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Systematic analysis of acid, neutral and basic drugs in horse plasma by combination of solid-phase extraction, non-aqueous partitioning and gas chromatography–mass spectrometry

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Abstract

A sample preparation method for mass chromatographic detection of doping drugs from horse plasma is described. Bond Elut Certify (1 g/6 ml) is used for the extraction of 4 ml of horse plasma. Fractionation is performed with 6 ml of $CHCl_3$–Me_2CO (8:2) and 5 ml of 1% TEA–MeOH according to its property. Simple and effective clean-up based on non-aqueous partitioning is adopted to remove co-eluted contaminants in both acid and basic fractions. Two kinds of 1-(*N*,*N*-diisopropylamino)-*n*-alkanes are co-injected with the sample into the GC–MS system for the calculation of the retention index. Total recoveries of 107 drugs are examined. Some data of post administration plasma are presented. This procedure achieves sufficient recoveries and clean extracts for GC–MS analysis. The method is able to detect ng/ml drug levels in horse plasma.

Keywords: Doping drugs; 1-(*N*,*N*-Diisopropylamino)-*n*-alkanes

1. Introduction

In doping control in horse racing, various drugs in broad therapeutic categories have been listed as prohibited substances. It is based on the recognition in the racing field, namely the use of any substance which can increase or decrease running performance of a horse is prohibited by law. The prohibited substances are categorized into several classes of drugs in accordance with their properties or pharmacological action such as acidic drugs, basic drugs, non-steroidal anti-inflammatory drugs (NSAIDs), anabolic steroids, etc. Post-race urine samples are collected every race and tested according to the method for each class of drugs. Urine has been generally used as the best testing specimen for the doping test, however, in a case where urine collection is unsuccessful or impossible within a specified time limit after the race, blood is collected and tested instead. After the sample collection is completed, an official veterinarian divides the sample into two sets (samples and B), and seals them hermetically. Sample A is used for routine drug test and sample B will be used for reconfirmation of the sample in the case of a positive result and remains securely in custody until re-analysis of the sample in the presence of a witness. In our laboratory, about 60 000 samples are been tested annually and about 7% of total testing

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specimens are blood. It is desirable that the doping test using horse plasma as the testing specimen should be applicable to a wide range of drugs as well as those obtained from urine. Tanaka et al. reported a study on a rapid and simple screening of doping drugs in horse plasma by high-performance thin-layer chromatography [1]. In the case where an unusual spot is found during the screening, a newly prepared sample is analyzed by more sophisticated methods for determining whether the prohibited substances are present in the sample or not.

Solid-phase extraction (SPE) provides wide applicability to various compounds. A single or multiple stage SPE has been applied for sample preparation methods in toxicological and environmental analyses [2–4]. In particular, a single mixed-mode solid-phase cartridge is reliable for the fractionation of acid, neutral and basic drugs from biological samples because the drug is collected separately by hydrophobic or ion-exchange interaction [5–7]. On the other hand, the detection method also must have high sensitivity and specificity, and applicability to a broad range of drugs and their metabolites which are present at low levels in the sample. In many cases of highly sensitive and selective methods of drug detection in biological samples, gas chromatography (GC) with specific detectors and gas chromatography–mass spectrometry (GC–MS) have been widely used for a broad range of drugs [8,9]. Mass chromatography (MC) is one of the effective techniques using GC–MS for the simultaneous analysis of a number of drugs in one analysis with little interference by contaminants because of the ion separation [10,11]. However, the removal of contaminants in the plasma extracts without loss of recoveries of drugs is desired to improve the chromatographic performance.

This paper describes optimization of SPE using a 6-ml column packed with 1 g of Bond Elut Certify to fractionate acid–neutral and basic drugs in 4 ml of horse plasma. Simple modification of Rapid Trace, an automated SPE system, adaptable to 6-ml size columns is briefly described. An effective clean-up method based on non-aqueous partitioning is given. Recovery data of 107 doping drugs and their metabolites from spiked plasma are presented. Moreover, practical application of mass chromatographic detection of some drugs in horse blood of post-administration is shown.

2. Experimental

2.1. Reagents and materials

Drug-free horse plasma was supplied from the blood-typing test section of our laboratory. Real plasma samples of racehorses were separated from whole blood which was collected into heparinized tubes (Nipro, Tokyo, Japan) after the race and were used after routine testing. The pure drugs were purchased from commercial suppliers; Sigma–Aldrich Japan (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), Tokyo Chemical Industry (Tokyo, Japan), Wako (Osaka, Japan) and Dainihonseiyaku (Osaka, Japan). Metabolites of some drugs were synthesized in our laboratory. Stock solutions of each drug (1 mg/ml in methanol) were prepared and stored below 5°C. The diluted standard solution (10–50 μg/ml in ethyl acetate) for GC–MS analysis was reconstituted from the stock solution. 1-(*N*,*N*-diisopropylamino)-*n*-alkanes (DIPA-alkanes) which were used as the marker substances for calculation of the retention index on MC were synthesized from corresponding aliphatic acids in three steps according to the usual manner [12,13]. Stock solutions of each DIPA-alkane (1 mg/ml in ethyl acetate) were prepared and stored below 5°C. Diluted solutions of a mixture of two DIPA-alkanes having different carbon numbers were reconstituted in ethyl acetate from the stock solution at a concentration of 2.5 ng/μl for small carbon numbers and 5.0 ng/μl for large ones. A mixture of DIPA-heptane (DIPA-C_7) and DIPA-tetracosane (DIPA-C_{24}) is used for MC screening. Other DIPA-alkane mixtures for confirmatory test are as follows; DIPA-C_7 and DIPA-hexadecane (DIPA-C_{16}) for condition A, DIPA-tetradecane (DIPA-C_{14}) and DIPA-C_{24} for conditions B, E and F, DIPA-*n*-heptadecane (DIPA-C_{17}) and DIPA-triacontane (DIPA-C_{30}) for conditions C and G; DIPA-C_7 and DIPA-docosane (DIPA-C_{22}) for condition D. Custom packaged columns of Bond-Elut Certify (1 g of the sorbent packed in an 8-ml syringe-type tube) were prepared by Varian Sample Preparation Products (Harbor City,

CA, USA). Deionized water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Acetonitrile, triethylamine and *n*-hexane were of analytical grade and were distilled before use. Other solvents and reagents used were of analytical grade or chromatographic grade unless otherwise specified.

2.2. Solid-phase extraction

The optimum conditions for SPE of drugs in horse plasma using Bond Elut Certify columns was examined with the vacuum manifold (Supelco, Bellefonte, PA, USA) and Rapid Trace system (Zymark, Hopkington, MA, USA). The conditions for the fractionation of the acid–neutral and the basic drugs in 4 ml of horse plasma; column size, pH, solvent system and the elution volume, were examined. A clean-up method for plasma extracts based on non-aqueous partitioning was examined to remove co-eluted plasma constituents and other contaminants. Total recoveries of drugs from spiked plasma throughout the method was examined. The Rapid-Trace system was modified to accommodate the 6 ml column.

