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Development of a method for sample preparation for subsequent identification and measurement of 1,2,3,4-tetrahydroisoquinolines and other potentially neurotoxic compounds by high-performance liquid chromatography with ultraviolet and fluorescence detection in blood plasma of Parkinson's disease patients

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Abstract

We developed sample preparation methods for the detection of various biogenic phenylethylamine derivatives such as 3,4-dihydroxyphenylalanine, and their cyclisation products with aldehydes, i.e., 1,2,3,4-tetrahydroisoquinoline derivatives in blood samples. 1,2,3,4-Tetrahydroisoquinolines are considered to play an essential role as neurotoxic compounds in the pathomechanism of Parkinson's disease. We used reversed-phase high-performance liquid chromatography with ultraviolet and fluorescence detection for separation and identification. Ultrafiltration, protein precipitation and solid-phase extraction were investigated for purification of blood samples and enrichment of various compounds with a wide range of hydrophilicity and hydrophobicity. Protein precipitation by methanol and perchloric acid is a fast method to separate the analytes from the plasma matrix. A higher yield of the analytes is attained with prior addition of an alkylsulfonic acid giving a fine-grained precipitate. With the addition of ion pairing compounds into the sample it is possible to enrich not only lipophilic compounds such as norharman, tryptamine and melatonin, but also hydrophilic ones such as 3,4-dihydroxyphenylalanine by reversed-phase solid-phase extraction. Ultrafiltration is not useful as a screening method.

Keywords: 1,2,3,4-Tetrahydroisoquinolines; Neurotoxic compounds

1. Introduction

Parkinson's disease (PD) is primarily caused by a degeneration of neuromelanin-containing neurons of the substantia nigra pars compacta (SNc). The origin of the nigral degeneration is unknown as yet. However, during the beginning of the 1980s, some consumers of illicit drugs, using a meperidin analogue intravenously, developed symptoms of an irreversible PD. In animal experiments it was shown that a contamination of this illicit drug, i.e., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (well-known during the past years as 'MPTP') was respon-

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sible for the loss of dopaminergic innervation of the relevant brain areas [1].

After the demonstration of neurotoxic properties of MPTP, scientific interest has focussed on two groups of biological compounds showing a high structural similarity, i.e., tetrahydro-β-carbolines [2,3] and tetrahydroisoquinolines [4,5]. Especially some of the derivatives of 1,2,3,4-tetrahydroisoquinoline (THIQ) showed a marked neurotoxic potential in vivo and in vitro [6–9]. However, on the other hand, a certain protective effect could be demonstrated in animal experiments in the case of 1-methyl-THIQ [10]. In the meantime, enzymes could be isolated which are able to synthesize enantioselectively (*R*)-salsolinol (6,7-dihydroxy-1-methyl-THIQ) and 1-methyl-THIQ [11,12].

However, it can be assumed that THIQs generally are formed by the so-called Pictet–Spengler reaction between compounds with a basic structure of 2-phenylethylamine and suitable aldehydes or α-keto acids. For example, salsolinol is generated from dopamine and acetic aldehyde or pyruvic acid with subsequent decarboxylation [13,14]. Fig. 1 represents the formation of salsolinol from dopamine.

Because of the fact that there are a considerable number of physiological precursor molecules with a basic structure of phenylethylamine, the number of THIQs which are possibly formed in the organism is extremely high. At least some of these compounds will exert toxic effects on neuromelanin-containing neurons.

Condensation and cyclization of 2-phenylethylamine and phenylacetic aldehyde, a metabolite of the former substance leads to 1-benzyl-THIQ after oxidation by monamine oxidase B (MAO B) [15,16].

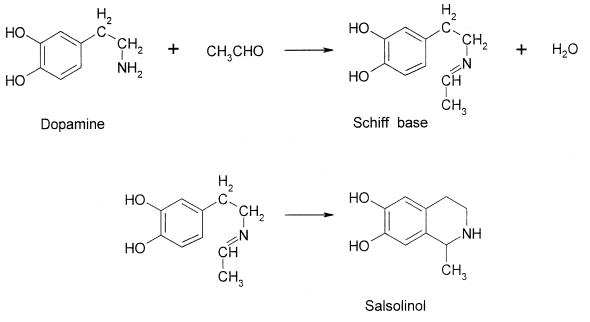

Fig. 1. Pictet–Spengler reaction of dopamine with acetaldehyde.

