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Toxicological analysis of whole blood samples by means of Bond-Elut Certify columns and gas chromatography with nitrogen–phosphorus detection

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Abstract

The application of Bond-Elut Certify solid-phase extraction columns to the systematic toxicological analysis of whole blood was evaluated. The reproducibility of the extraction was tested with thirteen drugs varying in physico-chemical properties. Analysis was performed with capillary gas chromatography with nitrogen-selective detection. The recoveries were reproducible, as long as other limiting factors, *e.g.*, chromatographic behaviour or volatility, do not play a significant role. The effect of limiting chromatographic behaviour was studied in more detail with the more sensitive mass spectrometry with selected-ion monitoring after converting the extracted morphine into its ditrimethylsilyl derivative.

1. Introduction

Solid-phase extraction (SPE) has mostly been applied to the highly selective extraction of individual analytes or groups or closely related analytes. Because the number of relevant parameters governing the extraction is larger than with liquid–liquid extraction, higher selectivity can be obtained. In the analytical screening for analytes that differ widely in physico-chemical properties such as acidic–basic nature, pK_a and polarity, SPE has not been considered the first choice.

The development of new types of stationary phases, *e.g.*, Bond-Elut Certify, has opened up new possibilities in this field. These columns possess both cation-exchange and hydrophobic properties [1,2].

After the reported good results with matrices such as plasma and urine [1,3] we considered the application to whole blood. Haemolysed whole blood is a matrix encountered in most cases in forensic toxicology. The preparation of serum or plasma from this blood is hardly ever possible. Pretreatment of whole blood before SPE is carried out either by precipitation of the proteins with a reagent such as zinc sulphate solution or trichloroacetic acid [4] or by dilution of the blood with an appropriate buffer solution. [1–3,5]. The dilution procedure has proved to yield better results and was employed in this study.

For this study, the dilution method was used followed by gas chromatography with nitrogen–phosphorus detection (NPD). Capillary GC–

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NPD combines high separation power, flexibility and selectivity. The last factor is based on the fact that most drugs encountered in forensic case work contain nitrogen. Gas chromatography–mass spectrometry is used for problems that cannot be solved using GC–NPD.

Whole blood was spiked with a mixture of drugs with various physico-chemical properties and GC behaviour. The individual substances chosen are listed in Table 1. Extraction recoveries were measured with the use of procaine as a chromatographic standard. For drugs with unfavourable chromatographic behaviour, morphine was selected as a model substance and recoveries were determined after silylation with N,O-bistrimethylsilyltrifluoroacetamide (BSTFA). Detection in this instance was performed using mass spectrometry in the selected-ion monitoring mode (MS-SIM).

2. Experimental

2.1. Materials

The extraction columns used were Bond-Elut Certify (Analytichem International/Varian, Harbor City, CA, USA), 3 ml, containing 130 mg of stationary phase. The packing material exhibits both hydrophobic and ion-exchange properties. The structural basis is silica.

The blood employed for experiments was outdated citrated whole blood from a blood bank and was stored at −18°C until used.

Methanol (HPLC grade) and chloroform and dichloromethane (glass-distilled grade) were obtained from Rathburn (Walkerburn, UK), acetone (Nanograde) from Prochem (Wesel, Germany), ethyl acetate, 2-propanol, 25% ammonia solution, potassium phosphate and potassium hydroxide (analytical-reagent grade) from Merck (Darmstadt, Germany) and BSTFA from Chrompak (Middelburg, Netherlands). Water was purified with a Milli-Q/Organex-Q system (Millipore, Milford, MA, USA). Amphetamine sulphate, codeine hydrochloride (dihydrate), morphine hydrochloride (trihydrate) and cocaine hydrochloride were purchased from Brocacef (Maarsen, Netherlands), phenobarbitone from Interpharm ('s-Hertogenbosch, Netherlands) and diphenhydramine hydrochloride, methaqualone and methadone hydrochloride from OPG (Utrecht, Netherlands). 6-Monoacetylmorphine hydrochloride was synthesized in our laboratory. Nalorphine hydrobromide and haloperidol were obtained from Bufa (Uitgeest, Netherlands) and strychnine from Merck. Oxazepam was kindly donated by Hoffman-La Roche (Mijdrecht, Netherlands).

2.2. Instruments and chromatographic conditions

GC–NPD was carried out using an HP 7673 autosampler and an HP 5890-II gas chromatograph equipped with a nitrogen–phosphorus detector and linked to an HP 5970C Chemstation (all from Hewlett-Packard, Waldbron, Germany).

