CHROMATOGRAPHIC DETERMINATION OF SOME CORTICOSTEROIDS, WITH SPECIAL REFERENCE TO HORSE DOPING

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ABSTRACT

Some chromatographic procedures which can be used to detect and determine certain corticosteroids in samples from racehorses, are described. These procedures include thin-layer gas and high pressure liquid chromatography.

THE DETECTION OF DOPING AGENTS IN BLOOD

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ABSTRACT

Gas chromatographic screening procedures have been evaluated which permit the detection of stimulants and sedatives in blood after administration of pharmacological doses. The techniques actually used in sample preparations and gas chromatographic work are presented as well as examples of pharmacokinetic studies and positive dope cases. The use of sensitive and selective detectors like the nitrogen-specific detector or a mass spectrometer is absolutely essential for routine work, as for non-specific detectors the number of "false positives" leads to an intolerable work load for the laboratory.

1. Screening Procedure for Doping Agents in Urine

The basis of our techniques for the detection of doping agents in urine is the screening procedures for volatile and slightly volatile drugs, devised for the XX Olympic Games, Munich, 1972. The procedure covers most of the common phenylethylamines and some neutral drugs like nikethamide, leptazol and caffeine.

1. Only one partition step: 5 ml of urine are extracted with 2 ml of ether after addition of 0.5 ml 5 N KOH, 3.5 g Na_2SO_4 and 25 μg of diphenylamine as internal standard. The ether phase is used for gas-chromatographic analysis without concentration.

2. Chromatography is achieved on Apiezon L on Chromosorb W, washed with KOH and coated with 2% Igepal CO880.

3. A nitrogen specific detector (N-FID, Hewlett-Packard) is used because of its high sensitivity and selectivity.

4. A temperature programme with a gradient of 20°C/minute is used to shorten analysis time. The analysis cycle – injection, GC-analysis, cooling, equilibration and injection – lasts only 15 minutes.

5. Automatic injection guarantees a high reproducibility of retention times, which is a prerequisite of automatic evalution of the analytical data by computer.

Fig. 1 (a-d) demonstrates the potential of this screening procedure.

2. Screening Procedure for Doping Agents in Blood

Three reasons exist for attempting to detect doping agents in blood, which are valid for both human and veterinary sport:

1. The direct relationship between blood concentration and pharmacological activity.

2. The impossibility of obtaining urine in some cases.

3. The improved analytical methods of today which make it possible to detect blood levels of doping agents.

The change from urine to blood as a dope detection fluid presented many problems. Finally, we succeeded in screening blood for many doping agents. The character-