Other aldehydes of biogenic origin suitable for these cyclization reactions are formaldehyde, malonic dialdehyde or acroleine. The latter is formed when fatty material is heated to temperatures at which they undergo thermal decomposition. (PD occurs in all human cultures as has been already observed in the last century: the heating of food has been a cultural similarity for a long time).

Malonic dialdehyde is well-known as a marker for oxidative stress, which is discussed as one of the reasons for the decay of the nigral cells [17,18]. Within this hypothesis, it is assumed that the metabolization of dopamine by MAO or the autoxidation leads to the formation of hydrogen peroxide which decomposes to hydroxyl radicals in the presence of iron-II [19]. Indeed, the SNc of PD patients accumulates iron to a much higher extent than that of control persons [20].

Similarly, a large number of isomeric compounds can be derived by variation of methyl, carboxy and hydroxy groups in the amine moiety of the precursor molecule, e.g., of tyramine and dopamine and their derivatives, respectively. The various isomeric hydroxy compounds can also be considered as being generated by the reactions of oxidative stress and the formation of hydroxy radicals occurring prior to or after the Pictet–Spengler cyclisation.

The formation of hydroxy radical adducts in a Fenton system and the significance of the formed products with respect to cause PD was already investigated in the case of MPTP [21]. In addition, hydroxylated dopamines could be demonstrated to occur in the urine of PD patients [22].

The number of THIQs which can be formed from simple physiological precursor molecules – phenylethylamine congeners and aldehydes mentioned above – is more than 4000. This means that it will be impossible to synthesize all of these compounds and to test them for a relevant neurotoxic effect with respect to SNc damage. Therefore, we decided to use another approach to solve this problem: the first step will be the screening of blood serum samples of de novo PD patients for the occurrence of potentially neurotoxic THIQ compounds not present in the blood of control persons. After identification, these compounds will be tested in the second step for neurotoxic effects in several appropriate test systems (cell cultures and animal models). This approach

requires the development of very sensitive and efficient analytical methods for the demonstration and identification of THIQ congeners and other potentially neurotoxic compounds expected to occur in minute concentrations in the organism of PD patients on the one hand and the synthesis of compounds suspicious for possible neurotoxic effects for further testing on the other.

The aim of the present work is to develop efficient analytical methods for the identification and measurement of THIQ congeners and other potentially neurotoxic compounds with relevance to the pathogenesis of PD by high-performance liquid chromatography with ultraviolet and fluorescence detection (HPLC–UV/FD) in the blood plasma of PD patients.

2. Experimental

2.1. Experimental device

The HPLC–FD system consisted of a Milton-Roy gradient pump (type: CM 4000), a Milton-Roy UV detector (type: SM 4000) (ThermoQuest, San Jose, CA, USA), a fluorescence detector from Shimadzu (RF-530) (Duisburg, Germany), a Rheodyne injection valve (Cotati, CA, USA) and an analytical column of 150 mm×4.6 mm I.D. packed with ODS-3, 5 μm (Dr. Maisch, Ammerbuch, Germany).

2.2. Reagents and standard compounds

All inorganic reagents were of analytical grade and were used without any further purification. Methanol (gradient grade) was from Merck (Darmstadt, Germany). Water from a Milli-Q device was used for the preparation of buffers and standard solutions. The sodium salt of 1-hexanesulfonic acid (HSA-Na^+) from Fluka (Buchs, Switzerland) was of puriss. grade (>99%; analytical-reagent grade for ion-pair chromatography). Ammonium dihydrogen phosphate from Aldrich was of 99.999% purity.

Salsolin and salsolidin were from Roth (Karlsruhe, Germany), 6-methyl-1,2,3,4-tetrahydroquinoline (6-Me-THQ) was from Lancaster (Eastgate, UK), and all other standard compounds were from Sigma (St. Louis, MO, USA).

2.3. Collection and pretreatment of samples

Blood samples were collected in polyethylene tubes containing 1.6 mg K-EDTA/ml blood. The samples were centrifuged for 15 min at 800 *g*. A 100-μl volume of 50 mmol/l ascorbic acid solution was added to 5–6 ml samples of plasma. The samples were stored at −28°C.

