TABLE II

EXTRACTION RECOVERIES OF AMBENONIUM FROM SERUM SAMPLES

Concentration added (ng/ml)	Recovery (mean ± S D, $n=6$) (%)
2 5	97 7 ± 4 4
10 0	100 0 ± 2 9

peak Under the above conditions, a maximum and constant recovery (97–100%) was obtained (Table II).

We tested the addition of several quaternary ammonium compounds to serum (1 μg/ml) using the proposed method: methylbenactyzium bromide, oxapium iodide, domiphen bromide, propanthline bromide, clocapramine hydrochloride, benzethonium chloride, benzalkonium chloride, neostigmine bromide, distigmine bromide and pralidoxime iodide Of these compounds, only the peak of methylbenactyzium bromide was near that of AMBC The retention time was 12 min, so it did not interfere with the determination of AMBC Drugs given concomitantly to patients with MG, such as prednisolone and atropine sulphate, did not interfere

Several quaternary ammonium compounds that had ester structures were examined as possible internal standards, but they are not recommended because of their instability in serum or aqueous solutions Timepidium bromide was very stable under the conditions adopted and the recovery was good, so it was selected as the internal standard.

We determined AMBC concentrations in patients' serum by the proposed method after oral administration of the drug. Serum concentration–time profiles were obtained in two instances (Fig 5). Patient A was given an oral dose of 10 mg of AMBC and patient B was given two doses of 40 and 30 mg in the sampling period. Patients A and B received previous doses of 10 mg at 4 h and 5 mg at 14.5 h before the beginning of sampling, respectively The profile of patient A showed a peak serum concentration (C_{max}) immediately after oral administration That in patient B also showed a C_{max} after the first dose, but not after the second The reason for the absence of a C_{max} was not elucidated The time to reach C_{max} was almost the same in both instances (1–1.5 h) C_{max} normalized with a dose was different in the two instances, being 0 84 ng/ml·mg in patient A and 0 20 ng/ml·mg in patient B Only one report of human serum AMBC concentrations has been published· Bloch et al. [4] reported C_{max} values of about 20 and 40 ng/ml with oral doses of 5 and 10 mg, respectively However, the concentration of AMBC in serum from patients was not as high in our study

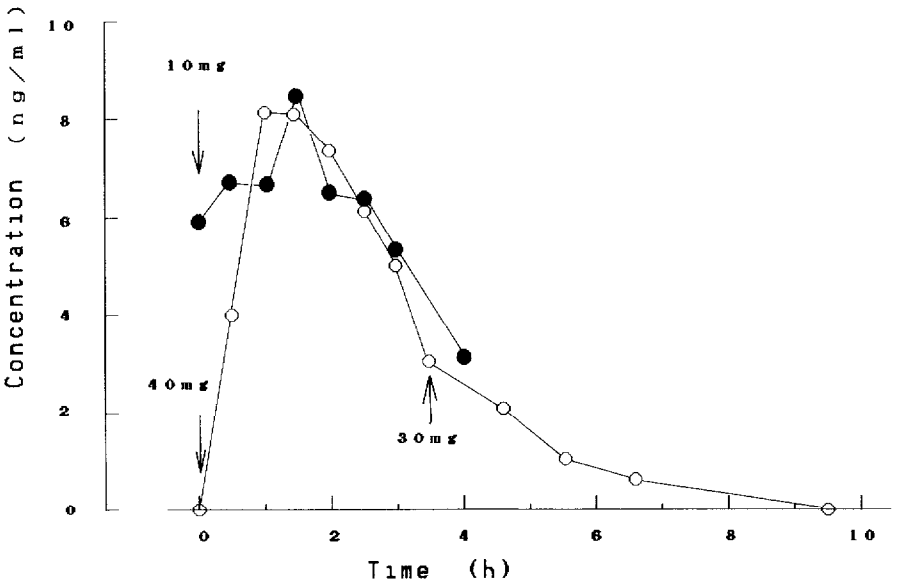

Fig 5 Serum ambenonium concentration-time profiles in two patients with myasthenia gravis under multiple oral therapy Patient A (●) received 10 mg of AMBC Patient B (○) received 40 and 30 mg at the interval shown Each value is the average of duplicate determinations Patient A (C K), female, age 33 years, height 149 cm, weight 65 kg, daily dose 50 mg, patient B (N T), female, age 42 years, height 158 cm, weight 55 kg, daily dose 80 mg