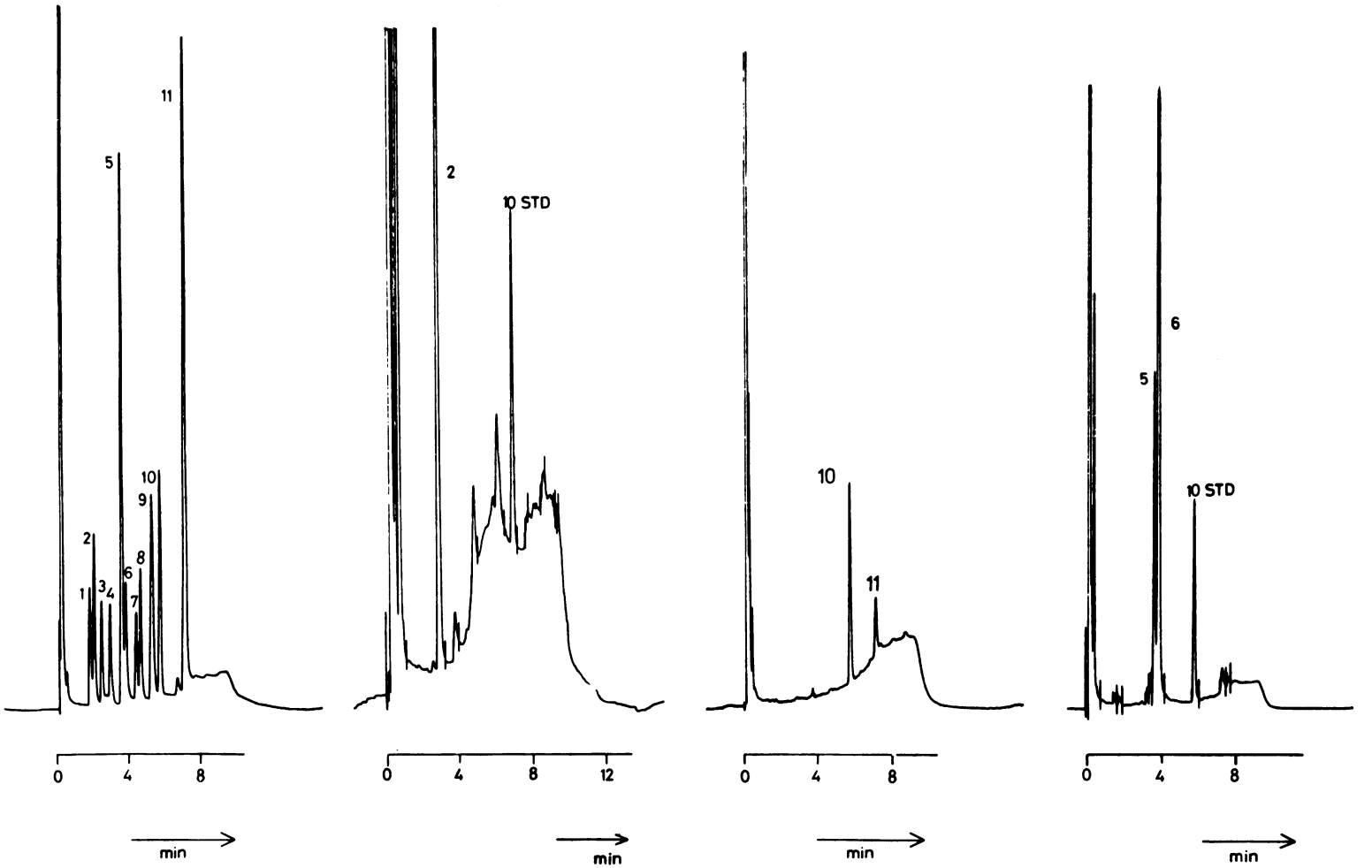


Fig. 1: Screening procedure for volatile doping agents in urine

a) Calibration curve (25 ng/injection)
1 = heptaminole, 2 = amphetamine, 3 = methamphetamine, 4 = dimethylamphetamine, 5 = nicotine, 6 = ephedrine, 7 = phenmetrazine, 8 = nikethamide, 9 = pentamethylentetrazole, 10 = diphenylamine (used as a standard), 11 = caffeine
Column Apiezon L, 130°-270°C, 20°C/min, 2 min at 270°C

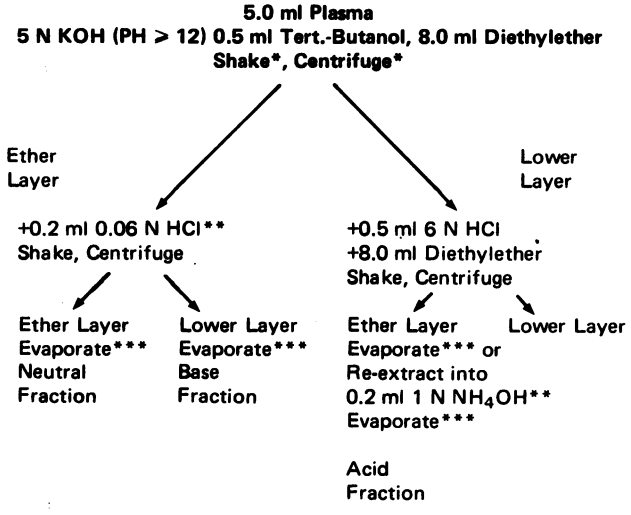
b) negative sample

c) positive sample: amphetamine (2)

d) positive sample: ephedrine (6)

istic features of the routine procedure are summarized in Table I.

TABLE I



* Shake: 15 min. centrifuge: 5 minutes at 2000 rpm
** Containing 10 μg methylorange
*** Evaporate: a) In vacuum
b) Over P_2O_5/KOH in a vacuum dessicator

Summary of routine screening procedure for detection of doping agents in blood.

5 ml of plasma are used with the assumptions that 1 ng/ml is the lowest effective plasma concentration of a doping agent and, that after dissolution of the residues of the basic, neutral or acidic fractions in 30 μl of methanol, a sample containing at least 1 ng can be injected. We have not yet done excretion studies for all doping agents in question but the following examples of either excretion studies or positive dope cases, demonstrate the capabilities of this scheme.

