the greater quantity of impurities in the extracts. This fact was confirmed during our routine examination of the urine samples for the horses of the Belgian and Dutch Racing Organisations. Of the nearly 200 samples examined during the past year most showed a neutral or even acid pH, probably as a result of the race performances. From these acid urines

a very pure extract was always obtained and the alkaloids eventually present were identified much more easily than in the alkaline ones from our experimental horses. Hence we came to the conclusion that similar experiments with injections of some drugs must be done on horses during race performances. Moreover it is well known that the urinary excretion of some basic substances in men and in several animal species is affected by the acid–base equilibrium of the body and the urinary pH so that it is greater in acid than in alkaline urine. Among others this is the case for the weckamine amphetamine[2,5] and the alkaloids cocaine and quinine in man[1,12]. Finally the use of urine offers the advantage that in cases with a doubtful result for which a definite conclusion is very difficult, the amount of material is mostly sufficient for a second analysis. The difficulty however is the collection of urine; a horse will not micturate to order. The collection of urine by catheter is not recommended and is rather difficult in male horses. At the same time attention must be drawn to the fact that by this method three spots always appeared on the chromatoplates, even for the control samples. They were probably alkaloids or alkaloid-like substances by nature and presumably normal metabolites. Under U.V. light at 254 mμ they were seen as absorbing spots and after spraying with Dragendorff's reagent they turned yellow. The first one has an R_F value in the neighbourhood of morphine, the second one in that of strychine and the third one lies between the reference spots for lobeline and heroin. A short time after the coloration however they disappear from the plates whereas the alkaloid spots keep their colour for several hours.

Nevertheless at the moment saliva is still the specimen of biological material chiefly used for doping control in race horses, from our results however it would seem that saliva is not as reliable as urine or even blood. Only the substances injected in the highest doses could be identified in the saliva. However it is difficult to say whether these unfavourable results should be attributed to a lower salivary secretion of these alkaloids or to the insufficient quantity of saliva obtained on the swabs during the collection. From our own experience we are convinced that the last factor is the principal cause since it is mostly very difficult to obtain a sufficient number of swabs properly drenched with saliva. Furthermore some of these stimulating alkaloids have an inhibiting action on the salivary secretion.

Only six alkaloids could be isolated from the blood. Except for lobeline, these were again the highest dosed alkaloids. An advantage of the use of blood is the very pure and clean extract. A disadvantage however is that many drugs are only found in the blood for a short time or in low concentrations, and that some cannot be detected at all in blood. Further there is always a risk of injury to the horse with the hazard of an action for damages against the veterinary surgeon and the stewards. A sample of blood can only be taken with the consent of the owner.

The alkaloids heroin, morphine and papaverine could never be isolated from the blood, saliva or the urine. In the case of heroin this is probably due to the rapid deacetylation of the heroin to morphine under influence of the liver- and kidney-deacetylase in the organism[25]. Since we were not able to isolate morphine even after its injection, it is acceptable that we could not detect the morphine resulting from the heroin deacetylation. It is well known that in the monkey, the dog and the rat, and especially in nontolerant animals morphine is excreted from the urine principally in the conjugated form[21,22,30]. It has been suggested that there may be at least two "bound" forms of morphine excreted in dog's urine and moreover that part of the

bound morphine is liberated by mild hydrolysis whereas part is freed only by autoclaving[31]. During our extraction procedure the urine samples were not hydrolysed and by analogy with other animal species this could probably be one of the reasons why we did not identify morphine in the horse. On the other hand morphine is only very readily extractable at an alkaline pH up to 8, whereas our extraction was always performed at pH 9–10. Presumably this was due to pH rather than solvent since the extraction with the mixture chloroform–isopropanol gave the same results. The fact that papaverine was not detectable in the biological fluids is probably related to the well known rapid metabolism of this alkaloid in different animals. Very little papaverine is excreted unchanged by man and rabbit[18]. In the rabbit only 0.4 % of an injected dose of papaverine was recovered in the urine over a 72 h period. The small excretion of unchanged papaverine indicates that it is almost completely destroyed in the body by a number of tissues, but most rapidly by the liver[28].

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