The correlation between dose and serum concentration was investigated in patients who had received AMBC under a diet or under fasting (Fig. 6) AMBC concentration was determined in serum collected 3 h after dosing, and dose was normalized with body weight We could not obtain a clear correlation in either group because of a large inter-individual variability of the serum concentrations The effect of diet before administration was investigated when the serum AMBC concentration was normalized with dose versus body weight The mean value ± standard deviation was 0.126 ± 0 164 ng/mg·kg ($n=10$) under a diet and 0 295 ± 0 214 ng/mg·kg ($n=17$) under fasting In view of the low bioavailability of reversible cholinesterase inhibitors, the serum concentration of AMBC was affected by various factors, involving ingestion of food and comedications [1] These effects remain to be confirmed by more studies

Pharmacokinetic studies may be advanced by the more intensive determination of serum concentrations of AMBC, and the proposed method should be useful for such studies

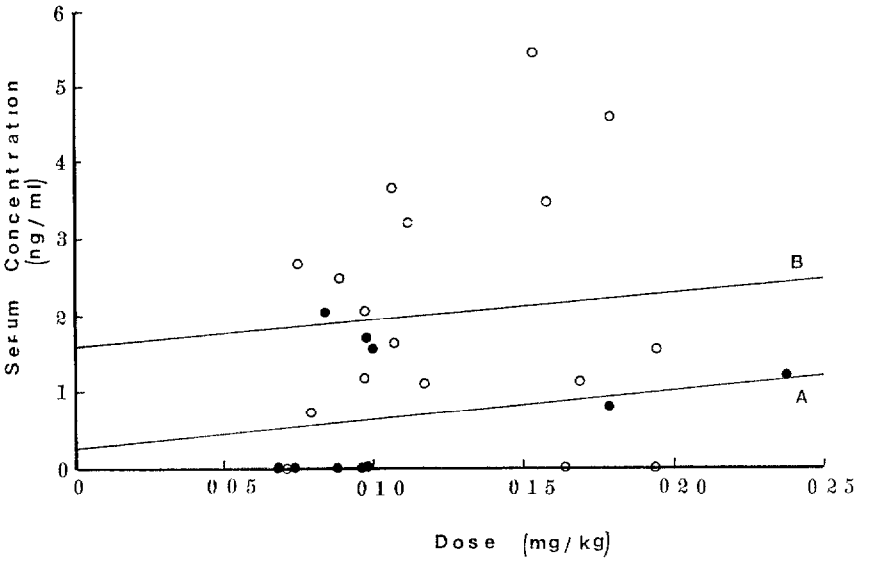

Fig 6 Relationship between serum ambenonium chloride concentration and normalized dose with body weight (mg/kg) The blood was collected 3 h after oral administration of ambenonium chloride under a diet (●) or fasting (○) Regression line A, under a diet ($y=3\ 7632x+0\ 3035$, $r=0\ 2407$, $n=10$), regression line B, under fasting ($y=3\ 5880x+1\ 5846$, $r=0\ 0950$, $n=17$) Patients under diet number, ten (five male, five female), age, 45 9 ± 14 0 years, body weight, 55 5 ± 8 4 kg, study dose, 6 0 ± 2 1 mg, serum concentration, 0 73 ± 0 83 ng/ml Patients under fasting number, seventeen (eight male, nine female), age, 42 7 ± 14 0 years, body weight, 58 4 ± 11 2 kg, study dose, 7 4 ± 2 6 mg, serum concentration, 2 04 ± 1 61 ng/ml

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THIN-LAYER CHROMATOGRAPHIC SCREENING PROCEDURE FOR SOME DRUGS IN HORSE PLASMA

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SUMMARY

A thin-layer chromatographic screening procedure for some basic, neutral and acidic drugs was developed using 3 ml of horse plasma Chloroform–2-propanol (95 5, v/v) was used as the extraction solvent The drugs were identified by a high-performance thin-layer chromatographic plate and spraying successively with some detection reagents In this study, the extraction recovery rates and the detection limits were determined at the same time

INTRODUCTION

The advantages of thin-layer chromatography (TLC) as a sensitive, simple and rapid method for the identification of organic compounds are well known Our laboratory has been applying it as a test of drugs on urine of racehorses [1] and we screen ca. 86 000 urine samples annually.