The residues are analysed on different columns according to the chemical nature of the expected compounds. At the moment the following gas chromatography procedures are used:

1. Basic fraction. (Phenylethylamines, local anaesthetics, antipyretics, phenothiazines, benzodiazepines, strychnine)

Column I: Apiezon L, 12.5% (w:w) on Chromosorb W, KOH washed and deactivated with 2% Igepal CO880, 1.06 m column length, 130°-270°C (4 min), 20°C min, N-FID

Column II: OV 101, 5% on Gas-Chrom Q, 80-100 mesh, 1.50 m column length

a) 120°-260°C (4 min) 20°/min, N-FID
b) 280°C isothermal, N-FID
c) 200°C isothermal, mass spectrometry (single ion detection) or N-FID

2. Neutral fraction. (Caffeine, leptazole, compounds of the amide type)

a) Column I (see above), 200°C (or 220°C) isothermal, N-FID
b) Column I, 130°-270°C, (4 min) 20°C/min N-FID

3. Acidic fraction. (e.g. Barbiturates, phenylbutazone)

Column II, 220°C isothermal N-FID
Column III, 10% OV 225 on Gas-Chrom Q, 80-100 mesh, 1.06 m column length, N-FID 100°-220°C (4 min) 20°C/min

In addition to columns I and II, the following columns are used to obtain further information on "nitrogen-positive" peaks not normally found in urine:

Column IV: 10% PEG 20 M on Chromosorb W, KOH washed, 1.50 m column length
Column V: 5% OV 17 on Gas-Chrom Q, 80-100 mesh, 1.50 m column length
Column VI: 2% OV 101 on Gas-Chrom Q, 1.06 m column length

To determine the Kovats indices the retention time of the substance is adjusted to about 4 min by the choice of column temperature. The selectivity of the N-FID is lowered to a value about 1:200 (Nitrogen:Carbon). Under these conditions n-alkanes at a concentration of 0.5 to 1.0 mg/ml are eluted as symmetrical peaks.

The significance of Kovats indices, determined on a polar and a non-polar column (PEG and Apiezon L) as a mean of identification for volatile doping agents was discussed recently (Donike & Stratmann, 1976).

Example 1

Blood was delivered with the comment: "Suspicious for *negative doping". The analysis showed the presence of nitrogen containing compounds only in the basic and neutral fraction (Fig. 2).

The Kovats indices measured on Apiezon L and PEG 20 M columns revealed, that ephedrine was present in the basic fraction and pentamethylentetrazole in the neutral fraction. Confirmation was obtained by mass spectrometry.

**Editor's note. Used to describe doping agents that impair performance, i.e. "nobbling".*

Example 2.

Two nitrogen-positive compounds appeared in the basic fraction analysed on the Apiezon L column (Fig. 3). The major compound proved to be identical with amidopyrine in retention time, retention index and mass spectrum. As amidopyrine is often combined with phenylbutazone, we screened for phenylbutazone on an OV 101 column in the acidic fraction (Fig. 3). Phenylbutazone was identified by its retention index on OV 101 and OV 17 as well as by mass spectrometry.

Example 3.

An excretion study after an i.m. dose of 5 g caffeine is demonstrated in Fig. 4, where the extracts were injected at 4 minute intervals. For quantitative work, the addition of an appropriate internal standard is necessary.

Example 4.

A study of phenylbutazone (Fig. 5) demonstrated that this compound can be detected in blood even after 72 h with the screening procedure described. (The samples were kindly provided by Dr. Schotman, Utrecht, The Netherlands).

Example 5.

Isopyrine administered in the same experiment as described in example 4 can only be detected for 24 h (Fig. 6).

Example 6.

Phenolalkylamines, which may be regarded as amphoteric substances, can be detected at therapeutic levels with a very simple extraction procedure: To 1.0 ml plasma 9 ml methanol, containing 2 μMol HCl/ml, are added. After mixing and centrifuging, the supernatant fluid is evaporated to dryness, trimethylsilylated and trifluoroacetylated and injected into a gas-chromatograph-mass spectrometer. The GC-eluate is monitored either by single or multiple ion detection or in the cyclic scan mode and the spectra stored on a tape for later reconstruction of the traces of the ions of interest. In Fig. 7, a study of etilefrine ("Effortil^R") is shown (Donike, 1975a).