Aliquots of 1 μl were injected in the split mode (1:9) on to an Ultra-1 column (12 m × 0.2 mm I.D.; 0.33 μm film thickness) (Hewlett-Packard). Helium was used as the carrier gas (2 ml/min). The detector and injector temperatures were 280°C. The oven temperature was programmed from 100 to 280°C at 5°C/min and kept at 280°C for 10 min. The bead current was adjusted to maintain a collector current of 20 pA at a 100°C oven temperature.

GC–MS was performed on a Model 5971A mass-selective detector (Hewlett-Packard). The autosampler, gas chromatograph, column and data system were as mentioned above. The temperature programme was as mentioned above, except that the programming rate was 10°C min. The injection port, transfer line and ion source temperatures were maintained at 280°C. Aliquots of 1 μl were injected in the splitless mode (purge time 0.5 min). The mass spectrometer was operated in the SIM at m/z 429.5 and 236.35 at the retention time of morphine and m/z 235.2 and 250.2 at the retention time of methaqualone.

2.3. Sample preparation and treatment

Spiking was done, on the day of analysis, in polypropylene tubes. The solutions of the drugs

in methanol were pipetted into the tube. Care was taken that the resulting spiked blood never contained more than 2% (v/v) of methanol. If necessary, the methanolic solution was evaporated to a sufficiently small volume. Before adding the blood to the contents of the tube, the methanolic solution was diluted with the same volume of water. In this way coagulation of the proteins by the methanol was prevented.

Samples were processed on a Vac Elut SPS 24 vacuum manifold (Analytichem/Varian). The vacuum pressure was adjusted to at least 15 mmHg and the flow through the columns was regulated with PTFE valves.

The columns were preconditioned with 2 ml of methanol followed by 2 ml of 0.1 *M* phosphate buffer (pH 6.0).

The vacuum was adjusted in such a way that a flow of about 2.0 ml/min was obtained through the columns. The columns were not allowed to dry before the sample was applied.

A volume of 1.00 ml of blood was mixed with 6.0 ml of 0.1 *M* phosphate buffer (pH 6.0) and the mixture was sonicated for 15 min and centrifuged for 15 min at 2000–3000 rpm (500–750 *g*). The supernatant was passed through the pretreated column at a flow-rate of about 1.5 ml/min. Care was taken that no precipitate was transferred. The columns were rinsed with 1.0 ml of water and 0.5 ml of 0.01 *M* acetic acid (pH 3.3). The columns were dried by suction of air at the maximum vacuum (>15 mmHg; 1 mmHg = 133.322 Pa) for 5 min. A 50-μl volume of methanol was added to the column and air (>15 mmHg for at least 1 min) was passed through in order to remove the last remaining liquid. Before elution the columns were placed on a clean outlet valve.

Acid–neutral fraction

The acid–neutral drugs were eluted with two portions of 2.0 ml of acetone–chloroform (1:1) in about 5 min.

Basic fraction

The basic drugs were eluted with 3 ml of ethyl acetate containing 2% (v/v) of 25% ammonia solution (freshly prepared before use). This solution passed through the column by gravity only (in about 4 min).

The two fractions were collected separately in silanized conical glass tubes and evaporated to dryness at 40°C under a stream of nitrogen. The residues were dissolved in 50 μl of a solution of procaine in methanol (5 μg/ml). The extracts were analysed by GC–NPD (standard procedure).

For the study of the recovery of morphine, the basic fraction was submitted to derivatization (see *Recovery of morphine*).

2.4. Determination of recoveries

Standard procedure

Ratios of the peak heights of the respective substances to the peak height of procaine were used for calculations. These ratios were compared with those obtained from injections of a mixture of the pure drugs and procaine (chromatographic standard) in methanol at known concentrations. The concentrations of the drugs in this solution were chosen in such a way that they equalled the concentrations in the reconstituted extracts when the recoveries would be 100%. This procedure was repeated five times for each concentration level (500, 250, 100 and 50 ng/ml) of the drugs in whole blood.

Recovery of morphine

In order to be able to discriminate between poor extraction recoveries and poor results originating from poor GC behaviour, a derivatization experiment was carried out. Morphine was chosen as an example of a substance with difficult GC behaviour.