As in the case with other methods, various improvements have been introduced to such high-performance TLC (HPTLC) plates [2] and TLC plates with a concentrating zone [3] Also, there are many reports of new types of TLC chamber. [4]

This paper describes a simple TLC screening method for the detection of thirty-four neutral and basic drugs and eighteen acidic drugs in horse plasma

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EXPERIMENTAL

Reagents and equipment

The reagent-grade solvents and chemicals were used without further purification. The drugs were commercial products, and each was confirmed by TLC to be free of impurities before they were used in the experiments.

Thin-layer plates (10 cm × 10 cm) were coated with silica gel 60 F_{254} with a concentrating zone (HPTLC plate, E. Merck). Before use, some gel was scraped off the plate using a thick needle to make slits at 5-mm and 3-mm intervals so that thirteen samples could be applied per plate A developing chamber for 10 cm × 10 cm glass plates, with two solvent tanks (Camag), was used A UV detector (Model C-70G, Ultra Violet Products, U S.A.) was used, set at 254 and 365 nm.

Horse blood samples were collected from the jugular vein using heparinized tubes (Nipro Neo-Tube, NT-HE 1000 Nipro, Japan) Immediately after collection, the samples were centrifuged for 5 min at 3000 *g* to obtain plasma. The plasma was frozen until use

Preparation of standard solutions

Each drug was dissolved in ethanol at a concentration of 5 μg/ml Each ethanol solution was used as it was or after dilution to an appropriate concentration.

Extraction of drugs from horse plasma

Thirty-four alkali-extractable drugs and eighteen acid-extractable drugs were added each to horse plasma sample at a concentration of 1 μg/ml, and extraction was performed in accordance with one of the following methods.

Alkali extraction A 3-ml volume of plasma was placed in a centrifuge tube (115 mm × 16 mm) with a universal stopper, and 0.2 ml of a 20% potassium carbonate solution and 4 ml of chloroform–2-propanol (95 5, v/v) were added followed by shaking for 5 min The mixture was centrifuged at 3000 *g* for 5 min at 5 °C The aqueous layer was aspirated off and the organic layer was transferred to a tapered test-tube (110 mm × 16 mm) through a filter paper on a glass funnel Two drops of a 5% acetic acid in ethyl acetate solution were added to this test-tube to protect low-boiling-point basic drugs from evaporating off, and the extract was evaporated to dryness under a stream of nitrogen gas The residue was dissolved in 0 1 ml of ethanol and applied to the TLC plate.

Acid extraction A 3-ml volume of plasma was placed in the centrifuge tube, and 0.2 ml of 0 25 *M* sulphuric acid and 6 ml of chloroform–2-propanol (95 5, v/v) were added. Extraction was performed as for alkali extraction, and the residue was dissolved in 0 1 ml of ethanol and applied to the TLC plate.

Thin-layer chromatography

Alkali-extracted drugs. The entire volume of the sample (0.1 ml) was applied to the plate using a microsyringe, and the plate was dried at room temperature The plate was developed to a height of ca. 6 cm using chloroform–methanol–ammonia (90 10 0 5, v/v) and dried at room temperature, and the spots were visualized under UV light at wavelengths of 254 and 365 nm. Next, ninhydrin reagent was sprayed, and the plate was heated at 100°C for 5 min and cooled to room temperature. Then Simon reagent, concentrated sulphuric acid, iodoplatinate reagent and Dragendorff reagent were sprayed, in that order.

Acid-extracted drugs The sample was applied to the plate as for the alkali-extracted drugs and developed using ethyl acetate–methanol–ammonia (100 18 0.5, v/v). After visualization at 254 and 365 nm, 0.1% diphenylcarbazone in ethanol, 1% mercurous nitrate in 1% nitric acid, Mandelin reagent and Dragendorff reagent were sprayed successively

Detection reagents

Ninhydrin reagent This consisted of 0.5% ninhydrin in butanol.

Simon reagent Solution A was 28% sodium carbonate solution, and solution B was 1% sodium nitroprusside solution. Solutions A and B were sprayed successively on the plate, which was then placed in a chamber saturated with acetaldehyde vapour.

Iodoplatinate reagent This reagent was made by adding 1 g of platinic chloride in 10 ml of water to 10 g of potassium iodide in 250 ml of water. The mixture was diluted to 500 ml with water.

Dragendorff reagent A 1.5-g amount of bismuth subnitrate in 4.5 ml of concentrated hydrochloric acid was added to 9 g of potassium iodide in 30 ml of water. The mixture was diluted to 150 ml with water.

