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Simultaneous analysis of homovanillic acid, 5-hydroxyindoleacetic acid, 3-methoxy-4-hydroxyphenylethylene glycol and vanilmandelic acid in plasma from alcoholics by high-performance liquid chromatography with electrochemical detection

Critical comparison of solid-phase and liquid–liquid extraction methods

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ABSTRACT

A method is described for the simultaneous determination of vanilmandelic acid, 3-methoxy-4-hydroxyphenylethylene glycol, 5-hydroxyindoleacetic acid, and homovanillic acid in a human plasma sample using reversed-phase high-performance liquid chromatography with column switching and amperometric detection. Two methods of sample preparation were tested. Liquid–liquid extraction yields better recoveries, is more selective and precise than solid-phase extraction and allows a shorter time of chromatographic analysis. Estimated plasma values of the metabolites from healthy controls are in good agreement with previously reported levels. Studies of alcoholics at the beginning of the delirium tremens provided different plasma levels of the metabolites, dependent on the different duration – and hence the severity – of the delirium.

INTRODUCTION

Dysfunctions of catecholaminergic and serotonergic neurotransmitter systems are implicated in many psychiatric disorders [1–6], especially in alcohol withdrawal syndrome with delirium [7–12]. Despite severe limitations, in clinical research the examination of monoamine metabolites in plasma and their relation to the clinical state is the most direct method currently available in living humans for providing information about central neuronal activity [13–15]. In recent years, only a few methods using high-performance liquid chromatography (HPLC) have been described for the analysis of acidic and neutral monoamine metabolites [16–20]. However, for the simultaneous determination of vanilmandelic acid (VMA), 3-methoxy-4-hy-

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droxyphenylethylene glycol (MHPG), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in plasma, problems of isolation and separation have still not been completely overcome. Generally, liquid–liquid extraction (LLE) has been the most widely used enrichment procedure. Recently, solid-phase extraction (SPE) has become an established method, because of its high selectivity, reproducibility, recovery and speed. Also for the determination of acidic or neutral monoamine metabolites good results were obtained with SPE [21–25]. To our knowledge, no separation procedure seems to exist for the simultaneous SPE of VMA, HVA, 5-HIAA and MHPG from human plasma prior to HPLC analysis.

We report here the results of a comparison of two extraction and preconcentration procedures (SPE and LLE) of acidic and alcoholic monoamine metabolites from a single human plasma sample, followed by simultaneous determination using reversed-phase HPLC with column switching and electrochemical detection (HPLC–ED). We used the method to determine the four monoamine metabolites in plasma samples from alcoholics at the beginning of the delirium tremens.

EXPERIMENTAL

Reagents

VMA and 5-HIAA were purchased from SERVA (Heidelberg, Germany), and MHPG and HVA from Aldrich Chemie (Steinheim, Germany). Earl's balanced salt solution was obtained from Sigma Chemicals (Deisenhofen, Germany). Water was purified with the HP 661-purifier from Hewlett-Packard (Waldbronn, Germany). All other chemicals we used were analytical or HPLC-grade from E. Merck, J.T. Baker (Gross-Gerau, Germany) or Aldrich Chemie.

Standards

Stock solutions of the four compounds (VMA, MHPG, HVA and 5-HIAA, each 1 mg/ml) were prepared monthly in a buffer (0.2 *M* KH_2PO_4, 0.26 m*M* EGTA, 5 m*M* red glutathione, pH 2.20) and stored at −80°C. The working standards were prepared daily from the stock solutions in eluent A or eluent X, respectively, to give a concentration of 100 ng/ml. Eluent A and X are different phosphate buffer-solutions (for preparation see *Chromatographic conditions*).

Sample collection and storage

A flexible cannula was placed into a peripheral vein, and 15–30 min later *ca.* 10 ml of blood were collected in a prechilled tube containing 200 μl of 0.2 *M* EDTA and 100 μl of 0.5 *M* $Na_2S_2O_5$. The tubes were closed, carefully and slowly tipped over and placed in ice-water for 10 min. Plasma was separated by centrifugation (20 min, 1500 *g*, 4°C), and transferred to a polyethylene tube containing 10 μl of 0.2 *M* EDTA and 10 μl of 0.5 *M* $Na_2S_2O_5$ per millilitre of plasma, and stored at −80°C.