Morphine was added to blood at concentrations of 500, 50, 10.0 and 5.0 ng/ml and the blood was extracted according to the standard procedure described above, with the following modifications. The basic fraction was evaporated to dryness after the addition of 250 ng (highest levels) or 10 ng (lower levels) of methaqualone dissolved in methanol as a chromatographic standard. To the residue 40 μl of acetone were added and the tube was vortex mixed for 15 s. Then 10 μl of BSTFA were added and after mixing the solution was transferred into a 100-μl

insert of an autosampler vial. The vial was sealed with a PTFE-lined septum and heated at 70°C for 30 mins, then 1.0 μl was injected splitless into the GC–MS system.

Recoveries were calculated by means of the peak areas with the ions of *m/z* 236.35 for morphine-di-TMS and *m/z* 235.2 for methaqualone, the so-called target ions. The qualifier ions of *m/z* 429.5 (for morphine-di-TMS) and 250.2 (for methaqualone) were used to confirm the identities of the substances. Only the chromatograms of extracts that showed satisfactory ion chromatograms of the qualifier ions were used for calculating the recovery of morphine. The criterion used was that the ratio of the peak areas of the target ion and the qualifier ion at the retention time of morphine-di-TMS should differ by not more than three times the standard deviation of the mean ratio obtained with the extracts of the spiked samples. An analogous criterion was used for the target/qualifier ratio of the internal standard.

The optimum conditions for the silylation were established as follows: a methanolic solution containing 0.5 μg of morphine and imipramine was evaporated to dryness in a silanized conical test tube. A 40-μl volume of acetone was added, followed by sonication for 5 s and then 10 μl of BSTFA were added. After vortex mixing, the mixture was transferred into an autosampler vial. The sealed vials were kept at temperatures of 40, 60, 80 and 100°C. The oven temperature was set isothermal at a temperature that separated morphine, morphine-di-TMS and the chromatographic standard (imipramine) within 10 min. The reaction can be monitored by injecting 1-μl aliquots into the GC–NPD system at 10-min intervals and calculating the ratio of the heights of the peaks of morphine-di-TMS and of the non-reacting imipramine.

3. Results and discussion

3.1. Standard procedure

The chromatograms of blank blood samples are very clean. The extracts of blank blood

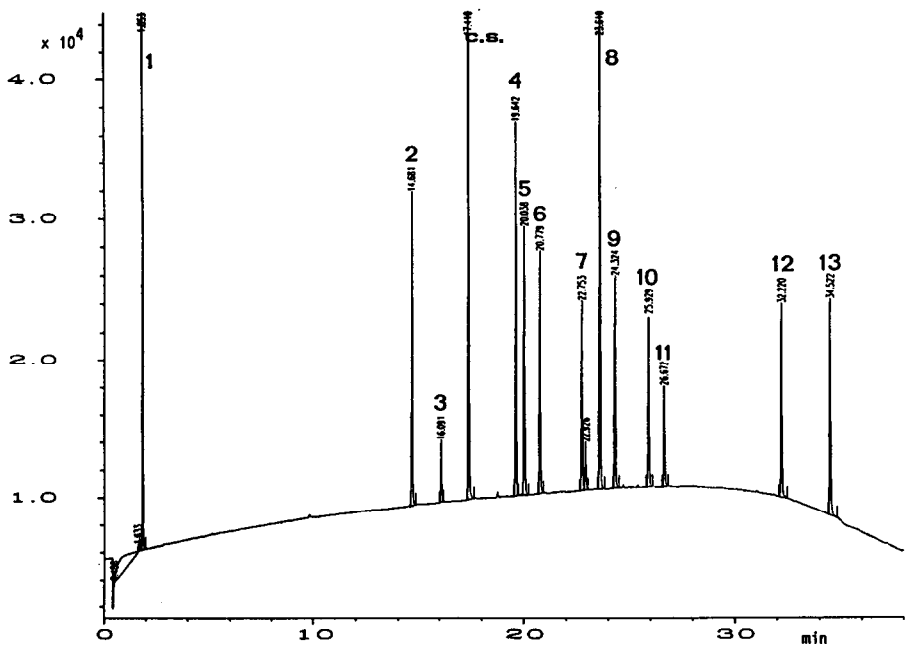

Fig. 1. GC–NPD of a solution in methanol containing the tested drugs and procaine. Volume injected, 1 μl containing 25 ng of each component. Peak numbers refer to the compounds in Table 1. C.S. = Chromatographic standard = procaine.