3. The Detection of "Non-volatile" Drugs, or Drugs of low Volatility

The problem of detecting drugs with poor gas-chromatographic properties and/or of an amphoteric nature, which are not to be found in the basic or acidic fractions is well known in dope analysis. Our approach is:

3.1 Isolation

The hydrochlorides of the drugs are isolated by either extraction with polar solvents such as ether/tert.-butanol, or ion exchange or methanolic HCl (compare Fig. 7).

3.2 Trimethylsilylation

The residues from 3.1 are treated with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (Donike, 1969). With excess reagent, hydroxyl groups react quickly to form O-trimethylsilyl-ethers, which may be analysed by gas chromatography (Donike *et al.*, 1970).

More common is the use of a large excess of MSTFA or another TMS-amide as silyl donor, which allows under drastic conditions (e.g. 1 h, 120°C) the trimethylsilylation of all N-H-functions of primary and secondary amines (Note: ω-NH_2 groups are converted to -$N(TMS)_2$) (Donike, 1970b).

With phenylethylamines and the phenylalkylamines the N,O-per-trimethylsilyl derivatives usually have good GC-properties (Fig. 8). The use of an N-FID is hindered (not excluded) by the excess TMS-reagents. The detection by GC-MS is facilitated, however, because the fragmentation pattern after electron impact of the N-TMS-amines gives rise to an ion of the amine fragment, which is found at m/e values 72 units higher than the free amine.

3.3 Selective O-Trimethylsilylation-N-Trifluoroacylation.

The observation that on some columns, N,O-per-TMS-derivatives showed poor GC-properties (Fig. 8) and that the stability of the calibration solution was poor (Donike, 1975a) prompted the search for a better derivatisation technique. It was found that the difficulties disappeared when the amine functions were acylated. N-Methyl-bis-trifluoroacetamide (MBTFA) derivatives (Donike, 1973) were synthesised, which allowed the N-trifluoroacylation of the trimethylsilylated amines (Fig. 8) (Donike & Stratmann, 1974).

This method offers four advantages over trimethylsilylation:

a) The reaction mixture may be injected directly.
b) The N-TFA-O-TMS derivatives are stable for weeks or months (moisture excluded).
c) The N-TFA-O-TMS derivatives have excellent GC-properties.
d) The derivatives of phenolalkylamines give rise to a characteristic fragmentation pattern.

The most interesting feature is that under electron impact the fragmentation pattern of these derivatives is completely altered compared to that of the amine or the N-TMS-amine. The main fragment, often the base peak, is the benzylic part of the molecule with m/e values of 91 for phenylethylamines, 179 for mono-hydroxy-phenolalkylamines and 267 for the catecholamines. A β-hydroxyl group adds 88 additional mass units to this value, for example m/e 179 for the ephedrines instead of m/e 91 (Donike, 1975a). After elimination of trifluoroacetamide (CF_3CO-NHR_3) as a neutral molecule an olefinic fragment appears in high yield which represents the carbon skeleton of the phenyl- or the phenol-alkylamine.

4. Derivatisation Techniques for Anabolic Steroids.

The anabolic steroids as well as their metabolites with hydroxyl functions are trimethylsilylated to improve their gas-chromatographic properties. The procedure consists of heating the steroid with MSTFA, which contains 2% (v:v) TMS-imidazole to 60°C for 10 to 20 min. Steroids with keto-groups in the 3- or 17-positions react with reagents in the above silylating mixture if an acidic catalyst is added. The following procedure is recommended:

The anabolic steroid (or the residue of an extraction) is heated for 15 min at 60°C with 100 μl of a mixture of MSTFA:TMSCI:TMS-imidazole (100:5:2; v:v:v). After tri-