Sample extraction

All preparation steps were performed only with silanized glass or polyethylene–polypropylene materials. If the extracts were injected directly they were stored for a short time at −80°C. Frozen human plasma samples were thawed at room temperature, supplemented with 10 μl of 0.5 *M* $Na_2S_2O_5$/ml plasma, and centrifuged (15 min, 1500 *g*, 4°C).

Solid-phase extraction

To 5 ml of plasma 1.5 ml of 1 *M* HCl were added. The solution was filtered through a 2-μm filter paper and divided into four 1.3-ml aliquots. Two of the aliquots were supplemented with 100 μl of eluent A, and the other two with 100 μl of the eluent A containing 10 ng of the standards. Then the solutions were sonicated for 10 min.

For plasma extraction, polypropylene columns (2-ml volume) containing 100 mg of Encapharm RP 18 (100 μm particle size) between two PE-frits (20 μm pore size) were used. The sorbent was conditioned by passing 5 ml of methanol and then 2 ml of 1 m*M* HCl through the columns. The prepared plasma solutions were then loaded on the columns. After passage, the columns were washed with 0.5 ml of purified water (0°C). The compounds were desorbed with 1.5 ml of methanol–water (80:20, v/v).

For complete passage of the solutions through

the columns in each step, we used pressure of highly purified nitrogen for 3–4 min with the aid of a suitable device ("Visiprep" with "Visidry", Supelco, Bad Homburg, Germany).

The eluates were evaporated at 37°C under nitrogen, redissolved in 200 μl of eluent A and, after vigorous vortex-mixing, filtered through a 0.45-μm Nylon-66 filter. At this point, the samples were ready for HPLC analysis. The injected volume was 150 μl.

Liquid–liquid extraction

A 2.2-ml volume of plasma was supplemented with 2.2 ml of Earl's balanced salt solution, briefly vortex-mixed, and divided into four 1-ml samples. Two of the aliquots were supplemented with 100 μl of eluent X, the other two samples with 100 μl of eluent X containing 10 ng of each standard. The further extraction procedure followed the method described by Gerhardt *et al.* [20] with some modifications: only 3 ml of ethylacetate were used for one extraction, and exactly 1 g of dry NaCl was added to one extraction vial to precipitate the proteins. After evaporation to dryness at 37°C under nitrogen, the residues were redissolved in 200 μl of eluent X, and filtered through 0.2-μm Millipore one-way filters (material, reg. cellulose; diameter, 13 mm; Schleicher & Schuell, Dassel, Germany) directly into the injection vials. The injection volume was 100 μl.

Chromatographic equipment

The HPLC system consisted of the following modules: a 1050 quaternary pump, a 1050 autosampler (with external cooling to 4°C) and a ChemStation Phönix (all from Hewlett-Packard), an isocratic pump 64, a six-port three-way switching valve with a pneumatic drive and a high pressure filter (both from Knauer, Berlin, Germany), an electrochemical detector Model 400 (dual channel, EG&G, München, Germany), and a column thermostat (W.O. Industrial Electronics, Vienna, Austria, temperature set to 35°C). All LiChrosorb/LiChrospher RP18 column materials were from E. Merck (Darmstadt, Germany), and the Encapharm RP18 material was obtained from Molnar Institut (Berlin, Germany). For details see Fig. 1.