2.3. GC–MS conditions

Capillary column GC–MS was performed with a Hewlett-Packard 6890A gas chromatograph coupled to a Model JMS BU20 mass spectrometer (JEOL, Tokyo, Japan). Mass spectral data were reduced on-line with a Shrader data system (JEOL). Several bonded phase wide-bore fused-silica capillary columns, DB-5 (15 m×0.53 mm I.D., film thickness 1.5 μm; J&W Scientific, Folsom, CA, USA), and SPB-20 (15 m×0.53 mm I.D., film thickness 1.0 μm; Supelco), DB-1, DB-17, DB-1701 (15 m×0.53 mm I.D., film thickness 1.0 μm; J&W Scientific) and XTI-5 (15 m×0.53 mm I.D., film thickness 1.0 μm; Restek, Bellefonte, PA, USA) were used and connected through an all glass jet separator coupling to the ion source. The interface temperature was controlled at 250°C. All sample injections were made on a direct injection mode at 250°C. The column temperature was programmed from 90 to 320°C at 15°C/min for DB-5 (MC screening); from 90 to 300°C at 10°C/min (condition A), 15°C/min (condition D) or from 170 to 300°C at 8°C/min (condition B) for SPB-20; from 140 to 280°C at 10°C/min for DB-17 (condition F) and DB-1701 (condition E); from 200 to 320°C at 8°C/min for DB-1 (condition C) and from 150 to 320°C at 15°C/min for XTI-5 (condition G). The ion source conditions were as follows: electron energy 70 eV, ionization current 300 μA, ion accelerating voltage 2.5 kV and ion source temperature 220°C.

3. Results and discussion

3.1. Solid-phase extraction

3.1.1. Column size

SPE using a single mixed-mode cartridge for the drugs of toxicological interest has been reported in detail by Chen et al. [5]. They used a commercially available SPE column (300 mg of Bond Elut Certify packed in a 3-ml tube) for the fractional extraction of acid–neutral and basic drugs from human urine and plasma. The results that we obtained, applying the same procedure to threefold dilution samples which corresponds to 4 ml of horse plasma spiking with some drugs gave poor recoveries for the neutral and acidic drugs whereas the basic drugs were collected satisfactorily. It is suspected that the adsorption of the drugs by hydrophobic interaction is strongly affected by the presence of large amounts of normally occurring plasma constituents. Namely the capacity of 300 mg packing is not sufficient for a 4 ml sample of horse plasma. The variation of recoveries of acid–neutral drugs from 4 ml of plasma spiked with caffeine, phenacetin, barbital and naproxen (0.1 μg/ml each) were plotted against the column contents varied from 300 to 1200 mg at 100 mg increments. As shown in Fig. 1, it was proved that the recoveries of acid–neutral drugs, except naproxen, increased in accordance with increasing column capacity and the recoveries finally reached a constant over 800 mg of the sorbent. Based on these results, we chose the Bond Elut Certify column (1 g/6 ml) for the fractional extraction of the acid–neutral and basic drugs in 4 ml of horse plasma.

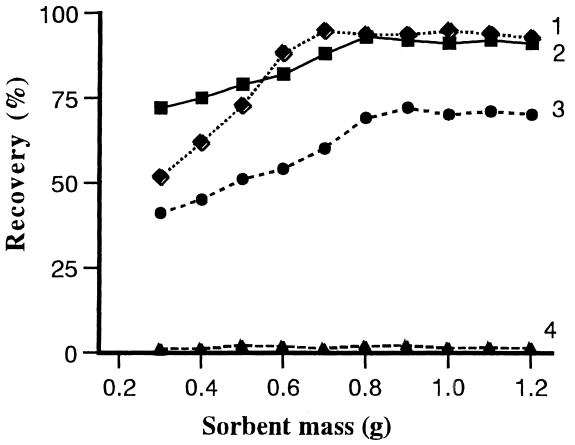

Fig. 1. Relation between recoveries of acid–neutral drugs and the sorbent mass. 1, Caffeine; 2, phenacetin; 3, barbital; 4, naproxen.

3.1.2. pH dependence on the recoveries of acidic drugs

Initially, pH dependence of the recoveries of acid–neutral drugs were examined to set the condition of charging the sample into the column. As shown in Fig. 2, the recoveries of the NSAIDs varied with the pH of the sample while those of other neutral and weak acidic drugs did not vary. Moreover, the recoveries of NSAIDs from the spiked plasma by elution with chloroform after charging the sample at pH 6 and rinsing the column with phosphate buffer (pH 3.0) were not improved appreciably. As is well known, dissociation of NSAIDs in the aqueous sample can be readily suppressed by adjusting the pH below their pK_a values, ca. 4–5 [14–16]. Thus the hydrophobic interaction between acidic drugs and the sorbent was enhanced by adjusting the pH below 4. In contrast to these drugs, lower pH tends to decrease the recoveries of the weak basic drugs in the acid–neutral fraction. This means that interaction between weak basic drugs and the sorbent by cation-exchange interaction was enhanced by adjusting to lower pH. Based on these results, the sample must be adjusted to pH 3.5 or below prior to apply it to the column. The pH adjustment of the plasma was accomplished by threefold dilution with 60 m*M* aqueous citric acid. The horse plasma (4 ml) which was distributed in the range of pH 6.98 to 8.12 could be adjusted to between 3.36 and 3.49 (3.43 ± 0.03, $n=140$) by dilution with 8 ml of 60 m*M* aqueous citric acid (pH 2.2).

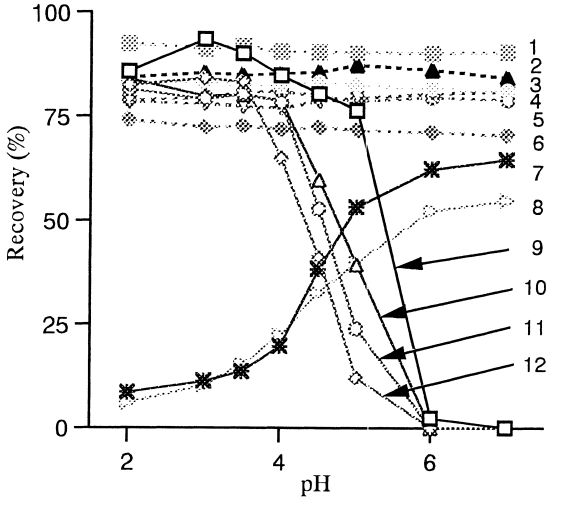

Fig. 2. pH dependence of the recoveries of acid–neutral drugs and weak basic drugs on the Bond Elut Certify column (1 g/6 ml). 1, Caffeine; 2, phenacetin; 3, theophylline; 4, amobarbital; 5, pentobarbital; 6, theobromine; 7, nikethamide; 8, antipyrine; 9, indomethacin; 10, piroxicam; 11, mefenamic acid; 12, ketoprofen.

3.1.3. Solvent system and elution volume

3.1.3.1. Acid–neutral fraction (fraction A). Although the Bond-Elut Certify column (1 g/6 ml) is obtainable from the manufacturer, little application data has been reported. We chose a modified solvent system consisting of chloroform and acetone as described by Chen et al. [5]. The ratio between the two solvents was examined to collect neutral and acidic drugs. While a quantity of the plasma constituents decreased in accordance with increasing the concentration of acetone in the solvent, the recoveries of NSAIDs also decreased. We finally decided to use 20% (v/v) acetone in chloroform as the eluent for fraction A. The column is rinsed with 6 ml of 10 m*M* acetic acid (pH 3) prior to the elution, because the column must be kept below pH 4 during the manipulation. The elution curves of 10 acid and neutral drugs in the elution of fraction A is shown in Fig. 3. The recoveries of these 10 acid and neutral drugs reached their maxima by the elution of 5 to 6 ml. Based on these results, we set the elution

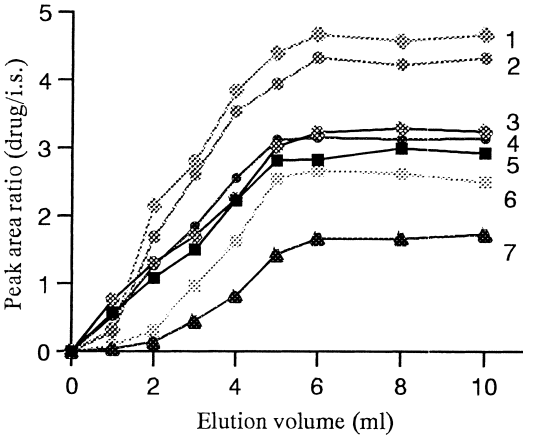

Fig. 3. Elution curves of acid–neutral drugs in fraction A. 1, Phenylbutazone; 2, pentobarbital; 3, amobarbital; 4, ketoprofen; 5, barbital; 6, theophylline; 7, theobromine.