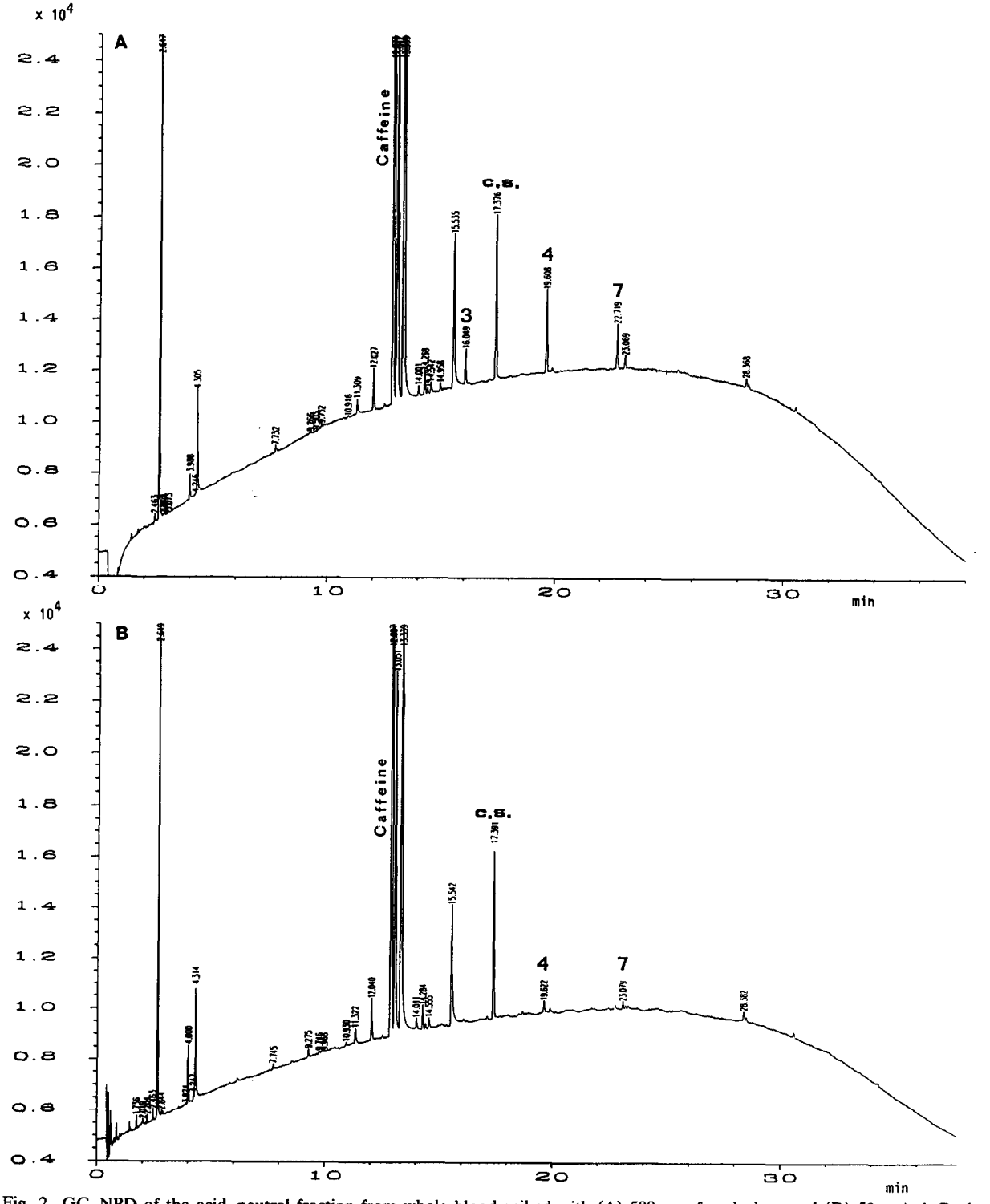

Fig. 2. GC–NPD of the acid–neutral fraction from whole blood spiked with (A) 500 ng of each drug and (B) 50 ng/ml. Peak numbers refer to the compounds in Table 1. C.S. = Chromatographic standard = procaine.