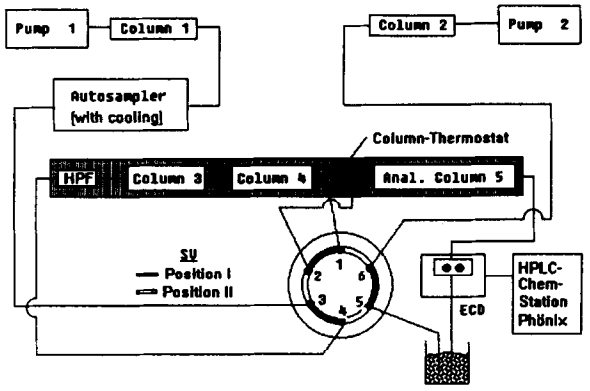

Fig. 1. Chromatographic system for analysis after SPE (for analysis after LLE the capillary connections on the switching valve must be exchanged: 1 with 4 and 2 with 3). Pump 1: quaternary gradient pump for eluents A, B, C and D (SPE) or X, Y and Z (LLE); Pump 2: isocratic pump for eluent D or Z; Column 1, Column 2: precolumns for eluent cleaning (30 × 4 mm I.D.), LiChrosorb RP18, 10 μm particle size); Column 3: guard column cartridge (5 × 4 mm I.D.) Encapharm RP18, 4 μm particle size (to protect column 4); Column 4: guard column (30 × 4 mm I.D.) Encapharm RP18, 4 μm particle size; Column 5: analytical column (250 × 4 mm I.D.) either Encapharm RP18, 4 μm particle size (for SPE) or LiChrospher 100 RP18 (endcapped), 5 μm particle size (for LLE); HPF: high-pressure filter with 0.2 μm Nylon-66 membrane filter; SV: six-port 3-way switching valve, position I is the normal position and position II is used for backflush (SPE) or for forward flush (LLE: connections not shown in this figure); ECD: Model 400 (two-channel detection with parallel arrangement of the electrodes) channel 1, +0.75 V (SPE) or +0.70 V (LLE); channel 2, +0.60 V; working electrode, glassy carbon; reference electrode, Ag/AgCl (3 *M* KCl).

Chromatographic conditions

Fig. 1 shows the chromatographic system used for analysis after SPE (for analysis after LLE the capillary connections on the switching valve must be exchanged: 1 with 4 and 2 with 3).

Preparation of the eluents. The corresponding amounts of the salts were dissolved in a volumetric flask with half the necessary volume of the water. After addition of the appropriate amount of methanol, the flask was filled with water to the calibration mark. The final adjustment of pH was done with few drops of either conc. H_3PO_4 or 2 *M* KOH. All eluents were degassed by helium sparging prior to use.

Eluents after solid-phase extraction. The composition of the buffer was 0.05 *M* KH_2PO_4, 0.26 m*M* EGTA, and 4.3 m*M* tetraethylam-

monium bromide. Eluent A was the buffer solution, containing no MeOH, at pH 1.95. Eluent B was the buffer solution, containing 15% (v/v) MeOH, at pH 3.10. Eluent C was the buffer solution, containing 15% (v/v) MeOH, at pH 4.80. Eluent D was the buffer solution, containing 30% (v/v) MeOH, at pH 6.00. The flow-rates were: for pump 1 (gradient pump), eluents A, B, C and D at 0.5–0.75 ml/min; pump 2 (isocratic pump), eluent D at 0.1 ml/min.

Eluents after liquid–liquid extraction. The composition of the buffer was 0.05 *M* KH_2PO_4 and 0.26 m*M* EGTA. Eluent X was the buffer solution, containing no MeOH, at pH 3.40. Eluent Y was the buffer solution, containing no MeOH, at pH 5.80. Eluent Z was the buffer solution, containing 30% (v/v) MeOH, at pH 6.00. The flow-rates were: pump 1 (gradient pump), eluents X, Y and Z at 0.6–1.0 ml/min; pump 2 (isocratic pump), eluent Z at 0.1 and 0.5 ml/min.

HPLC operating conditions after solid-phase extraction

Although we have performed some filtrations during SPE, additional filtration of the samples was necessary for trouble-free chromatographic analysis. We used a high-pressure filter, a guard column cartridge, a guard column and additional column-switching (Fig. 1). Furthermore, two precolumns for eluent cleaning were necessary to keep the system stable.

At the beginning of the analysis eluent A, the weakest eluent, provided a sufficient separation of VMA and MHPG. After 32 min the stronger eluent B accelerated the passage of HVA, as the last peak of interest, through column 4. After HVA had been eluted to the analytical column 5 (at 45 min), the valve was switched to position II and the later peaks of interference were cut off. While 5-HIAA and HVA were separated on the analytical column 5, pump 2 backflushed column 3, column 4 and the high-pressure filter with eluent D, the most effective eluent. Once the HVA signal had been detected, eluent D removed all interfering components from the analytical column 5. The analytical time was 75 min, and a further 15 min were required to re-equilibrate the system.

HPLC operating conditions after liquid–liquid extraction

The chromatographic conditions used after LLE were the same as those described for SPE with only one modification. After the valve had been switched to position II, columns 3 and 4 and the filter were not back-flushed but forward-flushed. This was done by exchanging the capillary connections on the switching valve 1 with 4, and 2 with 3 (Fig. 1).