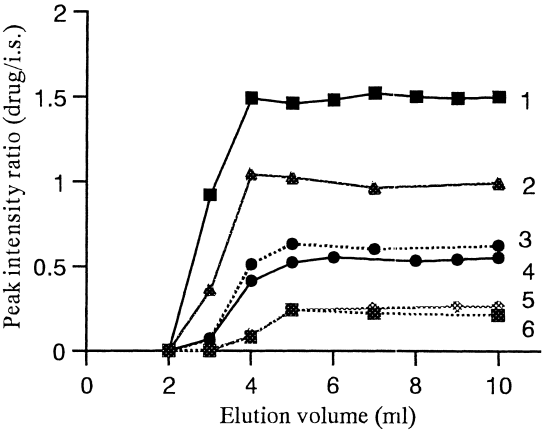

Fig. 4. Elution curves of typical basic compounds in fraction B. 1, Lidocaine; 2, methylphenidate; 3, methylephedrine; 4, monoethylglycinexylidide; 5, amphetamine; 6, ephedrine.

volume for fraction A at 6 ml of 20% (v/v) acetone in chloroform.

3.1.3.2. Basic fraction (fraction B). The column after the elution of fraction A is eluted with organic solvent containing small amounts of strong base to collect the basic compounds retained in the column. Several ammoniated solvent systems have been reported [17–19], but they must be prepared before use because of their instability, and a relatively large volume of the solvent was necessary to collect the basic drugs from the 1-g packed column. We examined 1, 2 and 3% (v/v) proportions of triethylamine (TEA) in methanol, ethyl acetate and acetonitrile instead of the addition of strong ammonia. The results indicated that the best solvent system on the recoveries and the elution volume was 1% TEA in methanol. This solvent is stable at a room temperature for at least 2 months. The elution curves of some typical basic compounds are shown in Fig. 4.

3.1.4. Clean-up

Plasma extracts contain large amounts of normally occurring fatty acids, cholesterol and its related compounds as well as other contaminants, further, these substances sometimes interfere with the detection of drugs even on MC. The retention time and the mass spectra of the contaminants found in both fractions were identical with those obtained from authentic fatty acids such as myristic acid, palmitic acid, linoleic acid and stearic acid. Besides these, dialkylphthlates from solvents, reagents, glassware, etc., were also detected. In the general process of sample clean-up, back extraction of the extracts, or washing the column with an appropriate volume of the solvent which does not elute the target compound can be used. However, rinsing the column with water contributes to poor recoveries not only for the acidic NSAIDs but also for weak basic drugs such as antipyrine and nikethamide. It is assumed the pH of the sorbent material is increased by rinsing with water. Moreover, fraction A in particular contains large amounts of plasma constituents and other co-eluted contaminants. Among them, the most problematic contaminants are fatty acids because they frequently affect on peak shapes of the substances and the chromatographic resolution. The fatty acids also give strong peaks on the ion trace of m/z 97 which is commonly used for the detection of aminopyrine, 3′-hydroxycyclobarbital and 3′-hydroxyhexobarbital by MC.

For the purpose of the removal of the contaminants, we attempted to adopt a non-aqueous partitioning with acetonitrile and *n*-hexane to the residue of the fraction. Acetonitrile and *n*-hexane are immiscible and the lipids and non-polar compounds are liable to be dissolved in *n*-hexane by the partitioning. Non-aqueous partitioning has been used as a part of

sample preparation in food analyses [20,21] and as a defatting process for the extraction of endogenous steroids in horse serum [22], however it is hard to find data concerning loss of recoveries of various drugs under these conditions. Therefore, the recoveries of acid–neutral and basic drugs using this partitioning process was examined. The loss of recoveries of the drugs on clean-up were determined by comparison with corresponding peak area to the substance on ion chromatograms which were obtained before and after the clean-up process. The clean-up method is as follows. The residue of fraction A is dissolved in 0.5 ml of acetonitrile then vigorously shaken twice with 2 ml of *n*-hexane for 1.5 min; after centrifuging at 3000 rpm for 1 min, the upper *n*-hexane layer is aspirated off; the acetonitrile phase is evaporated under N_2 at 40°C and the residue is reconstituted in ethyl acetate and subjected to GC–MS analysis. It was shown that the method is effective in removing the plasma constituents without loss of recovery (less than 10%). The recoveries on the clean-up process were more than 90% in all of 57 acid–neutral drugs including their metabolites except ibuprofen (45%) and mefenamic acid (74%). The comparative total ion chromatograms (TICs) of blank plasma for fraction A with and without purification are shown in Fig. 5. As can be seen in the figure, fatty acids and cholesterol were removed from the chromatogram by use of this clean-up process.

On the other hand, the clean-up of fraction B was also examined. The recoveries of 22 strongly basic drugs and their metabolites using the same process as with fraction A gave values of 20–60%. However, recovery of basic drugs was improved to be more than 90% using acetonitrile containing a small amount of acetic acid. It was shown that the contaminants in fraction B were effectively removed without loss of the drugs by adopting this clean-up operation. We decided to use the partitioning with 1% acetic acid in acetonitrile and *n*-hexane as the clean-up for the basic drugs. The clean-up by non-aqueous partitioning is really effective in the GC–MS analysis of the plasma extracts.

3.1.5. Modification of Rapid Trace

Rapid Trace, an automatically manipulated SPE extractor, can be sequentially operated with up to 10 samples per rack. In the proposed method as described above, a maximum number of the samples is five columns because two fractions must be collected per column. We modified some parts of the equipment to be adaptable to a 6-ml column. Namely, the column holders in the turret were removed and newly prepared springs were set above the holes instead. The column plunger for 6 ml column was assembled with the combination of that of the Autotrace equipment and the top fitting through suitable union. According to this simple modification, no other modification is necessary for 6-ml columns. This modification allows the use of 6 ml columns which is expected to be useful not only for drug analysis of racehorses but also for environmental analysis and other fields.

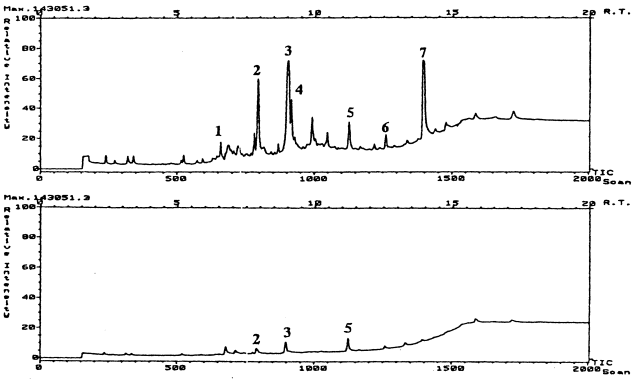

Fig. 5. Comparative total ion chromatograms of blank plasma in fraction A with (top) and without (bottom) purification. 1, Myristic acid; 2, palmitic acid; 3, linoleic acid; 4, stearic acid; 5, diethylhexylphthalate; 6, squalene; 7, cholesterol.