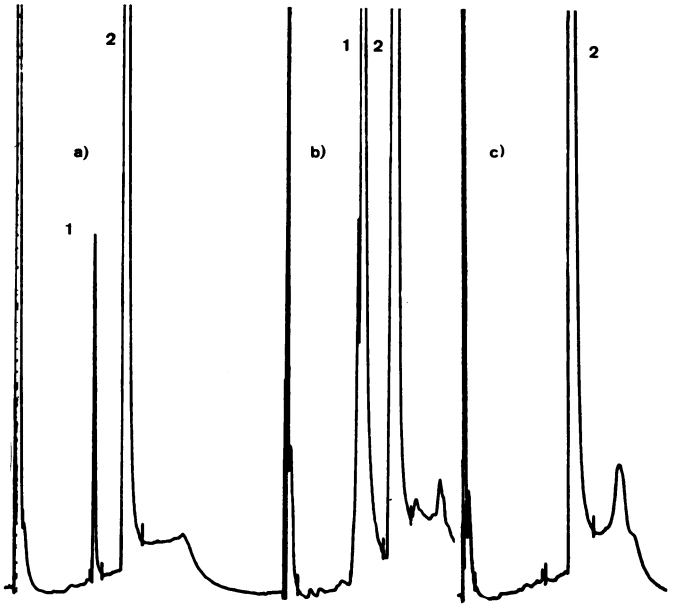


Fig. 2: Screening procedure for volatile doping agents in blood

a) Alkaline ether phase, without re-extraction into 0.6 N HCl
b) basic fraction
c) neutral fraction
1 = ephedrine
2 = pentamethylentetrazole

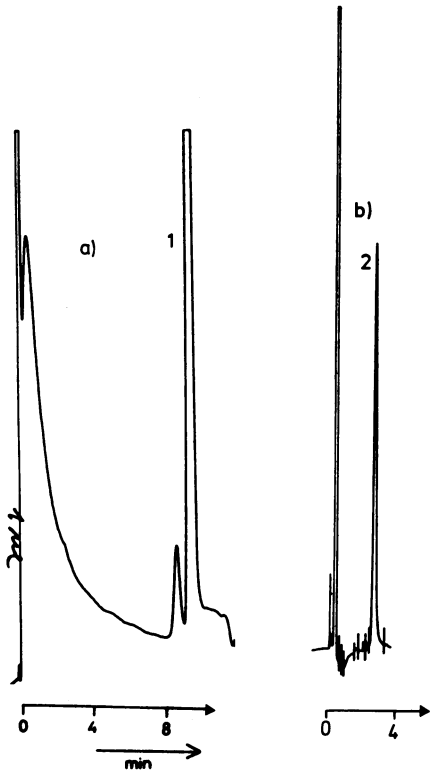


Fig. 4: Excretion study of caffeine, (5 g, i.v. 350 kg horse) Apiezon L, 240°C

The calculated values by computer are (hours = µg/ml): 0 = 0.2, 1 = 98, 2 = 15, 4 = 8, 6 = 6, 8 = 7, 10 = 2, 12 = 1.1, 24 = 1.0)

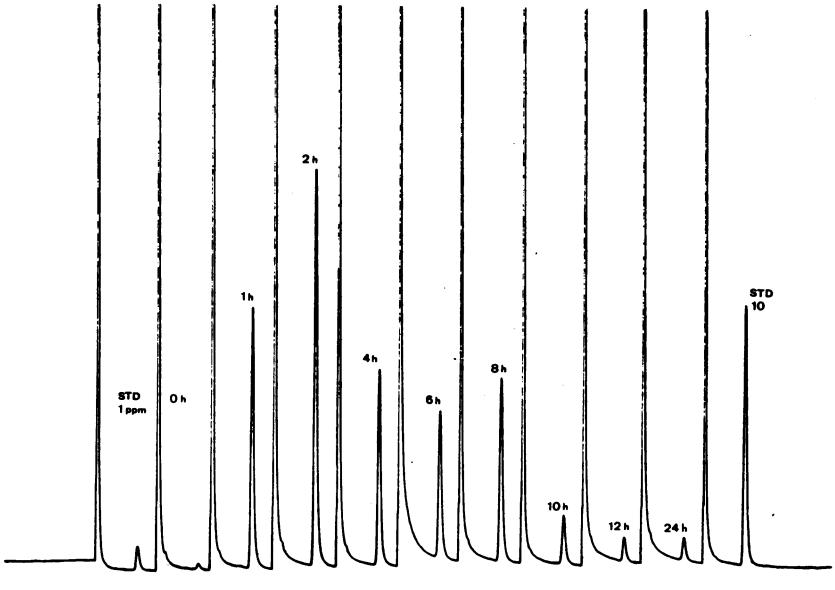


Fig. 3: Routine procedure: A positive dope case with

a) amidopyrin (1) in the basic fraction and
b) phenylbutazone (2) in the acidic fraction