For the HPLC separation of VMA eluent X was used. After 12 min, eluent Y was introduced to separate the other three substances. At 16.5 min the last peak of interest (HVA) left column 4, and the valve was switched to position II. During the separation of MHPG, 5-HIAA and HVA on the analytical column 5, pump 2 purged the columns 3 and 4 with eluent Z at a flow-rate of 0.5 ml/min. After HVA had been detected (at 35 min), eluent Z was also applied to the analytical column 5 to flush out interfering substances. The analytical time was 45 min, and the re-equilibration time was 10 min.

Data analysis and quantification

The compounds of interest were identified on the basis of retention times, the addition of standards, and the ratio of peak areas detected at two different potentials.

For quantification, the standard addition method [26] was employed based on peak-area measurements. Double estimations were carried out from all plasma samples with and without the standard addition of 10 ng/ml plasma.

RESULTS

Figs. 2 and 3 show typical chromatograms of standards and plasma extracts after the two different extraction procedures. The resolution of the analytes of interest from other plasma components was sufficient in all samples studied.

Recovery and precision

For the calculation of the recovery, plasma samples from patients were analysed ($n = 42$;

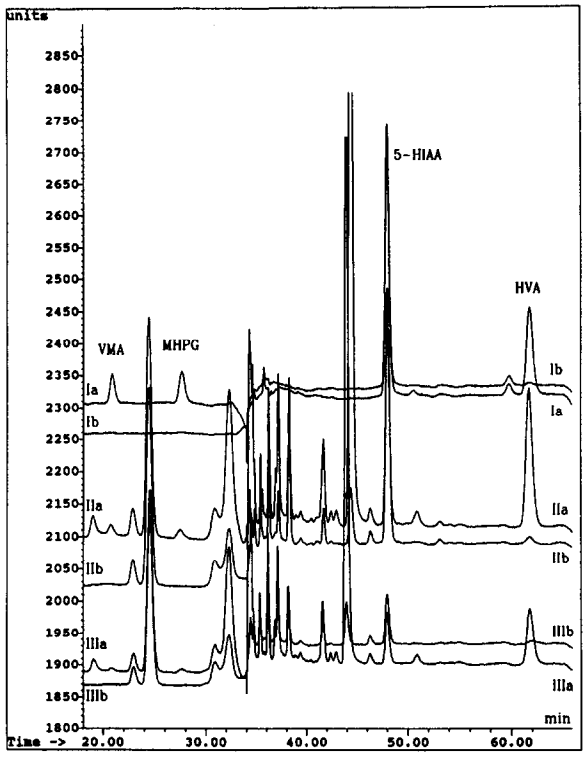

Fig. 2. Typical chromatograms of a standard mixture and plasma extracts with and without addition of standards after SPE. (Ia) Standard (each 7.5 ng), channel 1 (750 mV); (Ib) standard (each 7.5 ng), channel 2 (600 mV); (IIa) plasma spiked with standards, channel 1; (IIb) plasma spiked with standards, channel 2; (IIIa) plasma analysis without standards, channel 1; (IIIb) plasma analysis without standards, channel 2.