Table 1
Total recoveries (mean±SD%) of drugs throughout the method ($n=5$)

Drug	Spike (μg/ml)	Fraction	Total recovery (%)	RSD (%)	Drug	Spike (μg/ml)	Fraction	Total recovery (%)	RSD (%)
4-Aminoantipyrine	0.20	B	41.2±1.0	2.4	Metharbital	0.10	A	53.7±4.4	8.2
4-Methylamionantipyrine	0.50	B	52.4±2.0	3.8	Methoxyphenamine	0.20	B	89.7±1.9	2.1
Acebutolol	0.50	B	63.8±3.8	6.0	Methylephedrine	0.10	B	94.8±4.9	5.2
Acemethacine	0.20	A	90.4±4.6	5.1	Methylphenidate	0.10	B	88.9±1.7	1.9
Allobarbital	0.10	A	75.9±1.2	1.6	Metoprolol	0.50	B	77.6±3.3	4.3
Aminopyrine	0.10	B	74.6±1.5	2.0	Monoethylglycinexylidide	0.20	B	89.8±3.6	4.0
Amobarbital	0.10	A	76.2±4.2	5.5	Morphine	0.50	B	76.9±5.3	6.9
3′-Hydroxyamobarbital	0.50	A	61.8±1.9	3.1	Naproxen	0.20	A	62.4±3.1	5.0
Amphetamine	0.10	B	86.3±4.8	5.6	Nicotine	0.10	B	88.9±1.6	1.8
Antipyrine	0.10	B	48.6±1.7	3.5	Niflumic acid	0.20	A	76.4±3.7	4.8
Atropine	0.20	B	68.7±3.6	5.2	Nikethamide	0.10	B	54.0±2.2	4.0
Barbital	0.10	A	64.1±4.3	6.7	Noscapine	0.20	B	85.6±8.3	9.7
Benzocaine	0.10	B	91.2±2.8	3.1	Oxprenolol	0.50	B	73.8±3.7	5.0
Brucine	0.10	B	79.2±2.7	3.4	Oxyethyltheophylline	0.20	A	72.9±4.5	6.2
Caffeine	0.10	A	90.9±3.9	4.3	Oxyphenbutazone	0.50	A	84.3±3.6	4.3
Chlorpheniramine	0.20	B	80.8±1.6	2.0	Oxypropyltheophylline	0.20	A	75.9±2.0	2.7
Chlorpheniramine-*N*-oxide	1.00	B	83.5±2.1	2.5	Pemoline	0.50	A	78.3±2.6	3.3
Chlorpromazine	0.20	B	74.8±1.5	2.0	Pentazocine	0.20	B	84.7±4.6	5.4
Chlorpromazine sulfoxide	0.20	B	94.9±3.0	3.2	Pentetrazole	1.00	A	20.3±4.7	23.2
Cocaine	0.20	B	52.6±0.8	1.6	Pentobarbital	0.10	A	76.4±4.4	5.8
Cyclobarbital	0.10	A	74.5±5.4	7.3	3′-Hydroxypentobarbital	0.20	A	65.3±5.2	8.0
3′-Oxocyclobarbital	0.20	A	77.3±4.8	6.2	Phenacetin	0.20	A	85.4±5.6	6.5
3′-Hydroxycyclobarbital	0.50	A	15.1±1.3	8.6	Penobarbital	0.10	A	82.8±1.6	1.9
Cyproheptadine	0.20	B	87.4±2.8	3.2	Phenylbutazone	0.50	A	86.1±3.7	4.3
Cyproheptadine-*N*-oxide	0.50	B	83.9±3.3	3.9	Phenylethylmalonamide	0.20	A	48.5±6.3	13.0
Dibucaine	0.05	B	96.9±1.4	1.4	Phenylpropanolamine	0.50	B	89.7±3.0	3.3
β,γ-Dihydroxydibucaine	0.20	B	88.3±2.1	2.4	Pipradrol	0.50	B	84.5±4.1	4.9
Diclofenac	0.20	A	73.8±4.5	6.1	Piroxicam	1.00	A	67.5±3.1	4.6
Dihydroxypropyltheophylline	0.50	A	76.5±3.9	5.1	Primidone	0.20	A	79.9±5.4	6.8
Dimorpholamine	0.20	A	89.9±6.0	6.7	Procaine	0.10	B	84.1±2.9	3.4
γ-Hydroxydimorpholamine	0.20	A	84.8±6.7	7.9	Promazine	0.20	B	92.4±6.7	7.3
Diphenhydramine	0.10	B	84.7±3.2	3.8	Promazine sulfoxide	0.50	B	77.5±4.5	5.8
Des-methyldiphenhydramine	0.50	B	91.3±2.3	2.5	Propranalol	0.50	B	73.1±2.7	3.7
Ephedrine	0.50	B	62.6±1.1	1.8	Salbutamol	0.10	B	54.3±3.8	7.0
Flufenamic acid	0.20	A	80.6±2.9	3.6	Scopolamine	0.50	B	85.9±2.3	2.7
Flunixin	0.50	A	78.1±3.3	4.2	Secobarbital	0.20	A	73.2±2.0	2.7
Fluphenazine	0.20	B	76.3±3.5	4.6	3′-Hydroxysecobarbital	0.20	A	90.0±1.3	1.4
Hexobarbital	0.20	A	65.8±5.7	8.7	Strychnine	0.20	B	71.9±3.9	5.4
3′-Oxohexobarbital	0.20	A	69.6±5.5	7.9	Sulindac	0.20	A	86.5±3.9	4.5
3′-Hydroxyhexobarbital	0.20	A	42.6±1.9	4.5	Sulindac-sulfone	0.20	A	87.4±4.1	4.7
Ibuprofen	0.20	A	41.9±2.5	6.0	Tetracaine	0.20	B	75.8±3.2	4.2
Indomethacin	0.20	A	72.2±3.0	4.2	Tetracaine-*N*-oxide	0.20	B	67.3±3.4	5.1
Isoxsuprine	0.20	B	86.9±3.2	3.7	Theobromine	0.20	A	72.4±3.6	5.0
Ketoprofen	0.20	A	77.3±3.5	4.5	Theophylline	0.20	A	82.5±6.1	7.4
Hydroketoprofen	0.20	A	81.5±2.7	3.3	Thiamylal	0.50	A	78.4±3.8	4.8
Lidocaine	0.10	B	88.6±3.0	3.4	3′-Hydroxythiamiral	0.50	A	82.2±1.9	2.3
Loxoprofen	0.20	A	81.7±4.0	4.9	Thiopental	0.50	A	72.1±3.5	4.9
trans-Hydroloxoprofen	0.20	A	88.3±1.6	1.8	3′-Hydroxythiopental	0.50	A	68.7±2.9	4.2
Meclofenamic acid	0.20	A	80.2±2.4	3.0	Tiaramide	1.00	B	72.9±2.8	3.8
Mefenamic acid	0.20	A	58.3±4.1	7.0	Des-hydroxyethyltiaramide	1.00	B	69.1±3.0	4.3
Mephenesine	0.20	B	88.3±3.1	3.5	Timolol	0.20	A	77.2±3.2	4.1
Mephobarbital	0.10	A	63.0±5.7	9.0	Tolfenamic acid	0.20	A	75.7±2.8	3.7
Methapyrilene	0.10	B	88.4±8.4	9.5	Tolmetin	0.20	A	78.5±3.5	4.5
Methamphetamine	0.10	B	83.7±2.7	3.2					

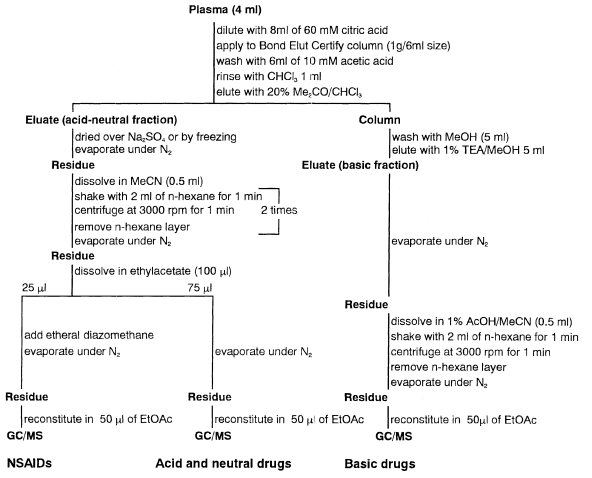

Fig. 6. Sample preparation method for doping drugs in horse plasma.