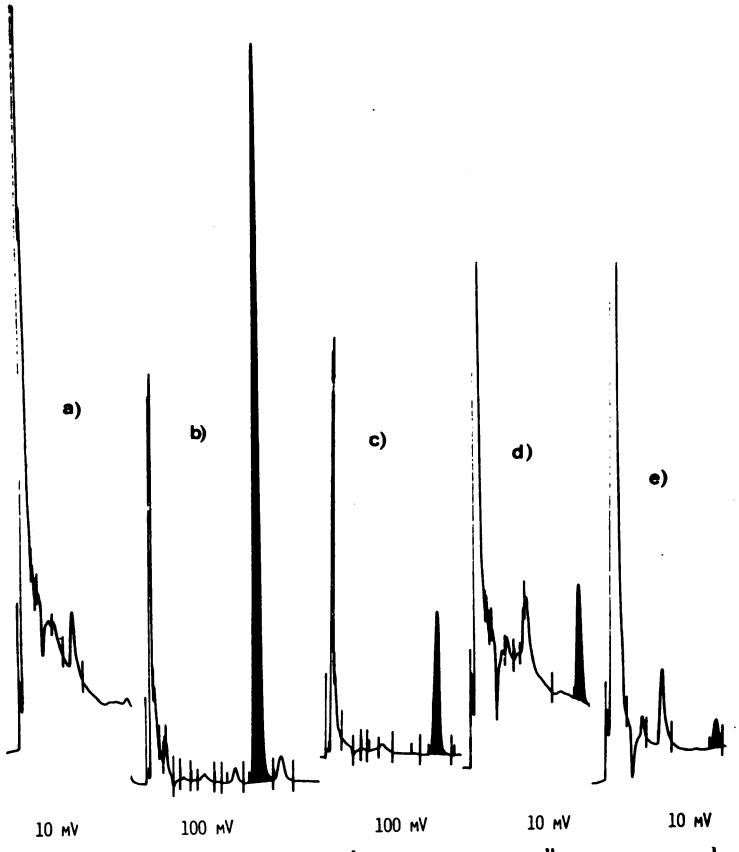


Fig. 5: Blood levels of phenylbutazone after 40 ml Tomanol^{R} to a horse. Acidic fraction 5% OV 101, 220°

a = 0 h = 0 µg/ml
b = 6 h = 29 µg/ml
c = 24 h = 5 µg/ml
d = 48 h = 0.4 µg/ml
e = 72 h = 0.1 µg/ml

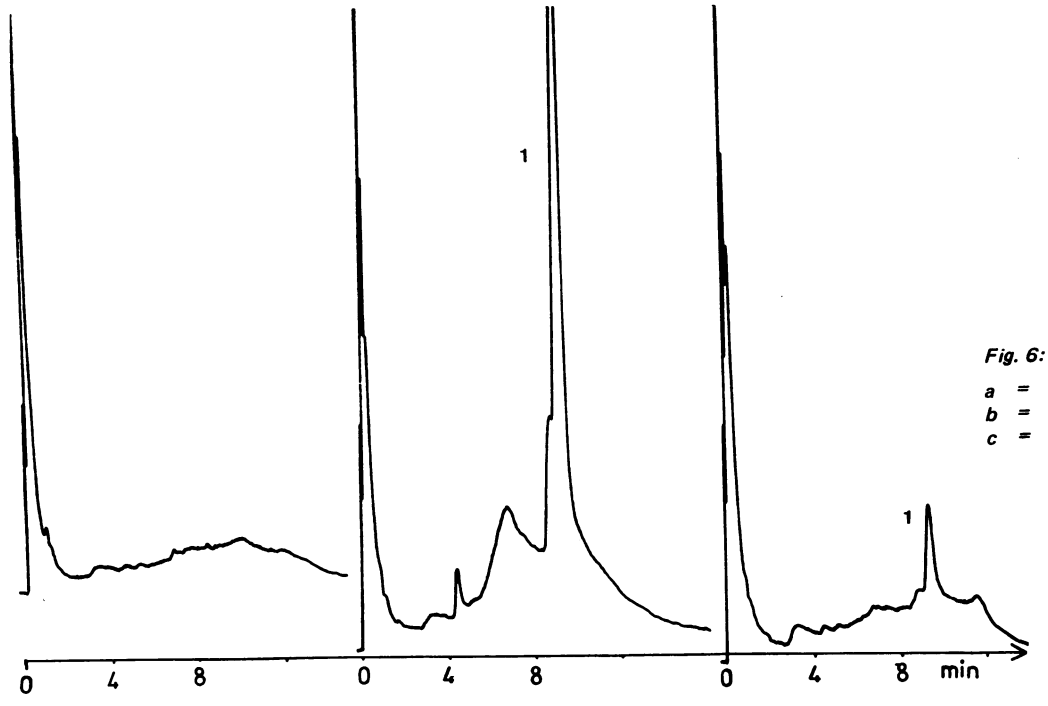


Fig. 6: Study with isopyrine (1)