2.4. HPLC separation and detection

Aliquots (25 μl) of the samples were injected for the analysis by reversed-phase (RP) HPLC–FD or UV, respectively, with ion-pair reagent. Gradient elution was performed at a flow-rate of 0.7 ml/min. The aqueous mobile phase consisted of 12 mmol/l $(NH_4)H_2PO_4$, 3 mmol/l HSA-Na^+ and 5% methanol. The pH value was adjusted to 3 by phosphoric acid. Methanol was added during the run as described in Table 1. The excitation wavelength was 285 nm and the emission wavelength 315 nm. The areas of the integrated peaks of standard reference solutions were used for quantification.

In order to enable an attribution of a certain peak to a compound on account of its retention time (t_R), tyrosyl tyrosine was added as a reference compound to all samples. This compound was eluted in a range where no other signals are expected. The attribution was performed on account of its relative retention time, defined as t_R(analyte)/t_R(reference compound). This value differs only by ±0.02 units from one run to another for each analyte.

In order to obtain any information about a possible coelution of analytes, the ratio of the absorbance at the UV wavelengths of 215 and 240 nm was used as

Table 1
Gradient used in HPLC: A=12 mmol/l $(NH_4)H_2PO_4$, 3 mmol/l HSA-Na^+ and 5% methanol, B=methanol; linear gradient

Time (min)	A (%)	B (%)
0	100	0
5	100	0
40	55	45
42	0	100
46	0	100
52	100	0
60	100	0

an identifying characteristic. In addition, the ratio of fluorescence intensity/UV absorbance was determined for the same purpose.

For the detection of analytes, we used the selective UV detector and the potent fluorescence detector. At 210 and 240 nm, all compounds mentioned below could be detected efficiently. THIQs and other physiological compounds could be detected with very high sensitivity using an excitation wavelength of 285 nm and an emission wavelength of 340 nm. The quantification was realized by the more sensitive FD.

2.5. Recovery of standard compounds: calibration, precision, accuracy

The validation of the three different methods for plasma sample preparation was performed using a mixture of six physiological compounds with a wide range of different physico–chemical properties, i.e., DOPA, norsalsolinol, 3-methyl-DOPA (3-Me-DOPA), salsolinol, salsolin and salsolidin.

The calibration curves for each compound were constructed with seven concentrations ranging from 10 to 500 ng/ml. Up to eight measurements were performed for one single concentration. The resulting calibration curves showed linearity over the tested range of concentration. The coefficients of determination (r^2) were calculated as follows: DOPA: r^2= 0.9906, norsalsolinol: r^2=0.9954, 3-Me-DOPA: r^2= 0.9976, salsolinol: r^2=0.9906, salsolin: r^2=0.9953, and salsolidin: r^2=0.9967.

The robustness of the method was tested with the standard test mixture by intra- and inter-day accuracy and precision for concentrations of 25, 50, 250 and 500 ng/ml. The intra-day accuracy and precision was carried out with five injections for each concentration within a single day (n=5). For the inter-day variation each concentration was measured twice a day on 4 different days (n=8). The mean calculated concentrations of every standard compound as well as the values of intra- and inter-day precision and accuracy are summarized in Tables 2–7.

3. Results and discussion

Three different methods were taken into consideration for the pretreatment of the samples and the enrichment of THIQ analytes present in the samples: ultrafiltration, precipitation of serum proteins and enrichment of the analytes on a solid-phase extraction (SPE) column.

3.1. Ultrafiltration

In a first step, the suitability of an ultrafiltration of the sample combined with a subsequent reduction of the volume by evaporation was tested. However, testing the recovery of five compounds – salsolinol, salsolin, DOPA, salsolidin and melatonin – yielded acceptable recovery rates only in two cases, i.e., salsolinol and salsolin, when the standard compound was added to the sample 5 min prior to ultrafiltration.

Table 2
Intra- and inter-day accuracy and precision of DOPA

	Actual concentration (ng/ml)	Mean calculated concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (n=5)	25	16.8	34.5	32.7
	50	36.7	9.8	26.6
	250	240.1	5.6	1.6
	500	480.6	4.1	3.9
Inter-day (n=8)	25	14.3	33.6	42.8
	50	39.5	18.2	21.1
	250	232.0	5.6	7.2
	500	439.5	16.3	12.1

[a] Precision=(SD/mean calculated concentration)×100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]×100.

Table 3
Intra- and inter-day accuracy and precision of norsalsolinol

	Actual concentration (ng/ml)	Mean calculated concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (n=5)	25	20.7	26.2	17.3
	50	36.2	24.0	27.7
	250	228.0	8.6	8.8
	500	425.0	15.3	15.0
Inter-day (n=8)	25	19.0	28.7	24.1
	50	46.8	12.9	6.5
	250	209.2	9.3	16.3
	500	394.3	14.1	21.1

[a] Precision=(SD/mean calculated concentration)×100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]×100.