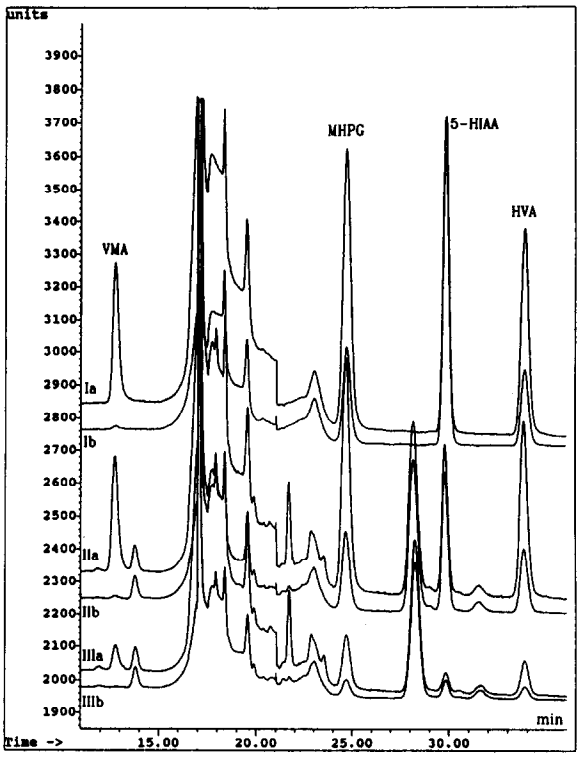

Fig. 3. Typical chromatograms of a standard mixture and plasma extracts with and without addition of standards after LLE. (Ia) Standard (each 5 ng), channel 1 (700 mV); (Ib) standard (each 5 ng), channel 2 (600 mV); (IIa) plasma spiked with standards, channel 1; (IIb) plasma spiked with standard, channel 2; (IIIa) plasma analysis without standards, channel 1; (IIIb) plasma analysis without standards, channel 2.

probability $p = 95\%$). The concentrations of the analytes were in the physiological and pathophysiological range (see Tables II and III).

The calculation was:

$$\frac{A_{SA} - A}{A_{ES}} \times 100\%$$

where A_{SA} is the area of the peak with the addition of 10 ng of standard, A is the peak area without the addition of standard and A_{ES} is the peak area of 10 ng of the external standard. The resulting recoveries of the two extraction procedures are summarized in Table I. LLE provided better recoveries for VMA and MHPG than SPE.

For the intra- and inter-assay variability, pooled plasma from ten patients was analysed eight times. The measured concentrations of the four metabolites were at the lower end of the physiological ranges. The coefficients of variation (C.V.) were calculated from the resulting concentrations of the samples.

After SPE, the C.V. for the intra- and inter-assay variabilities were 13.2% and 27.8% for VMA, 23.4% and 26.3% for MHPG, 13.1% and 24.4% for 5-HIAA and 13.2% and 12.0% for HVA, respectively. After LLE, the C.V. for intra- and inter-assay variabilities were 12.4% and 10.3% for VMA, 10.9% and 8.1% for MHPG, 25.8% and 24.5% for 5-HIAA and 14.3% and 12.3% for HVA, respectively. In addition, it should be noted that the reproducibility of the peak areas of the pure standard solutions (each 5 ng) was *ca.* 2%.

TABLE I

RECOVERY AFTER SPE AND LLE

Pooled plasma samples were used; $n = 42$ in each case, and $p = 95\%$.

Compound	SPE		LLE	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
VMA	20 ± 2	20	77 ± 2	7
MHPG	23 ± 2	24	72 ± 2	8
5-HIAA	56 ± 3	17	58 ± 4	18
HVA	79 ± 2	8	77 ± 2	7

To estimate the detection limit, the ratio of the signal peak height to the height of the baseline noise of plasma samples was calculated. The limits of detection (expressed per ml of plasma) at S/N = 3 were 610 pg (SPE) and 100 pg (LLE) for VMA, 1600 pg (SPE) and 110 pg (LLE) for MHPG, 120 pg (SPE) and 90 pg (LLE) for 5-HIAA and 130 pg (SPE) and 100 pg (LLE) for HVA.

The linearity can be described in terms of the square of the correlation coefficient r. A more exact evaluation provides the variance s^2 (residual sum of squares). For the linearity of the detector response, we found the best results with linear fit in the range from 0.1 to 50 ng injected for VMA ($s^2 = 158\,236$, $r^2 = 0.9992$), for MHPG ($s^2 = 51\,737$, $r^2 = 0.9997$) and for HVA ($s^2 = 33\,508$, $r^2 = 0.9997$). For 5-HIAA in the range 0.1–20 ng (injected amount – at concentrations more than 20 ng the detector was overloaded in that sensitivity range), that linear regression did not give the best results, *i.e.* the smallest variance ($s^2 = 922\,901$, $r^2 = 0.9917$). For this substance, non-linear regression with the least-squares method showed a better fit for quadratic ($s^2 = 45\,012$, $r^2 = 0.9996$), cubic ($s^2 = 4346$, $r^2 = 0.999\,98$) and exponential ($s^2 = 76243$, $r^2 = 0.9995$) functions.