3.1.6. Sample preparation

Following the experimental results described above, we assembled the extraction method as shown in Fig. 6. Horse plasma (4 ml) is diluted with 8 ml of 60 m*M* citric acid. After setting the sample on a shuttle rack of the Rapid Trace, the extraction is manipulated automatically as described below. The column (1 g/6 ml) is primed with successive, methanol (6 ml), deionized water (6 ml), and 5 m*M* aqueous citric acid (3 ml) at 20 ml/min. Diluted sample is applied onto the column at 1.5 ml/min, the column is rinsed with 10 m*M* acetic acid (6 ml) at 10 ml/min and followed with 1 ml of chloroform to eliminate the remaining moisture in the sorbent. After drying the column with N_2 gas for 2 min, the column is eluted with 20% (v/v) acetone in chloroform (6 ml) at 1.5 ml/min (fraction A), and the eluate is collected in a test tube. The column is

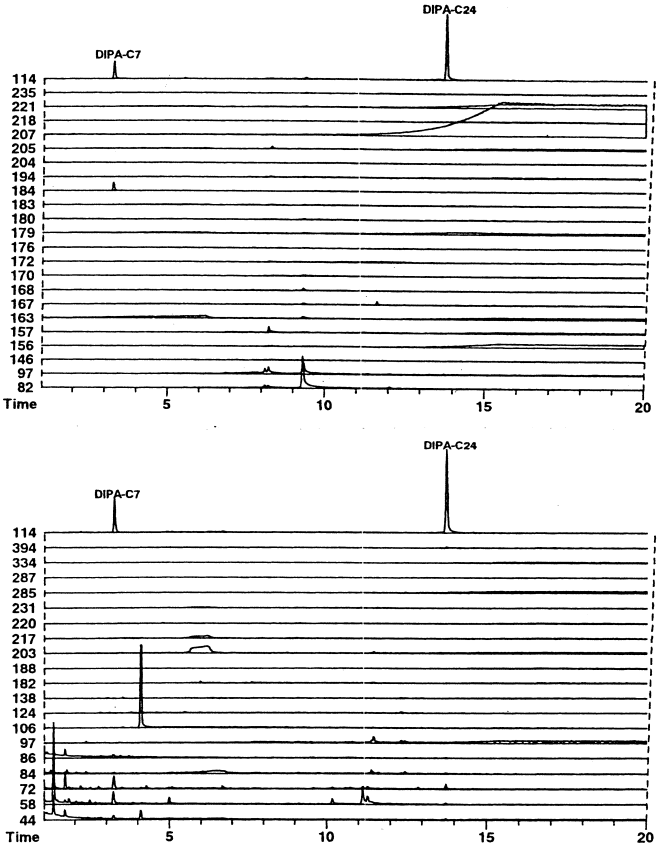

Fig. 7. Ion chromatograms of blank plasma extracts in fraction A (top) and fraction B (bottom). Column: DB-5 (15 m×0.53 mm, I.D., 90–320°C, 15°C/min; DIPA-C_7, 1-(*N*,*N*-diisopropylamino)-*n*-heptane; DIPA-C_{24}, 1-(*N*,*N*-diisopropylamino)-*n*-tetracosane.

rinsed with methanol (6 ml) at 10 ml/min then eluted with 5 ml of 1% (v/v) triethylamine in methanol at 1.5 ml/min (fraction B), and the eluate is collected in another tube.

Both fractions A and B are evaporated to dryness under a nitrogen stream at 40°C. Fraction A is dried by freezing in a cryo-bath at −40°C. After evaporation, the residues are dissolved in 0.5 ml of acetonitrile and 0.5 ml of 1% acetic acid in acetonitrile for fractions A and B, respectively. Then the solution is shaken with 2 ml of *n*-hexane as a final clean-up. The partitioning should be repeated twice for fraction A. Afterwards, fraction A is reconstituted in ethyl acetate and one fourth of the volume is taken for analysis of NSAIDs after derivatizing as methyl ester. Total recoveries in most drugs were ranged from 70 to 95% throughout the method whereas the lowest was about 42% (Table 1). Typical ion chromatograms of blank plasma extracts in fractions A and B are shown in Fig. 7.

3.2. GC–MS

A drug detection method for a doping test must be applicable to a wide range of drugs and their metabolites which are present at low nanogram levels. In our routine work, the process of confirmation can be classified into four steps, namely sample preparation, second screening, drug identification and reconfirmation. After the first screening, positive samples are subjected to a second screening by MC to determine whether the prohibited substances are present or not. This MC screening is applicable to the simultaneous screening for 35 acid–neutral drugs including their metabolites using 22 specified ions (fraction A), 14 NSAIDs using 11 specified ions (fraction A methylated) and 51 basic drugs using 19 characteristic ions (fraction B). Additional drugs can be detected by addition or change of the selected ions to the ion chromatograms reconstruct from the mass spectra. A typical ion chromatogram of an extract of horse plasma spiked with ephedrine (50 ng/ml) is shown in Fig. 8. Mass spectral data of doping drugs and related compounds are shown in Table 2.

In the case of a positive on MC screening, the sample is subjected to the drug identification test by GC–MS again. Alternatively, the sample is derivatized by acetylation, trimethylsilylation or trifluoroacetylation. After completing the measurement, the retention index of the peak corresponding the drug is calculated with the retention time of DIPA-alkanes as

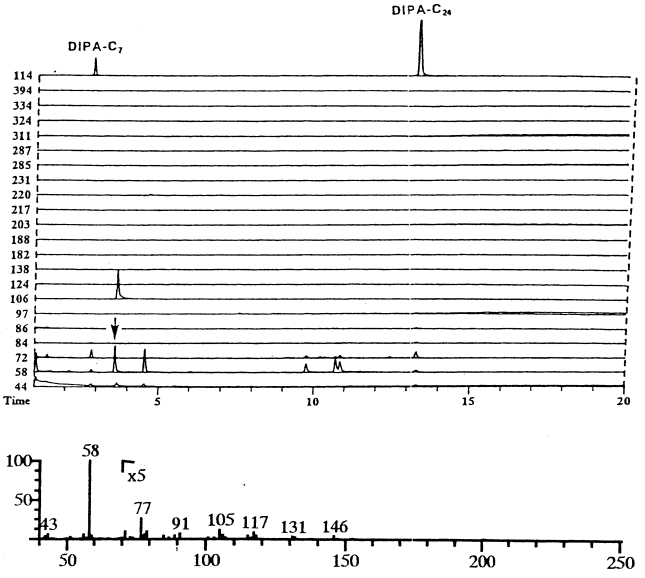

Fig. 8. Ion chromatogram and mass spectrum of ephedrine obtained from spiked plasma (50 ng/ml). DIPA-C_7, 1-(*N*,*N*-Diisopropylamino)-*n*-heptane; DIPA-C_{24}, 1-(*N*,*N*-diisopropylamino)-*n*-tetracosane.