Table 4
Intra- and inter-day accuracy and precision of 3-Me-DOPA

	Actual concentration (ng/ml)	Mean calculated concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (n=5)	25	30.6	10.1	−22.3
	50	46.6	10.5	6.8
	250	262.6	7.5	−5.0
	500	488.4	1.3	2.3
Inter-day (n=8)	25	30.9	12.7	−23.5
	50	48.4	10.6	3.2
	250	253.8	3.2	−1.5
	500	487.6	3.2	2.5

[a] Precision=(SD/mean calculated concentration)×100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]×100.

Table 5
Intra- and inter-day accuracy and precision of salsolinol

	Actual concentration (ng/ml)	Mean calculated concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (n=5)	25	25.5	35.5	1.9
	50	35.9	21.7	28.2
	250	234.5	5.8	6.2
	500	439.5	13.8	12.1
Inter-day (n=8)	25	16.9	22.6	32.2
	50	45.2	14.4	9.6
	250	214.2	9.2	14.3
	500	397.2	20.6	13.9

[a] Precision=(SD/mean calculated concentration)×100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]×100.

Table 6
Intra- and inter-day accuracy and precision of salsolin

	Actual concentration (ng/ml)	Mean calculated concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (n=5)	25	30.1	28.1	−20.5
	50	47.1	8.8	5.7
	250	260.2	4.9	−4.1
	500	481.7	1.3	3.7
Inter-day (n=8)	25	21.7	7.5	13.1
	50	46.4	8.1	7.2
	250	242.0	5.6	3.2
	500	473.6	2.8	5.3

[a] Precision=(SD/mean calculated concentration)×100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]×100.

Table 7
Intra- and inter-day accuracy and precision of salsolidin

	Actual concentration (ng/ml)	Mean calculated concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (n=5)	25	31.2	29.4	−24.6
	50	54.9	4.3	−9.8
	250	27.6	5.6	−11.9
	500	499.4	1.3	0.1
Inter-day (n=8)	25	24.5	12.3	2.2
	50	55.0	5.2	−10.1
	250	265.5	6.5	−6.2
	500	490.3	3.2	1.9

[a] Precision=(SD/mean calculated concentration)×100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]×100.

In the case of DOPA, salsolidin and melatonin, the recovery rate was 30% and below under the same conditions (Table 8).

However, when the test compounds were added approximately 12 h prior to the ultrafiltration (T= 4°C), the recovery rates were considerably lower. Possibly, the test compounds were adsorbed on the surface of plasma proteins with relative molecular masses above 5000, and consequently lost within the ultrafiltration step. Furthermore, at a detection wavelength of 240 nm, the ultrafiltration method yielded a lower number of signals than the precipitation method described below, i.e., a lower number of compounds could be detected.

Table 8
Recovery of the amount added for five reference compounds after ultrafiltration of plasma samples[a]

Compound	Recovery (%)	
	5 min	12 h
DOPA	4	2
Salsolinol	67	14
Salsolin	95	51
Salsolidin	30	27
Melatonin	9	4

[a] Incubation with standard solution for 5 min and 12 h, respectively, prior to sample preparation (n=3).

The very limited suitability of the ultrafiltration procedure within a general screening method was demonstrated also in other cases of toxicological analyses: e.g., methadone showed a recovery rate of less than 10% [23]. Another disadvantage was the fact that the ultrafiltration procedure did not lead to a complete removal of all proteins from the sample [24]. Apparently, this procedure is only a useful supplementation in special cases.

Therefore, the following two methods had to be developed for the pretreatment of the samples and the enrichment of THIQ analytes present in the samples: precipitation of serum proteins and enrichment of the analytes on an SPE column.

3.2. Precipitation of serum proteins

3.2.1. Development of the precipitation method

For the pretreatment of plasma samples several precipitation procedures have been proved to be successful. Acetone, methanol, trichloroacetic acid and perchloric acid have been used as precipitation reagents [25–27]. Within our study, we added 800 μl methanol to 400 μl plasma. After thorough mixing (vortex), the precipitated protein was removed by centrifugation. However, the precipitation exclusively by methanol did not result in a complete removal of the protein matrix. This could already be seen by the amount of the precipitated pellet. When the pellet material was extracted for determining the amount of coprecipitated analytes, the protein pellet dissolved again completely. Furthermore, the recovery rates after protein precipitation of four test compounds with various degrees of hydrophobicity, i.e., DOPA, 3-Me-DOPA, 3-NO_2-tyrosine (3-NO_2-Tyr) and salsolin, was insufficient (see Table 9).