Because the standard addition method for quantification is based on a linear response, we proceeded as follows. In practice, the complete working range has never been used for quantification, so we only took a segment of the range and tested it for linearity. A linear regression in the ranges 5–20 ng, 1–10 ng or 0.5–5 ng (all injected amounts) provided substantial lower variances. The F-test of these variances with those for all points of exponential regression yielded no significant differences between variances ($P = 95\%$). Therefore, quantitation based on linear regression is possible without systematic error. Furthermore, our studies showed that, *after* the extraction procedure with plasma, the non-linear response is less clear-cut than it is with the pure standards. Because of the additional extraction step and the increased scattering of experimental points, the differences between the variances of linear and non-linear regressions were lower than for the standards. Therefore, no significant differences existed and we could justifiably work with the linear fit. In the range 3–45 ng/ml plasma, measured under analytical conditions, (5-HIAA, 1–41 ng/ml plasma) and using the peak areas for SPE and LLE, the following values for r^2 for linear regression were obtained: VMA, 0.9983 and 0.9995; MHPG, 0.9999 and 0.9995; 5-HIAA, 0.9986 and 0.9994; and HVA, 0.9990 and 0.9996.

Plasma concentrations in healthy volunteers and alcoholics at the beginning of delirium tremens

The mean values of VMA, MHPG, HVA and 5-HIAA in plasma samples obtained from six normal volunteers on three consecutive days without restriction in diet and activities before blood sampling are shown in Table II. The average values are in good agreement with reported results from other studies.

We determined the four metabolites in a single plasma sample from alcoholics at the beginning

TABLE II

CONCENTRATIONS OF VMA, MHPG, HVA AND 5-HIAA IN PLASMA FROM HEALTHY HUMAN VOLUNTEERS AFTER SPE, AND NORMAL RANGES PREVIOUSLY REPORTED

Day	Concentration (mean ± S.D., $n = 6$) (ng/ml)			
	VMA	MHPG	HVA	5-HIAA
1	11.58 ± 5.01	3.94 ± 2.08	13.90 ± 5.86	7.52 ± 1.85
2	11.65 ± 5.98	5.25 ± 4.05	16.35 ± 3.72	7.60 ± 1.73
3	10.60 ± 3.95	4.22 ± 1.46	14.48 ± 6.46	6.63 ± 1.31
Normal ranges				
Minimal	8.34 ± 1.53 [30]	3.00 ± 1.20 [29]	6.80 ± 2.30 [28]	12.80 ± 4.90 [20]
Maximal	9.30 ± 1.40 [20]	4.60 ± 1.40 [20]	2.6–14.1 [28]	15.50 ± 3.40 [27]

of delirium tremens and during the course of the delirium under pharmacotherapy. Preliminary results from blood samples collected just prior to commencement of pharmacotherapy are shown in Table III.

DISCUSSION

Sample preparation for SPE and subsequent chromatographic conditions

The determination of catecholamine metabolites in human plasma is difficult, not because of their very low concentrations but because of the poor selectivity of the whole procedure (the HPLC analysis as well as the sample extraction). SPE is generally a rapid and inexpensive method that provides high selectivity and recovery, so we tested it for the extraction of the four metabolites of interest from human plasma.

Because ion-exchange materials are not applicable to the simultaneous extraction of acidic and neutral substances, C_{18} phases were used. The best recoveries for the four metabolites were obtained using the Encapharm C_{18} material with a particle size of 100 μm. Additionally, this phase is excellent to handle (*e.g.* flow behaviour). In an effort to improve the selectivity and recovery of the procedure, especially for VMA and MHPG, many parameters were tested with the following results.

TABLE III

LEVELS OF VMA, MHPG, 5-HIAA AND HVA IN PLASMA FROM ALCOHOLIC PATIENTS AT THE BEGINNING OF DELIRIUM COMPARED WITH HEALTHY VOLUNTEERS AFTER SPE

Subject group[a]	Concentration (mean ± S.D.) (ng/ml)			
	VMA	MHPG	HVA	5-HIAA
Alcoholics (short)	12.85 ± 8.91	11.09 ± 7.36[c]	7.69 ± 3.58	42.96 ± 21.41[d]
Alcoholics (middle)	7.45 ± 4.53[b]	7.34 ± 3.77[b]	3.74 ± 1.37[b]	14.66 ± 9.20
Alcoholics (long)	6.55 ± 3.89[b]	8.00 ± 5.90	5.56 ± 3.63[b]	15.06 ± 15.08
Controls	11.27 ± 4.98	4.47 ± 2.53	7.25 ± 1.03	14.91 ± 5.35

[a] The alcoholics were divided into three groups according to the duration of the delirium: short, less than 2 days; middle, 2–4 days; long, more than 4 days. ($n = 8$ subjects per group). The controls ($n = 6$) were measured on three consecutive days. Statistical analysis of the comparison with controls was done by the *t*-test.