Table 2
Mass spectral data and DIPA retention indices of doping drugs and their related compounds and selected ions for mass chromatography

Compound	M_r	Base peak m/z (%Σ)	Other prominent ions m/z (relative intensity %)					Confirmation condition	DIPA-RI
Acid–neutral drugs									
Allobarbital	208	124(10.5)	80(81.2)	106(43.6)	143(47.8)	**167**(88.8)	**179**(12.1)	D	1245
Amobarbital	226	**156**(23.1)	55(25.1)	98(17.8)	141(95.6)	**157**(38.6)	197(10.2)	D	1363
3′-Hydroxyamobarbital	242	59(14.1)	71(15.6)	141(38.1)	**156**(62.5)	**157**(71.7)	227(11.4)	D	1583
Barbital	184	**141**(25.3)	69(8.9)	83(12.6)	98(17.7)	112(17.5)	**156**(89.8)	D	1118
Caffeine	194	**194**(37.4)	55(18.4)	67(11.9)	**82**(13.0)	109(39.4)	193(13.9)	D	1453
Cyclobarbital	236	**207**(29.2)	67(37.4)	81(19.2)	141(41.3)	147(9.3)	164(7.8)	D	1687
3′-Hydroxycyclobarbital	252	**97**(24.7)	67(6.8)	79(10.5)	141(6.3)	**205**(31.3)	223(3.4)	D	1920
3′-Oxocyclobarbital	250	**221**(22.6)	95(18.5)	123(12.2)	136(8.6)	150(20.4)	250(13.7)	D	1988
Dihydroxypropyl-	254	223(11.4)	95(28.0)	109(38.0)	123(23.8)	137(12.7)	166(39.3)		
Theophylline (DPT)			**180**(88.2)	193(44.4)	**194**(92.6)	236(25.2)	254(54.1)		
DPT-TMS	398	73(8.9)	129(42.3)	147(59.7)	**252**(53.5)	**308**(38.2)	**398**(9.4)	B	1884
Dimorpholamine	398	**114**(25.0)	70(28.7)	169(37.8)	199(27.1)	212(23.5)	213(17.9)	C	2210
γ-Hydroxydimorpholamine	414	**114**(16.9)	70(37.0)	84(28.8)	169(36.9)	**157**(22.6)	199(20.2)	C	2400
Hexobarbital	236	**221**(18.4)	81(72.3)	155(41.5)	**157**(42.6)	236(4.5)		D	1566
3-Hydroxyhexobarbital	252	**97**(20.1)	79(18.2)	**156**(12.5)	219(14.7)	234(3.3)		D	1800
3′-Oxohexobarbital	250	95(13.4)	**156**(15.3)	193(18.7)	**207**(18.9)	**235**(93.6)	250(26.1)	D	1858
Metharbital	198	155(23.3)	83(20.8)	98(20.1)	112(34.9)	126(12.7)	**170**(81.5)	D	1036
Mephobarbital	246	**218**(24.5)	117(51.5)	**146**(32.7)	**161**(18.0)	175(17.6)	246(4.5)	E	1650
Oxyethyltheophylline	224	**180**(19.8)	68(10.8)	95(40.5)	109(22.6)	123(25.3)	151(5.8)	B	1740
			193(21.0)	**194**(22.5)	206(15.6)	224(60.7)			
Oxypropyltheophylline	238	**194**(15.2)	68(5.2)	81(11.4)	95(15.7)	109(57.8)	123(16.7)	B	1782
			137(26.3)	165(8.1)	**180**(88.5)	193(55.4)	238(44.4)		
Pemoline	176	**176**(16.5)	70(25.9)	77(29.2)	89(39.7)	90(52.6)	107(88.5)		
POD-Me	191	**105**(30.2)	77(41.7)	89(37.4)	90(80.4)	106(57.9)	**191**(53.9)	D	1301
Pentetrazol	138	**82**(20.5)	54(33.7)	55(94.9)	68(11.3)	109(24.4)	138(27.4)	A	1225
Pentobarbital	226	141(25.2)	71(9.3)	98(12.1)	112(7.3)	**156**(96.4)	**157**(18.5)	D	1400
3′-Hydroxypentobarbiral	242	**156**(17.3)	69(53.4)	141(93.5)	**157**(44.6)	195(10.2)	197(12.8)	D	1647
Phenacetin	179	108(20.3)	80(10.8)	81(12.9)	109(93.5)	137(63.6)	**179**(88.5)	D	1390
Phenobarbital	232	**204**(23.1)	77(15.4)	117(42.1)	**146**(12.7)	161(14.9)	232(7.8)	E	1822
Phenylbutazone	308	**183**(23.1)	77(49.6)	105(15.2)	**184**(41.6)	252(15.6)	308(53.7)	D	2085
Phenylethylmalonamide	206	148(23.3)	91(30.2)	103(39.8)	117(15.4)	120(20.1)	**163**(89.6)	E	1749
Primidone	218	**146**(17.3)	117(65.3)	118(42.3)	161(25.8)	190(81.9)	**218**(3.1)	E	2237
Secobarbital	238	**168**(17.1)	**97**(29.7)	108(10.9)	124(52.6)	**167**(98.3)	195(16.8)	D	1454
3-Hydroxysecobarbital	254	**168**(12.0)	70(38.3)	**97**(40.5)	124(18.4)	**167**(59.4)	169(42.5)	D	1692
Theobromine	180	**180**(34.4)	55(24.1)	67(18.7)	**82**(17.1)	109(24.6)	137(15.5)	B	1607
Theophylline	180	**180**(34.9)	68(23.7)	95(41.4)	123(14.5)	151(6.7)		B	1738
Thiamylal	254	**184**(16.1)	**97**(12.3)	169(21.6)	**183**(44.9)	185(43.1)	212(11.8)	D	1586
3′-Hydroxythiamyral	270	**184**(8.1)	**97**(27.1)	171(42.6)	**183**(53.3)	185(61.3)	229(8.4)	D	1822
Thiopental	242	**157**(17.1)	69(21.2)	**97**(17.4)	**172**(93.5)	173(62.8)	242(5.1)	D	1537
3′-Hydroxythiopental	258	**172**(13.6)	69(49.3)	**97**(12.7)	**157**(68.9)	173(95.5)	258(5.2)	D	1779
*NSAIDs**									
Acemethacin	429	**139**(27.5)	111(23.3)	141(33.4)	312(18.9)	429(24.5)	431(8.2)	G	2742
Diclofenac	309	**214**(17.0)	215(35.4)	216(38.5)	242(51.3)	309(26.1)	311(16.9)	F	2131
Flufenamic acid	295	**263**(28.3)	166(10.8)	207(13.5)	235(14.2)	243(8.5)	295(47.6)	F	1586
Flunixin	310	295(15.6)	235(11.7)	251(40.1)	**263**(80.5)	277(17.1)	310(39.8)	F	
Ibuprofen	220	**161**(26.9)	121(38.4)	134(58.7)	177(28.5)	194(25.1)	220(23.8)	A	1070
Indomethacin	371	**139**(28.5)	111(25.3)	141(32.8)	312(12.5)	371(18.1)	373(6.4)	G	2427
Ketoprofen	268	**209**(23.7)	77(56.2)	105(89.1)	191(25.3)	210(21.3)	268(37.6)	F	2036
Hydroketoprofen	270	105(14.2)	**165**(61.2)	183(13.2)	191(25.1)	210(12.4)	211(25.1)	F	2054
Loxoprofen	260	**201**(18.0)	117(63.4)	177(33.6)	200(26.1)	242(9.4)	260(36.9)	F	1927
trans-Hydroloxoprofen-TFA	262	117(19.5)	177(21.4)	185(27.9)	244(17.3)	299(51.4)	358(10.2)	F	1616
Mefenamic acid	255	**223**(18.6)	180(24.3)	194(20.3)	208(58.9)	222(27.2)	255(64.3)	F	1938