Precipitation by means of perchloric acid in the presence of methanol (400 μl plasma+100 μl methanol+100 μl 1 mol/l $HClO_4$) did not result in an increase of the recovery rates. The addition of 200 μl HSA-Na^+ (0.5 mol/l) prior to the precipitation by methanol and perchloric acid only resulted in a slight increase of the recovery rates.

Table 9
Recovery of the amount added to 400 μl plasma for reference compounds and sample analytes (*) after protein precipitation (n=3)[a]

Compound	Recovery (%)		
	A	B	C
DOPA	16–33	30–35	95–100
Tyr*		40–50	85–95
3-Me-DOPA	20–38	38–41	110–120
3-NO_2-Tyr	20–34	33–38	88–93
Salsolin	19–39	34–39	96–100
Trp*		40–50	85–95

[a] Precipitation conditions: A=800 μl methanol; B=200 μl HSA-Na^+ (0.5 mol/l), 400 μl methanol, 200 μl $HClO_4$ (3 mol/l); C=200 μl HSA-H^+ (0.5 mol/l), 400 μl methanol, 200 μl $HClO_4$ (3 mol/l).

In order to shift the equilibrium into the direction of an increased ion-pair formation, the addition of the H^+-form of HSA (HSA-H^+) was tested. The conversion of the HSA-Na^+ into the free acid was performed on a cationic exchanger (Amberlite CG-120-II). The exchanger is purified by 0.5 mol/l NaOH, water, 1 mol/l hydrochloric acid, water, methanol and equilibrated with water. Then, a solution of 100 mg/ml HSA-Na^+ is added. Measurement of the pH value indicated the appearance of HSA-H^+ showing a pK value of 1. Eluates with a pH 1 were used for the protein precipitation experiments.

In order to test the efficiency of an addition of HSA-H^+, protein precipitations were performed in the presence and absence of the acid using the two test compounds DOPA and 6-Me-THQ (n=4). The results showed significantly higher recovery rates by using HSA-H^+, i.e., for DOPA 50–70% without HSA-H^+ and 65–95% with the ion pair reagent as well as for 6-Me-THQ 25–65% and 65–81%, respectively.

In a second series of experiments we compared the effects of HSA-Na^+ and HSA-H^+ as protein precipitation reagents. The use of HSA-H^+ instead of HSA-Na^+ resulted in a marked increase of the recovery rates both for test compounds added to the plasma prior to precipitation, as well as for physiological compounds (Table 9).

In both precipitation procedures, the recovery rates showed a large range of variation (Table 9). However, the protein precipitation by means of the free acid was superior to that without HSA-H^+. In order to find out whether the variation of the recovery values was due to the composition of the plasma itself, the same experiments were performed using bovine serum albumin as matrix.

The use of bovine serum albumin as matrix reduced the variation of the values significantly (Table 10). Also in this case the use of HSA-H^+ is superior to precipitation without it. Only melatonin shows insufficient recovery rates under both conditions. Apparently, binding of melatonin to proteins, especially to albumin, is stronger than the binding of all other analytes.

For the HPLC analysis the samples had to be concentrated carefully after protein precipitation.

Table 10
Recovery of the amount added for six reference compounds after precipitation ($n=3$) in the presence of bovine albumin as protein matrix (7 g albumin/100 ml)[a]

Compound	Recovery (%)		
	A	B	C
DOPA	50–60	84–87	65–95
Norsalsolinol			100–140
Salsolinol	54–61	95–105	95–110
Salsolin	44–48	68–72	90–112
Salsolidin	45–48	70–74	110–130
6-Me-THQ	40–49	73–80	

[a] Sample volume: 400 μl (A/B); 2000 μl (C). Precipitation conditions: A=400 μl methanol, 200 μl $HClO_4$ (3 mol/l); B=200 μl HSA-H^+ (0.5 mol/l), 400 μl methanol, 200 μl $HClO_4$ (3 mol/l); C=500 μl HSA-H^+ (0.5 mol/l), 2000 μl methanol, 500 μl $HClO_4$ (3 mol/l).