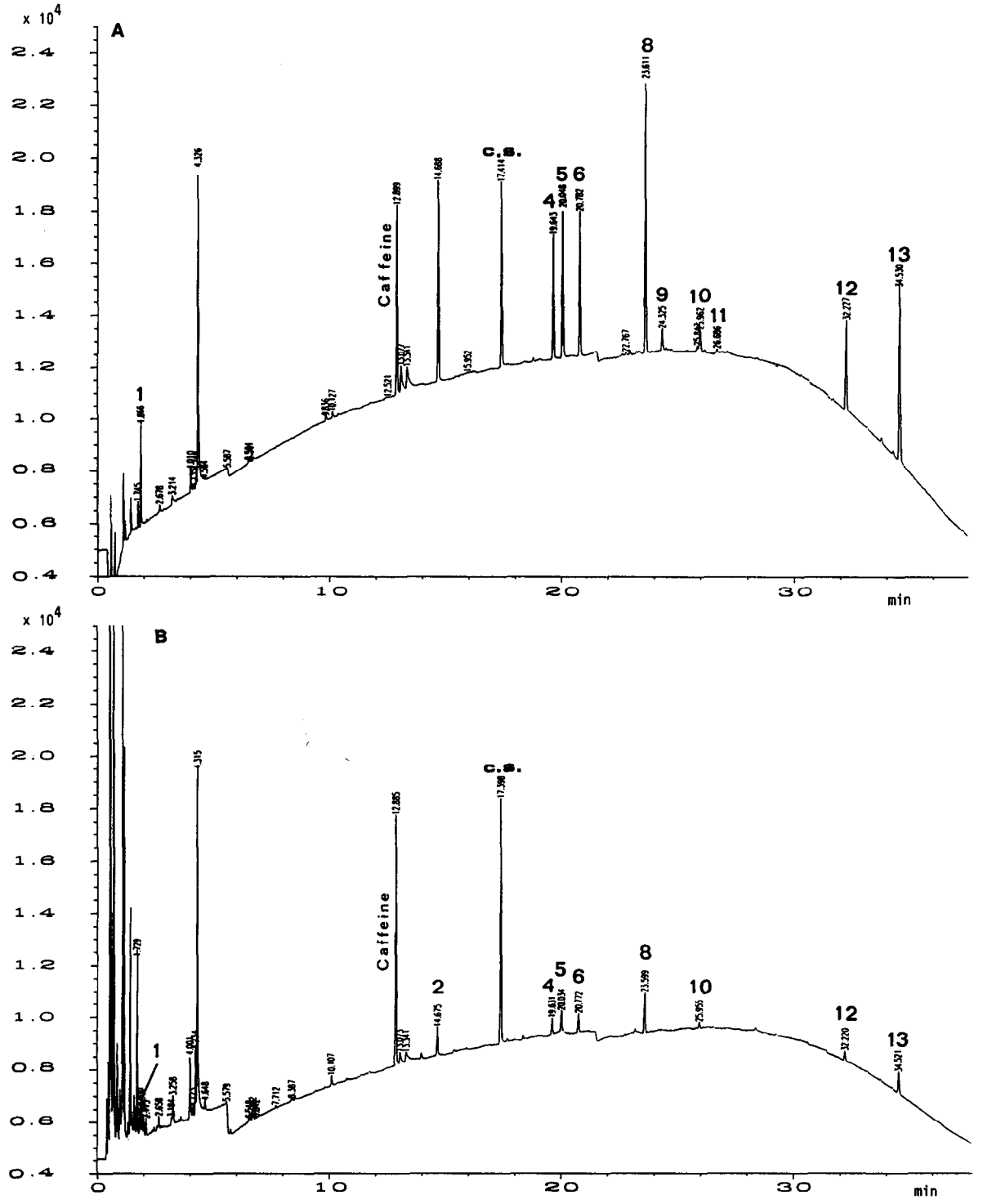

Fig. 3. GC–NPD of the basic fraction from whole blood spiked with (A) 500 ng of each drug and (B) 50 ng/ml. Peak numbers refer to the compounds in Table 1. C.S. = Chromatographic standard = procaine.