Recovery studies

A 1-μg amount of each drug was added to 3 ml of horse plasma in order to examine the efficiency of the extraction procedure. The recovery of drugs was determined by gas chromatography or high-performance liquid chromatography. The mean recovery was the average value from five spiked samples.

Detection limit

An aliquot of each drug solution in ethanol was added to horse plasma and diluted stepwise by blank horse plasma. After the extraction, the extracts were applied to the TLC plate. The detection limit was determined from five positive results on five spiked samples.

RESULTS AND DISCUSSION

Extraction

The pH of horse plasma separated from the blood collected in heparinized tubes was 8.25 ± 0.30 ($n = 190$) It was decided to adjust the pH to 10 for alkali extraction and 5 for acid extraction, since extensive emulsion formation occurred at strongly acidic pH. Therefore, a study was made to find appropriate buffers. As a result, it was found that addition of 0.2 ml of 20% potassium carbonate to 3 ml of plasma for alkali extraction and 0 2 ml of 0 25 *M* sulphuric acid to 3 ml of plasma for acidic extraction would bring the pH to 10.23 ± 0.12 ($n = 190$) and $5.44 \pm 0\ 31$ ($n = 33$), respectively Pentz and Shutt [5] used a chloroform–acetonitrile–ethyl acetate solution (4 3 2) for both alkali and acid extraction of drugs in human plasma, and analysed the extracts by gas chromatography. When this solvent was used for horse plasma, plasma components interfered with the detection of drugs by TLC for both the alkali and acid extracts. Thus, other solvents were tested, and chloroform–2-propanol (95 5, v/v) was selected. The amount of solvent added to 3 ml of plasma was optimized, with the purpose of establishing a simple and rapid analytical method To do this, the volume of the test-tube, the centrifugation time and the recovery were studied, and the volume of solvent was fixed at 4 ml for alkali extraction and 6 ml for acid extraction.

Recovery

The recoveries of drugs from horse plasma are shown in Tables I and II.

In the case of alkali extraction, caffeine, cocaine and methylephedrine showed high recoveries of 97 0, 97.2 and 97.9%, respectively. The recovery was 51 4% for ephedrine, 32.8% for theobromine and only 6 4% for theophylline. The recovery of morphine was also low, 6.9%. This is because the pH range suitable for its extraction is narrow and its optimal extraction pH is 9. The low recovery of 8 9% for dyphylline is thought to be due to its high water solubility

In the case of acid extraction, the recoveries of barbiturates were all 60% or higher except for metharbital (36.0%). These recoveries are close to those reported by Bailey and Kelner [6], who carried out diethyl ether extractions from human plasma at pH 1–2. Since our method does not use diethyl ether, it is thought to be safe and useful as a routine method. Flufenamic acid and mefenamic acid, both anti-inflammatory drugs, showed 79.6 and 79.2% recovery, and phenylbutazone exhibited a 65.2% recovery Theophylline, which gave a low recovery in the alkali extraction, showed a better recovery of 58 1% in the acid method

Thin-layer chromatography

There have been many studies [7–11] of developing solvents and detection reagents for TLC of neutral, basic and acidic drugs. The solvent systems were

TABLE I

RECOVERIES OF ALKALI-EXTRACTABLE DRUGS FROM HORSE PLASMA

Drug	Recovery (mean ± S D , $n=5$) (%)	Drug	Recovery (mean ± S D , $n=5$) (%)
Aminopyrine	83 8 ± 1 1	Morphine	6 9 ± 0 6
Antipyrine	93 7 ± 8 0	Nicotine	92 8 ± 8 4
Caffeine	97 0 ± 1 5	Nikethamide	91 1 ± 4 6
Chlorpromazine	48 0 ± 1 3	Oxyethyltheophylline	42 2 ± 1 4
Chlorpromazine-sulphoxide	72 4 ± 5 1	Oxypropyltheophylline	82 8 ± 1 2
		Pentazocine	82 1 ± 3 0
Cocaine	97 2 ± 0 8	Phenacetin	36 2 ± 0 9
Dibucaine	88 7 ± 5 3	Procaine	83 0 ± 1 7
Dyphylline	8 9 ± 0 8	Promazine	96 4 ± 1 9
Ephedrine	51 4 ± 2 6	Strychnine	87 8 ± 4 1
Lidocaine	70 5 ± 2 5	Tetracaine	53 3 ± 6 7
Methamphetamine	81 1 ± 1 6	Theobromine	32 8 ± 5 4
Methapyrilene	81 9 ± 3 6	Theophylline	6 4 ± 0 5
Methylephedrine	97 9 ± 3 8		