[b] $p < 0.05$.

[c] $p < 0.01$.

[d] $p < 0.001$.

Sorbent conditioning. Conditioning of the sorbent with a wide range of different suitable solutions had no effect on either the recovery or the selectivity.

Sample treatment. The influence of the pH of the plasma samples for loading the columns was different among the substances of interest. At pH 6, for example, the recovery of MHPG was 63% but that of VMA was only 5%. The best results for all four metabolites were obtained when plasma samples were adjusted to pH 3–4 before being loaded on the columns.

Plasma dilution before column loading reduced drastically the recoveries of VMA and MHPG. No differences were observed for 5-HIAA and HVA.

Washing procedure. The weak adsorption behaviour of VMA and MHPG limited the washing procedure used to remove interfering plasma components. After different inorganic and organic solutions had been tested, the minimal effect on the metabolites was observed when 0.5 ml of purified water (0°C) was used to wash the column.

Sample elution. Because the elution step was expected to improve the selectivity of the separation between the four metabolites and the interfering plasma components, it was studied in particular detail. Solutions with different polarity and pH, as well as a second extraction procedure with an SPE-DIOL phase, were intensively tested, but showed no significant effect. When all the boundary conditions were taken into consideration, methanol–water (80:20, v/v) was found to be the optimal eluent.

To dry the SPE columns in each step, the use of nitrogen is necessary to prevent loss of the substances of interest. In an effort to improve the recovery and purity of the plasma extracts, other solid phases, such as C_8, CN and PH, were tested. The only improvement was a 5% increase of recovery for VMA and MHPG on a CH phase. However, because it was easier to work with the 100-μm material of Encapharm C_{18}, we chose to use this phase for sample preparation.

Sample preparation by SPE had only a low efficiency, and thus a very high selectivity of the chromatographic separation was essential. Isocratic separation was insufficient, in spite of the use of a long analytical column after each plasma injection it was necessary to elute late interfering peaks by the injection of five 100-μl volumes of pure MeOH. However, this resulted in stability problems with the working electrode of the electrochemical detector.

In the literature, the column-switching technique has sometimes been reported to improve the separation and stability of the chromatographic procedure [31,32]. Therefore we applied gradient elution in combination with column switching.

The main separation problem was the occurrence of an unknown peak with the same retention time as the neutral MHPG. Variation of pH of the eluent was unfruitful, an organic modifier was not present, and a change of the buffer salt was not successful. Thus an ion-pair method was applied to increase the selectivity of the system. The best results were obtained with NEt_4Br. An eluent with a low pH at the beginning of the chromatographic run provided a satisfactory separation of VMA and MHPG. In this way, we obtained a great range of possible variations for the later peaks.

The column-switching technique proved successful. After the analysis cycle a clean baseline was obtained. The complete chromatographic run was 90 min. However, only this analysis time guaranteed a satisfactory separation and identification of the peaks of interest.

Use of a two-channel detector and the possible calculation of peak-area ratios was very advantageous, because of increased specificity. This was essential, since various plasma samples provided different patterns of interfering peaks.

For quantitation we had a choice between daily calibration and the standard addition method. The latter required larger amounts of plasma and more chromatographic runs. However, these disadvantages were outweighed by the use of the same plasma sample for calibration and for sample analysis, so this was our preferred method.

Sample preparation for LLE and subsequent chromatographic conditions

Sample preparation with LLE using EtOAc for plasma extraction provided better recoveries

TABLE IV

EFFECT OF THE AMOUNT OF NaCl ADDED FOR PROTEIN PRECIPITATION ON THE RECOVERIES OF THE ANALYTES

Compound	Recovery (%)		
	0.5 g NaCl	1.0 g NaCl	2.0 g NaCl
VMA	67	80	64
MHPG	59	75	67
5-HIAA	34	55	26
HVA	61	80	66

for VMA and MHPG, and gave chromatograms with fewer interfering peaks than those obtained after plasma extraction by SPE on C_{18}. The method of Gerhardt *et al.* [20] was modified only insofar as 3 ml of EtOAc per plasma extraction were added. This permits the use of 6.5 ml PP-tubes, which were easy to handle. The recoveries were not diminished.