Table 1
Extraction recoveries (means of five determinations) of the drugs extracted from spiked whole blood

No.	Compound	500 ng/ml				250 ng/ml				100 ng/ml				50 ng/ml			
		A/N (%)	B (%)	Total (%)	R.S.D. (%)	A/N (%)	B (%)	Total (%)	R.S.D. (%)	A/N (%)	B (%)	Total (%)	R.S.D. (%)	A/N (%)	B (%)	Total (%)	R.S.D. (%)
1	Amphetamine	ND	47.9	47.9	10.8	ND	68.3	68.3	7.8	ND	72.1	72.1	10.2	ND	50.4	50.4	13.3
2	Difenhydramine	ND	80.8	80.8	2.3	ND	94.4	94.4	3.5	ND	88.4	88.4	1.7	ND	93.6	93.6	6.1
3	Phenobarbitone	138.2	ND	138.2	10.3	117.0	ND	117.0	15.8	–[a]	ND	–	–	–[a]	ND	–	–
4	Methaqualone	42.8	49.8	92.6	3.9	49.7	50.5	100.2	8.0	48.5	52.5	101.0	3.1	40.5	54.8	95.3	7.7
5	Methadone	ND	85.7	85.7	3.9	ND	95.8	95.8	3.0	ND	85.6	85.6	2.7	ND	89.5	89.5	6.6
6	Cocaine	ND	88.8	88.8	3.5	ND	95.1	95.1	3.1	ND	84.8	84.8	3.0	ND	88.5	88.5	5.9
7	Oxazepam	63.0	16.4	79.4	12.0	55.5	29.3	84.8	7.6	47.5	45.9	93.4	5.0	41.0	–[a]	41.0	5.5
8	Codeine	ND	83.9	83.9	4.6	ND	81.6	81.6	3.2	ND	86.4	86.4	4.4	ND	93.0	93.0	11.8
9	Morphine	ND	171.3	171.3	66.0	ND	56.3	56.3	26.8	ND	–[a]	–	–	ND	–[a]	–	–
10	6-Acetylmorphine	ND	57.6	57.6	33.3	ND	26.4	26.4	3.4	ND	58.3	58.3	16.6	ND	90.7	90.7	20.7
11	Nalorphine	ND	135.2	135.2	120.2	ND	–[a]	–	–	ND	–[a]	–	–	ND	–[a]	–	–
12	Haloperidol	ND	80.1	80.1	8.7	ND	98.3	98.3	4.3	ND	94.0	94.0	5.5	ND	112.4	112.4	8.1
13	Strychnine	ND	87.2	87.2	6.3	ND	114.6	114.6	3.0	ND	107.0	107.0	7.8	ND	103.3	103.3	11.1

A/N = Acid–neutral fraction; B = basic fraction; ND = not detected.
[a] Not detected owing to limiting chromatographic behaviour.

contain only the peaks not labelled with the numbers referring to the spiked substances in Figs. 2 and 3, except for caffeine, which is present in almost all blood samples. A chromatogram of a mixture of the pure drugs is shown in Fig. 1.

The extraction recoveries at the 500, 250, 100 and 50 ng/ml levels are given in Table 1. They are high, reproducible and independent of the concentration provided that the chromatographic behaviour (morphine), volatility (amphetamine) and stability (6-monoacetylmorphine, oxazepam) are not limiting in the determinations. Drugs for which the chromatographic behaviour is not the limiting factor in detection can be identified on the chromatogram when present in the blood at concentrations down to at least 50 ng/ml (Fig. 2) using GC–NPD.

Substances that exhibit problematic behaviour during GC (*e.g.*, morphine) cannot always be detected at toxicologically relevant concentrations using the standard procedure, *i.e.*, without derivatization.

The procedure is at least suitable for reliable qualitative analysis, as can be concluded from Table 1.

3.2. Recovery of morphine

The study of the extraction of morphine with derivatization clearly shows that the analytical technique rather than the SPE limits the detectability in the concentration range studied. The results are presented in Table 2.

Blank blood does not show significant interfering peaks down to at least 5 ng of morphine per millilitre of blood. Dissolution in acetone of the dry residue before adding BSTFA is necessary in order to obtain a sufficient reaction rate. Also, the BSTFA burden on the NPD bead is limited by the diluting effect of acetone. The conversion of morphine into the di-TMS derivative is complete after 30 min at 70°C. Benzoylecgonine is not detected with the GC–MS-SIM procedure.

Table 2
Recoveries (means of five determinations) of morphine by GC–MS after silylation

Concentration of morphine added (ng/ml)	Recovery (%)	R.S.D. (%)
500	95.4	6.6
50	74.8	10.8
10	86.2	12.0
5	92.4	11.5

Probably a more polar solvent is needed for elution. This is in accordance with the results of Wernly and Thormann [6], who prepared three fractions, including a further more polar basic fraction with a more polar eluent in which benzoylecgonine was detected. Addition of an extra fraction and/or replacement of the eluent with a more polar eluent for the basic fraction is currently being studied.