TABLE II

RECOVERIES OF ACID-EXTRACTABLE DRUGS FROM HORSE PLASMA

Drug	Recovery (mean ± S D , $n=5$) (%)	Drug	Recovery (mean ± S D , $n=5$) (%)
Allobarbital	60 6 ± 1 1	Metharbital	36 0 ± 3 2
Amobarbital	78 7 ± 3 2	Pentobarbital	72 3 ± 2 2
Barbital	66 7 ± 3 0	Phenobarbital	93 8 ± 2 1
Cyclobarbital	66 5 ± 2 0	Phenylbutazone	65 2 ± 1 9
Flufenamic acid	79 6 ± 2 2	Primidone	48 0 ± 1 8
Hexobarbital	83 4 ± 1 7	Secobarbital	87 8 ± 2 1
Mefenamic acid	79 2 ± 3 0	Theophylline	58 1 ± 2 4
Mephobarbital	68 6 ± 2 3	Thiopental	87 8 ± 2 1

studied for their ability to separate each drug from other drugs and to separate each drug from plasma components Our investigations led to the use of the solvent systems chloroform–methanol–ammonia (90 10 0 5, v/v)and ethyl acetate–methanol–ammonia (100 18 0.5, v/v) for alkali-extracted and acid-extracted drugs, respectively.

A 3-μg amount of each drug was applied to the TLC plate, and after development the spots were detected by UV light and by an overspray method consisting of a combination of detection reagents Tables III and IV show R_F values

TABLE III

R_F VALUES, UV ABSORPTION, REACTION TO SPRAY REAGENTS AND DETECTION LIMITS OF THE ALKALI-EXTRACTABLE DRUGS IN TLC

Drug	R_F (×100)	Detection[a]					Detection limit ($n=5$) (μg/ml)
		UV	Color of Spot				
			Ninhydrin	Simon	Iodoplatinate	Dragendorff	
Aminopyrine	56	+	Pink			Orange	0 05
Amphetamine	24	+	Pink			Orange	0 90
Antipyrine	41	+			Purple	Orange	0 05
Atropine	6				Purple	Orange	0 10
Brucine	34	+	Light pink		Purple	Orange	0 10
Caffeine	67	+	Light pink			Orange	0 05
Chlorpromazine	50	+			Purple	Orange	0 05
Chlorpromazine sulphoxide	25	+			Purple	Orange	0 05
Cocaine	76					Orange	0 30
Dibucaine	60	+			Purple	Orange	0 10
Dyphylline	21	+				Orange	0 80
Ephedrine	5		Pink			Orange	0 05
Lidocaine	81	+				Orange	0 50
Methamphetamine	20		Light pink	Blue		Orange	0 05
Methapyrilene	54	+			Purple	Orange	0 10
Methylephedrine	12					Light orange	0 20
Methylphenidate	59					Orange	0 30
Morphine	13	+			Purple	Orange	0 90
Nicotine	57	+			Blue	Orange	1 00
Nikethamide	71	+				Orange	0 30
Oxyethyltheophylline	36	+				Orange	0 05
Oxypropyltheophylline	46	+				Orange	0 05
Pentazocine	32				Purple	Orange	0 20
Pentetrazol	73					Yellow-orange	3 00
Phenylbutazone	43	+				Orange	1 00
Phenacetin	52	+					0 10
Pipradrol	52	+	Pink		Purple	Orange	0 50
Procaine	46	+			Blue	Orange	1 00
Promazine	45	+			Purple	Orange	0 05
Scopolamine	44				Purple	Orange	0 20
Strychnine	30	+			Purple	Orange	0 10
Tetracaine	56	+			Purple	Orange	1 50
Theobromine	48	+				Orange	0 30
Theophylline	36	+				Orange	0 10

[a]A 3-μg amount of each drug was applied to the plate UV detection was at 254 and 365 nm

in alkali-exobtained with each solvent system. These developing systems led to R_F values larger than 0 2, except for atropine, ephedrine, morphine and methylephedrine in alkali-extracted drugs and flufenamic acid and mefenamic acid in acid-extracted drugs. Drugs were also separated from plasma compo-