Table 2. Continued

Compound	M_r	Base peak m/z (%Σ)	Other prominent ions m/z (relative intensity %)					Confirmation condition	DIPA-RI
Naproxen	244	**185**(34.6)	141(11.3)	154(10.2)	170(13.8)	186(12.3)	244(37.5)	F	1865
Piroxicam	345	121(9.1)	162(60.3)	**250**(62.8)	280(27.8)	330(30.1)	345(5.8)	G	2381
Sulindac	370	**233**(14.9)	247(32.1)	248(38.6)	281(25.3)	354(29.4)	370(22.1)	G	2733
Sulindac sulfone	386	**233**(17.7)	220(10.2)	247(21.3)	248(40.2)	312(36.8)	386(27.8)	G	2767
Basic drugs									
4-Aminoantipyrine	203	**203**(15.4)	56(63.2)	57(40.9)	83(25.4)	84(64.0)	123(6.5)		
4-Aminoantipyrine-TFA	299	56(19.4)	83(55.0)	**230**(36.9)	231(10.2)	**299**(34.8)		B	1625
Aminopyrine	231	56(21.8)	77(12.0)	**97**(62.1)	111(25.4)	112(17.3)	**231**(56.3)	B	1522
Amphetamine	135	**44**(54.9)	65(4.8)	77(3.6)	91(9.2)	120(5.6)			
Amphetamine-TFA	231	**140**(26.0)	91(61.3)	92(12.8)	117(22.8)	118(92.8)	119(13.0)	A	805
Antipyrine	188	**188**(21.8)	56(37.8)	77(62.0)	96(87.5)	105(26.6)	187(14.8)	B	1496
Atropine	289	**124**(24.7)	82(32.5)	83(30.9)	94(20.3)	96(13.7)	289(5.5)	B	1806
Brucine	394	**394**(22.2)	207(17.5)	281(23.2)	356(5.6)	379(29.2)		C	2943
Chlorpromazine	318	**58**(19.9)	**86**(46.3)	214(15.2)	233(16.6)	246(33.2)	318(12.1)	C	1910
Chlorpromazine sulfoxide	334	**58**(6.8)	**86**(36.3)	214(12.2)	246(24.2)	318(10.9)	**334**(5.2)	C	2250
Cocaine	303	**82**(17.3)	83(43.1)	96(21.2)	105(22.2)	**182**(52.8)	**303**(10.3)	B	1822
Cyproheptadine	287	96(13.7)	70(37.7)	189(27.0)	215(52.0)	229(24.9)	**287**(76.6)	B	1946
Dibucaine	343	**86**(66.0)	**58**(5.7)	87(6.8)	99(3.8)			C	2120
β,γ-Dihydroxydibucaine	375	**86**(63.9)	**58**(4.3)	99(2.1)	116(1.8)	215(2.3)	330(1.2)		
β,γ-Dihydroxydibucaine-TMS	519	**86**(48.1)	73(3.0)	87(5.0)	117(3.3)	147(3.5)		C	2523
Ephedrine	165	**58**(47.3)	77(6.6)	105(4.6)	117(6.9)	146(3.5)			
Ephedrine-TFA	357	**154**(49.8)	77(3.2)	91(2.9)	110(32.5)	155(7.4)		A	887
Lidocaine	234	**86**(56.5)	**58**(10.5)	87(6.3)	120(4.1)	234(2.3)		A	1468
Methamphetamine	149	**58**(56.9)	91(5.0)	134(4.9)					
Methamphetamine-TFA	245	**154**(40.4)	91(20.2)	110(49.0)	118(30.3)	155(8.7)		A	930
Methapyrilene	261	**58**(25.5)	71(24.5)	72(26.4)	**97**(73.2)	191(10.1)	261(3.4)	B	1581
Methoxyphenamine	179	**58**(68.5)	65(2.8)	77(2.2)	91(6.5)	121(2.6)			
Methoxyphenamine-TFA	275	**154**(29.2)	91(25.5)	110(47.4)	121(13.5)	**148**(48.5)		A	1115
4-Methylaminoantipyrine	217	56(16.0)	83(39.0)	92(9.3)	98(19.2)	123(19.7)	**217**(95.6)		
4-Methylaminoantipyrine-TFA	313	**216**(18.2)	56(40.3)	123(85.6)	193(8.3)	217(12.6)	**313**(58.4)	B	1762
Methylephedrine	179	72(54.2)	77(4.0)	105(2.7)	118(2.6)				
Methylephedrine-Ac	221	**72**(58.0)	56(4.1)	73(4.9)	117(3.8)			A	930
Methylphenidate	233	**84**(47.6)	56(4.6)	91(9.6)	118(3.8)	150(4.5)	172(2.3)		
Methylphenidate-TFA	329	**180**(50.5)	67(24.7)	91(8.5)	126(6.6)	150(6.4)	181(6.5)	A	1488
Monoethylglycinexylidide	206	**58**(30.8)	**106**(8.3)	120(11.4)	121(12.9)	163(15.3)	206(4.1)	A	1422
Morphine	285	**285**(14.1)	124(12.1)	162(23.2)	215(21.7)	228(6.4)	268(12.0)		
Morphine-TMS	429	**429**(8.0)	196(37.2)	**236**(78.2)	287(25.1)	401(25.1)	**414**(41.2)	B	2102
Nicotine	162	**84**(31.9)	42(12.3)	86(18.2)	119(10.0)	133(25.2)	**162**(13.3)	A	868
Nikethamide	178	**106**(35.8)	51(27.9)	78(50.5)	107(8.7)	177(33.7)	178(11.7)	A	1124
Noscapine	413	**220**(53.3)	**205**(24.4)	221(12.7)				C	9578
Pentazocine	285	**217**(10.5)	110(28.2)	173(17.4)	202(36.7)	284(27.3)	**285**(34.1)		
Pentazocine-TMS	357	**289**(8.1)	73(67.4)	244(37.4)	245(35.4)	**342**(35.8)	**357**(18.7)	B	1807
Pipradrol	267	**84**(58.4)	56(9.5)	77(7.5)	85(6.3)	105(6.9)		B	1757
Procaine	236	**86**(45.1)	**58**(13.0)	71(5.5)	92(6.3)	99(24.0)	120(17.6)	B	1645
Promazine	284	**58**(13.4)	**86**(34.3)	180(17.5)	199(16.1)	238(14.3)	**284**(18.4)	C	1737
Promazine sulfoxide	300	**58**(12.0)	**86**(26.0)	180(20.7)	199(19.6)	212(23.8)	284(6.7)	C	2100
Scopolamine	303	94(11.2)	**97**(21.0)	108(51.9)	138(74.2)	154(26.0)	**303**(12.5)	B	1991
Strychnine	334	**334**(15.0)	107(25.9)	120(37.4)	143(23.6)	144(28.0)	162(24.9)	C	2554
Tetracaine	264	**58**(32.9)	**71**(63.2)	72(7.9)	150(11.7)	**176**(12.0)	193(4.6)	B	1825

* Measured as methyl ester. Ac, Acetyl derivative; TFA, trifluoroacetyl derivative; TMS, trimethylsilyl derivative; POD, 5-phenyl-2,4-oxazolidinedione. Bold face shows the selected ions for MC screening: acid–neutral drugs: m/z 82, 97, 146,156,157,163,167,168,170, 172,176,179, 180, 183, 184, 194, 204, 205, 207, 114 (I.S.); NSAIDs: m/z 139, 161,165, 185, 201, 209, 214, 223, 233, 250, 263, 114 (I.S.); basic drugs: m/z 44, 58, 72, 84, 86, 97, 106, 124, 138, 182, 188, 203, 217, 220, 231, 285, 287, 334, 394, 114 (I.S.). Confirmation and reconfirmation: A: SPB-20 (15 m×0.53 mm, film thickness 1.0 μm); 90–300°C (10°C/min); I.S. DIPA-C_7 (2.5 ng) and DIPA-C_{16} (5 ng). B: SPB-20 (15 m×0.53 mm, film thickness 1.0 μm); 170–300°C (8°C/min); I.S. DIPA-C_{14} (2.5 ng) and DIPA-C_{24} (5 ng). C: DB-1 (15 m×0.53 mm, film thickness 1.5 μm); 200–320°C (8°C/min); I.S. DIPA-C_{17} (5 ng) and DIPA-C_{30} (10 ng). D: SPB-20 (15 m×0.53 mm, film thickness 1.0 μm); 90–300°C (15°C/min); I.S. DIPA-C_7 (2.5 ng) and DIPA-C_{22} (5 ng). E: DB-1701 (15 m×0.53 mm, film thickness 1.5 μm); 140–280°C (10°C/min); I.S. DIPA-C_{14} (2.5 ng) and DIPA-C_{24} (5 ng). F: DB-17 (15 m×0.53 mm, film thickness 1.0 μm); 140–280°C (10°C/min); I.S. DIPA-C_{14} (2.5 ng) and DIPA-C_{24} (5 ng). G: XTI-5 (15 m×0.53 mm, film thickness 1.0 μm); 150–320°C (15°C/min); I.S. DIPA-C_{17} (5 ng) and DIPA-C_{30} (10 ng).