Case example

Fig. 4 shows the chromatogram of the basic fraction of a blood sample obtained at a forensic autopsy (suicide). After screening with the method described here, strychnine (at the lethal concentration of 6.0 mg/l), amitriptyline and nortriptyline (at a therapeutic concentration, 0.1 mg/l) were detected, quantified and confirmed using HPLC–scanning UV detection, as described previously [7].

4. Conclusions

Clean extracts are obtained by the procedure described without the need for back-extractions from the organic phase into acid or base and re-extraction into an organic phase.

High and reproducible recoveries, independent of the concentration in the range under study, make this method very suitable for qualitative and/or semi-quantitative applications.

The extracts do not contain much material that contaminates the injection port of the gas chromatograph, thus making this method very suitable for routine work.

Our experience with benzoylecgonine indicates that not all substances of potential interest are recovered. Further validation and fine-tuning of the procedure will be undertaken.

This relative simple solid-phase extraction method shows the potential and reliability of the

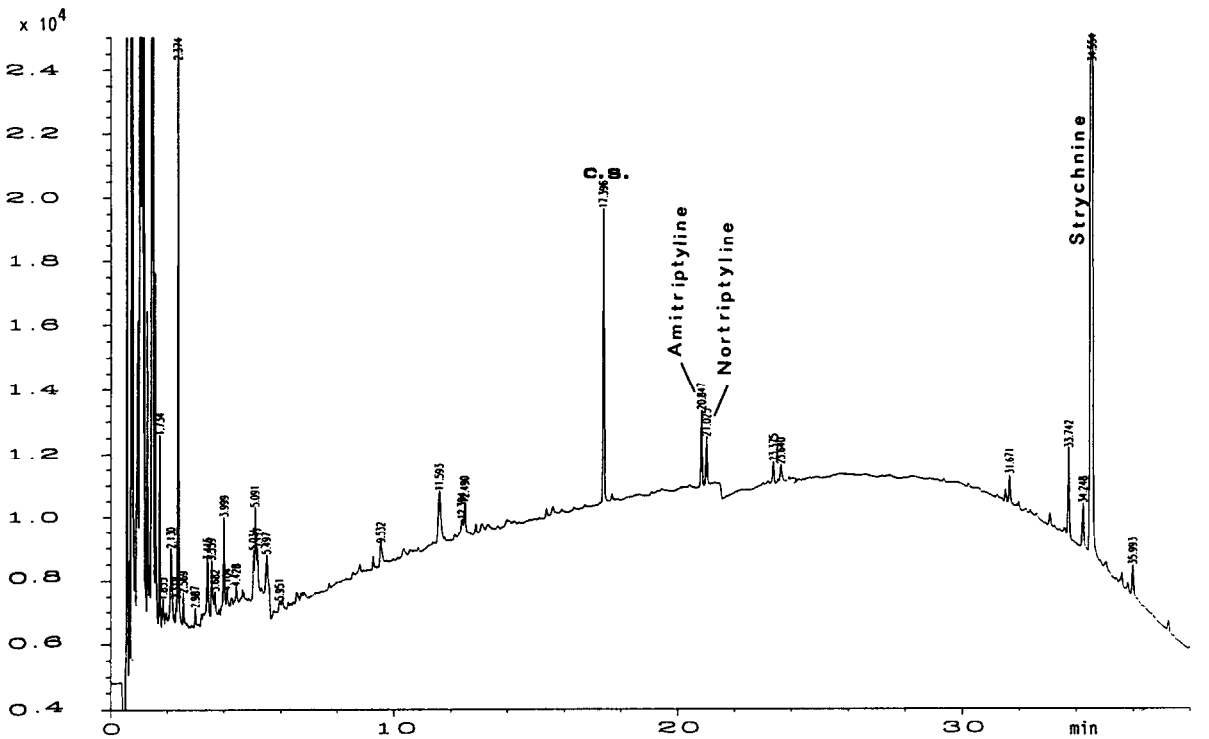

Fig. 4. GC–NPD of an extract of blood obtained at a forensic autopsy (suicide). Detected are strychnine (6 mg/l), amitriptyline and nortriptyline (each *ca.* 0.1 mg/l).

most elaborate comprehensive extraction procedures available today.

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