TABLE IV

R_F VALUES, UV ABSORPTION, REACTION WITH SOME SPRAY REAGENTS AND DETECTION LIMITS OF THE ACID-EXTRACTABLE DRUGS IN TLC

Drug	R_F (×100)	Detection[a]				Detection limit ($n=5$) (μg/ml)
		UV	Colour of spot			
			DPC + $HgNO_3$[b]	Mandelin	Dragendorff	
Allobarbital	75	W	Pink			0 05
Amobarbital	77	W	Pink			0 10
Barbital	71	W	Pink			0 10
Cyclobarbital	73	W	Pink			0 10
Flufenamic acid	19	+	Blue	Blue		0 10
Hexobarbital	78	W	Pink			5 00
Mefenamic acid	20	+	Blue	Blue		0 10
Mephobarbital	79	W	Pink			0 50
Metharbital	78	W	Pink			2 00
Pentobarbital	77	W	Pink			0 20
Phenobarbital	69	W	Pink			0 10
Phenylbutazone	33	+	Blue		Yellow-orange	1 00
Primidone	59	W	Pink			5 00
Secobarbital	80	W	Pink			0 10
Theobromine	41	+	Blue		Orange	0 10
Theophylline	24	+	Blue		Orange	0 10
Thiamylal	74	W	Pink			1 00
Thiopental	83	W	Pink			0 50

[a]Amounts applied were 10 μg per spot for hexobarbital and primidone and 3 μg per spot for others UV detection at 254 and 365 nm W represents weak UV absorption
[b]Diphenylcarbazone reagent followed by $HgNO_3$ reagent

nents, many of which stay at the baseline The R_F value of each drug changed slightly from day to day owing to small differences in the solvent composition and the activity of TLC plates. The values given in the tables are representative

Many alkali-extractable drugs could be detected under UV light, but basic drugs such as atropine, cocaine, ephedrine, methamphetamine, methylephedrine, methylphenidate, pentazocine, pentetrazol and scopolamine, could not be detected under UV light in this amount Almost all these drugs absorb weakly at this wavelength. An overspray technique, consisting of successive sprays of ninhydrin reagent, Simon reagent, iodoplatinate reagent and Dragendorff reagent, resulted in specific colouration of each drug (Table III) In this study, chlorpromazine and morphine did not react with ninhydrin reagent, but Dutt and Teng Poh [7] reported that colouration was observed when 10 μg of chlorpromazine hydrochloride and 5 μg of procaine hydrochloride were used After successive overspraying of Simon reagent and Dragendorff reagent, we sprayed concentrated sulphuric acid This is because we expected enhancement of the colouration to increase the sensitivity of the detection

Of the acid-extracted drugs, barbiturates showed the weakest absorptions of UV light at these amounts. However, by overspraying with 0.1% diphenylcarbazone in ethanol, 1% mercurous nitrate in 1% nitric acid, concentrated sulphuric acid and Dragendorff reagent, we achieved specific colouration of each drug That is, 0.1% diphenylcarbazone reagent and 1% mercurous nitrate reagent caused barbiturates to turn pink and blue with flufenamic acid and mefenamic acid, and subsequent spraying of Dragendorff reagent caused no reaction in the colour of these drugs.

Barbiturates migrated to the same narrow range between R_F values 0.6 and 0.8, so only poor separation was achieved. However, the chemical classification of barbiturates can be established on the basis of the characteristic colour reaction. Therefore, this procedure is thought to be useful for screening these drugs in horse plasma.

Detection limit

The detection limits of drugs from horse plasma are also shown in Tables III and IV. The detection limits for alkali-extracted drugs were 0 05–0.1 μg/ml for seventeen drugs, 0.2–0.5 μg/ml for nine drugs and 0 8–1 0 μg/ml for five drugs. The detection limits of tetracaine and pentetrazol were 1 5 and 3.0 μg/ml, respectively. The poor detection limit of tetracaine might be due to hydrolysis by cholinesterase in plasma. Kalow [12] and Foldes [13] reported that the ester-type local anaesthetic agents were hydrolysed relatively rapidly by plasma cholinesterase Thus the relationship between tetracaine and the plasma cholinesterase in horse plasma should be studied. The case of pentetrazol might be also considered, since it has poor sensitivity to both UV light and locating reagents.

In the case of acid-extracted drugs, the detection limit was 5 0 μg/ml for hexobarbital and primidone, 2 0 μg/ml for metharbital and 1 0 μg/ml or lower for the other drugs The limit of detection was 0.1 μg/ml for both theobromine and theophylline The limit of detection of theobromine was improved by the acid-extraction method.

Using this procedure, we successfully established a rapid and simple testing method, which does not require any special purification This method may be useful to test for drugs in racehorses.

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