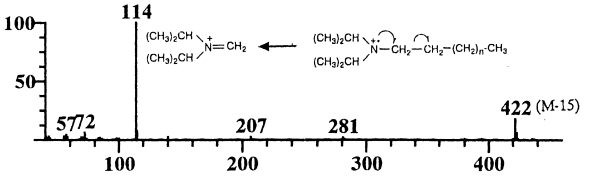

Fig. 9. Mass spectra and typical fragmentation of 1-(*N*,*N*-diisopropylamino)-*n*-tetracosane (DIPA-C_{24}).

marker substances [13]. As shown in Fig. 9, the mass spectral patterns of DIPA-alkanes are simple, and a fragment ion at m/z 114 which is produced by alpha cleavage of amino alkyl chain, appeared commonly as a base peak in the electron impact (EI) mass spectra of all DIPA-alkanes. This fragment ion of m/z 114 is an uncommon fragment and does not interfere with fragments from pharmaceutical compounds. Moreover, the 114 ion rarely appears on the background spectra of blank plasma.

The retention index (RI) system based on a homologous series of *n*-alkanes was first proposed by Kovats [23], and it has been used in the widespread field of drug analysis [24–26]. A modified equation for the calculation of RI value under the temperature-programmed condition was published by Van Den Dool and Krats [27], and it has been useful for the substances having a wide range of boiling points. The retention index on temperature-programmed GC was calculated using the following equation:

$$I = 100(z - y)\frac{t_R(s) - t_R(y)}{t_R(z) - t_R(y)} + 100y$$

where y and z represent the carbon numbers of the earlier and later eluting *n*-alkanes or DIPA-alkanes, respectively; $t_R(s)$ is the retention time of the drug; $t_R(y)$ and $t_R(z)$ are the retention times of *n*-alkanes or DIPA-alkanes of carbon number y and z that are respectively smaller and larger than $t_R(s)$. The carbon number for DIPA-alkane is defined by the number of carbon atoms in an *n*-alkyl chain of the diiso-

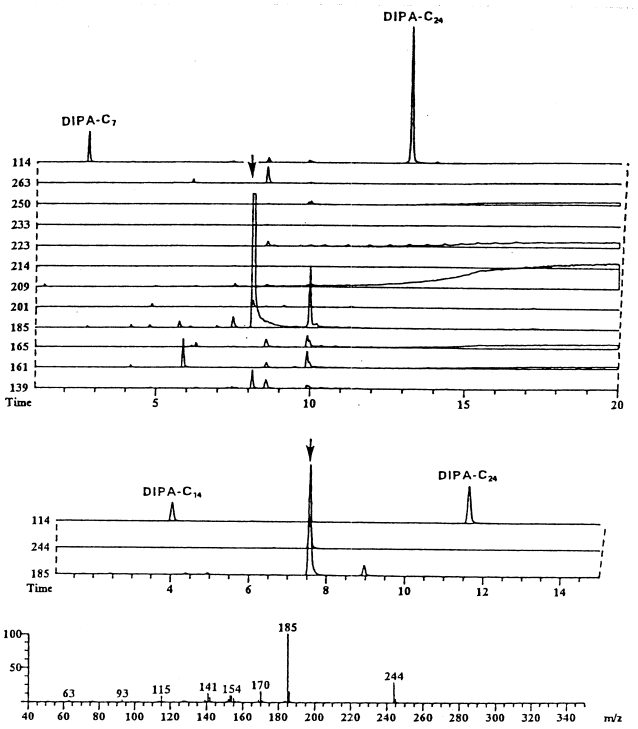

Fig. 10. Ion chromatograms for the screening of NSAIDs (top) and the data of the drug identification test of naproxen (middle and bottom) in horse plasma collected 24 h after administration of naproxen (6 mg/kg, p.o., condition F is shown in Table 2).

propylamino group. The index values found from DIPA-alkanes (DIPA-indices) were evaluated by comparing the data found from *n*-alkanes (Kovats-indices). DIPA-alkanes showed linear distribution in order of the number of methylene units of *n*-alkyl chain with temperature-programmed gas chromatography. There is a linear correlation between the Kovats-indices and the DIPA-indices on several capillary columns examined [13].

The retention indices of DIPA-alkanes in four kinds of columns showed linearity, and the equations to convert them to Kovats retention index are as follows: DB-1, $KI = 1.012D + 572.241$ ($r = 0.9999$); DB-5, $KI = 1.017D + 565.748$ ($r = 0.9999$); SPB-20, $KI = 1.014D + 575.065$ ($r = 0.9999$); and DB-17, $KI = 1.016D + 580.993$ ($r = 0.9999$). Where KI is the Kovats index (found value) and D is the DIPA-index for each drug. The Kovats indices are led from DIPA-indices by placing the DIPA-index value in the place of D in the respective equations above. Full mass spectral data of the peak are compared with those obtained from the authentic compound. The cut-off value on mass chromatographic screening was regarded as a lower limit of a drug detection on the ion chromatogram when a peak height of the specified ion peaks of a drug showed approximately 20% compared with that of the peak of DIPA-tetracosane at m/z 114 used as an internal standard.

When positive results are obtained in the confirmatory test, the drug confirmation must be done again with the split sample in the presence of a competent witness with appropriate training and experience. The final decision of a positive result should be completely matched all analytical data obtained from both samples and the authentic compound. Mass chromatographic screening data and confirmatory test data of the extracts of horse plasma collected 24 h after administration of naproxen (6 mg/kg, p.o.) to a thoroughbred horse (gelding, 5 years old, body mass 505 kg) are shown in Fig. 10. The method provides sufficient sensitivity to detect low ng/ml levels of the drugs in horse plasma.

4. Conclusion

A Bond Elut Certify column of 1 g/6 ml size is suitable to extract 4 ml of horse plasma. To collect the acidic drugs, it is desirable that the column must be kept below pH 3.5 during the extraction. The basic drugs are effectively collected with a small volume of 1% TEA in methanol solution. The plasma constituents (fatty acids and cholesterol) and dialkylphthalates are dramatically removed by non-aqueous partitioning with acetonitrile and *n*-hexane (for fraction A) and 1% (v/v) acetic acid in acetonitrile and *n*-hexane (for fraction B). The method is well suited to sample preparation for GC–MS analysis of doping drugs in horse plasma. Selected ion monitoring, a highly sensitive detection, is used to avoid the influence of contaminants. Total recoveries for most of drugs ranged from 70 to 95% throughout the method, the lowest recovery was about 42%. Cut-off value on the mass chromatographic screening ranged from 20 to 100 ng/ml. This procedure achieves both good recoveries and clean extracts for GC–MS analysis.

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