a = 0 h
b = 6 h
c = 24 h

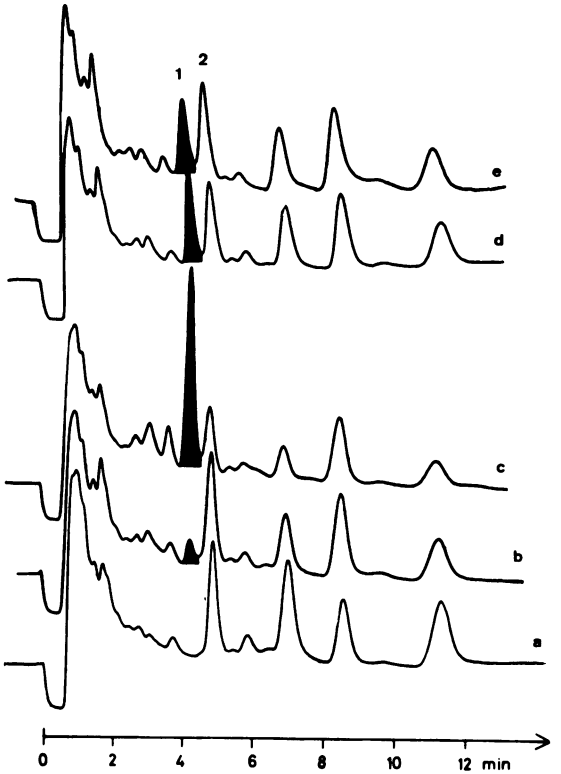


Fig. 7: Plasma concentrations of etilefrine (2-ethylamino-1-(3'-hydroxyphenyl)ethanol), 40 mg/75 kg

5% OV 17, mass specific detection (m/e = 267)

1 = etilefrine, 2 = p-hydroxy-norephedrine (internal standard).

a = 0 h = 0 ng/ml
b = 1 h = 37 ng/ml
c = 2 h = 597 ng/ml
d = 4 h = 225 ng/ml
e = 6 h = 142 ng/ml