Furthermore, exactly 1 g of dry NaCl was added to the plasma samples for protein precipitation, because a clear dependence of the recovery on the amount of NaCl was observed (Table IV).

Moreover, the amount of HCl added to the plasma samples before extraction had marked influence on the recovery of all compounds (Table V). Unfortunately, the trend of this dependence for 5-HIAA and MHPG was opposite to that for VMA. The best compromise for all four substances was to add 100 μl of 1 *M* HCl, in agreement with the previous report [20]. Gerhardt *et al.* [20] used a coulometric detector with a guard cell, a pre-analytical cell and a normal analytical cell. Our purpose was to establish conditions that would allow us to estimate VMA, MHPG, HVA and 5-HIAA with amperometric detection. We used almost the same apparatus as described for post-SPE analysis. LLE with EtOAc is a relatively unspecific extraction method. However, with our equipment it was impossible to remove quantitatively some interfering peaks, we used a long analytical column (250 × 4 mm I.D.) and applied gradient elution with column switching.

The chromatographic system (Fig. 1, with forward-flush) proved successful again. The forward-flush column switching method prolonged the stability of the precolumns. In addition, there were no problems of pressure, and we noted a longer lifetime of the guard-column cartridge (Column 3) and the HPF. We built a potentiometer to control pump 2. The complete analysis time between two injections was 55 min. Although with an amperometric detector it is impossible to clean up the injected samples online, this analysis is only 10 min longer than that described by Gerhardt *et al.* [20].

Comparison of SPE and LLE with identical plasma samples

Determination of identical plasma samples with both the described extraction methods provided good results in spite of the disadvantages of SPE. For HVA, we observed the same recoveries and variances for both methods. For 5-HIAA, the recoveries were identical with both methods, and the intra- and inter-assay C.V.

TABLE V

INFLUENCE OF THE AMOUNT OF HCl ADDED TO THE PLASMA SAMPLES ON THE RECOVERIES OF THE METABOLITES

Compound	Recovery (%)				
	20 μl HCl	50 μl HCl	100 μl HCl	200 μl HCl	400 μl HCl
VMA	10	40	75	80	85
MHPG	90	80	73	56	44
5-HIAA	86	65	55	33	11
HVA	74	77	77	80	85

showed the highest variances, reflecting the particular sensitivity of this substance.

Indeed, we would expect similar values with the two procedures because of the quantification method used (standard addition). However, in practice this is not so in each case. The influence of the matrix, and hence the effects of preconcentration and clean-up procedures, cause retention time shifts, peak broadening, overlapping peaks, poor recoveries, etc. This can easily lead to different results, even within one analysis method. It is remarkable, despite all the advantages of the standard addition method, that this quantification method in principle gives rise to lower accuracy of the analysis, because two peaks must be analysed for one result.

CONCLUSION

For simultaneous determination of VMA, MHPG, 5-HIAA and HVA in human plasma, solid-phase extraction is unsatisfactory. Contrary to our expectations, the recovery and selectivity and also the precision of the SPE procedure are insufficient. To compensate for these disadvantages, column switching and two-channel detection were necessary, as well as a longer time for chromatographic analysis and an elaborate quantification method.

Though liquid–liquid extraction is a time-consuming method, it yielded better recoveries of the substances of interest and allowed shorter chromatographic analysis. It is therefore preferable for simultaneous estimation of these four metabolites in human plasma.

In the present study we found that the mean levels of the catecholaminergic metabolites at the beginning of the delirium tremens are different depending on the duration and hence the severity of the delirium tremens. These findings are important not only for more rational treatment strategies of the delirium tremens but also for understanding the pathophysiology of the delirium tremens. Till now the delirium tremens was considered as a uniform clinical picture, and it was generally supposed that the severity of the withdrawal symptoms correlates positively with the amount of norepinephrine released. However, the present data for alcoholics at the beginning of the delirium tremens, prior to the onset of pharmacotherapy, showed the highest plasma levels of catecholaminergic metabolites in patients with short-duration delirium tremens. Particularly severe delirium tremens is prolonged for *ca.* 4 days.

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