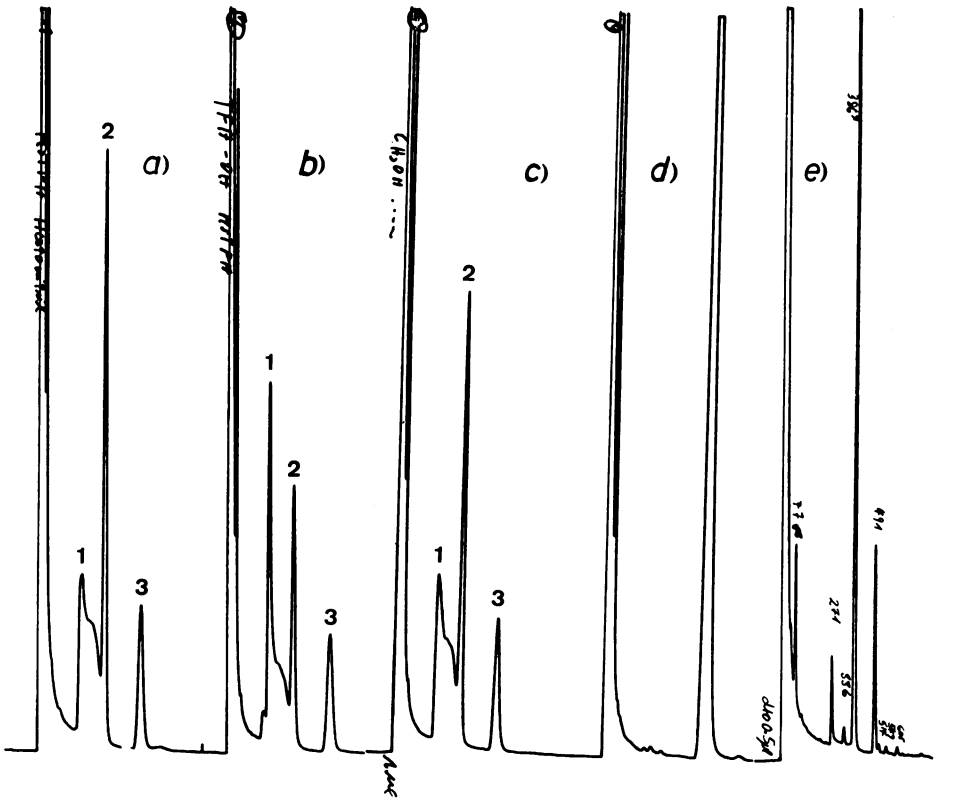


Fig. 8: The improvement of GC-properties by N-trifluoro-acetylation of N.O-TMS-pholedrine (para-hydroxy-methamphetamine)

a-c) Pholedrine silylated by different methods:

a) The sulphate was heated with CH_3CN-MSTFA (1:1, v:v) for 1 hour at 80°C

b) The sulphate was dissolved in 0.1 ml CF_3CO_2H in 0.9 ml.

c) The free base of pholedrine was treated as in b)

d) An aliquot of 0.1 ml MBTFA was added and heated 10 min to 80°C

e) The reaction mixture b) injected onto a column with better performance.

Columns a-d) 5% OV 17 on Chrom G AW-DMCS,
Column e) 2% OV 101 on Chromosorb W AW-DMCS (laboratory quality).

methylsilylation, 25 µl of N,N-dimethylhydrazine are added and heating is continued for 30 min. This reaction mixture may be used directly for GC or MS studies.

For biological samples, especially when the advantageous detection by the N-FID is intended, a partition step is necessary to remove the nitrogen containing silylating reagents. Iso-octane (3 ml) and water (1 ml) are added and the sample is shaken and centrifuged. The organic phase is dried in vacuo and the residue dissolved in n-hexane for GC with the N-FID.

For GC-MS-analysis, the residue is taken up in a mixture of n-hexane, MSTFA, TMS-imidazole (80:19:1, v:v:v) to protect the O-TMS-functions for longer periods of time. The fragmentation pattern under electron impact of the O-TMS-dimethylhydrazones of the anabolic steroids is the most favourable of all derivatives we have hitherto investigated (Donike & Stratmann, 1976).

REFERENCES

Donike, M. 1975a. 'N-Trifluoracetyl-O-Trimethylsilyl-Phenolalkylamine Darstellung und massenspezifischer gaschromatographischer Nachweis" *J.Chromatogr.* **103,** 91.

Donike, M. 1969. "N-Methyl-N-trimethylsilyl-trifluoracetamid, ein neues Silylierungsmittel aus der Reihe der silylierten Amide" *J.Chromatogr.* **42,** 103.

Donike, M. 1973. "Acylierung mit Bis (Acylamiden) N-Methyl-bis(trifluoracetamid) und Bis(trifluoracetamid), zwei neue Reagenzien zur Trifluoracetylierung" *J.Chromatogr.* **78,** 273.

Donike, M. 1975b. "Zum Problem des Nachweises der anabolen Steroide: Gas-chromatographische und massenspezifische Möglichkeiten" *Sportarzt und Sportmedizin,* **26,** 1.

Donike, M. & Stratmann, D. 1976. "Gas-chromatographische Identifizierung von Stimulantien der Phenyläthylaminreihe mit Hilfe der Retentions-Indices" *Z.Anal.Chem.* **279,** 2, 129.

DISCUSSION

CARTONI: By using ether as the extraction solvent for horse urine you cannot be sure you can extract all the compounds, as if you were using chloroform.

DONIKE: We have not yet tried extractions in all cases but if you do not have amphoteric compounds like opium or ritalinic acids, I believe that nearly all the compounds you wish to detect are extracted in sufficient quantity in the ether because the partition coefficient of most of these compounds is very high because there is present a lot of sodium sulphate. Ephedrine for example, if you do not add sodium sulphate you do not get a good yield.

CARTONI: Morphine is a difficult drug.

DONIKE: First we do an acidic hydrolysis then we extract, not with chloroform, but with a mixture of ether and t-butanol which is sufficiently polar to extract sufficient morphine for GC screening and other specific tests, but this is a difficult group of substances because also it contains substances such as pemoline.