Therefore we removed the methanolic layer and parts of the aqueous layer in a nitrogen stream under slightly elevated temperatures (40°C). However, complete removal of water from the sample has to be avoided. A 2500–5000-μl volume of aqueous sample solution can be concentrated to approximately 200 μl with losses of analytes below 10%.

Faced with the various problems of plasma sample pretreatment described before, we developed a complete procedure based on protein precipitation, represented in Fig. 2. Starting with a plasma volume of 2000 μl, the sample volume of 5000 μl can be concentrated to an end volume of 200 μl. Under these conditions a limit of detection (LOD) of approximately 500 pg/ml can be reached in the case of salsolinol, the compound being characterized best within the group of potentially neurotoxic THIQs. In the literature levels of salsolinol in the range of 10 pg/ml–4 ng/ml have been reported [28–30].

3.2.2. Validation of the precipitation procedure

To validate the presented method, bovine serum albumin was spiked with the six standard compounds of the test mixture, i.e., DOPA, norsalsolinol, 3-Me-DOPA, salsolinol, salsolin and salsolidin, and processed as shown in Fig. 2. The recovery rates and the standard deviation (SD) for each standard compound were determined for spike concentrations of 90.9 ng/ml and 454.5 ng/ml. The recovery rates (±SD) were at least 77.0±13.5% in the case of DOPA for the smaller concentration ($n=5$) and at least

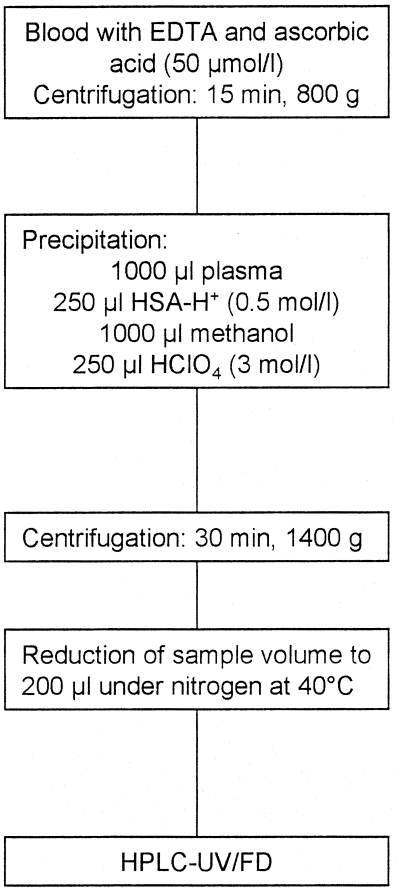

Fig. 2. Processing of plasma samples based on precipitation of plasma proteins.

87.4±3.0% in the case of 3-Me-DOPA for the higher concentration ($n=5$). The values for each component of the test mixture are represented in Table 11.

The selectivity of our method for the tested standard compounds, i.e., DOPA, norsalsolinol, 3-Me-DOPA, salsolinol, salsolin and salsolidin, is acceptable. In the artificial matrix of bovine serum albumin all analytes are baseline separated and can be detected simultaneously. Furthermore, the method shows also a high selectivity when applied to human plasma (see below).

The SDs of the recovery rates of the precipitation method (Table 11) are nearly within the range of variation of the precision and accuracy values for the standard compounds (Tables 2–7), especially in the case of the inter- and the intra-day values of the

Table 11
Recovery and SD of the precipitation method at two different concentrations of the reference compounds ($n=5$)

Compound	Spike (ng/ml)	Recovery (%)	SD
DOPA	90.9	77.0	13.5
	454.5	88.3	5.1
Norsalsolinol	90.9	104.4	13.9
	454.5	111.9	6.5
3-Me-DOPA	90.9	82.8	5.5
	454.5	87.4	3.0
Salsolinol	90.9	99.6	15.9
	454.5	104.6	4.2
Salsolin	90.9	81.0	5.9
	454.5	88.0	4.4
Salsolidin	90.9	88.7	7.1
	454.5	88.4	4.6

accuracy and precision at 25 ng/ml standard concentration. Apparently, the variation range of the recovery values of the precipitation method is mainly due to technical properties of the analytical device, but not to the variation of the method procedure itself. This indicates a comparatively high robustness of the precipitation method as a screening method to determine a series of serum components with considerably different polarity.

3.3. Enrichment by solid-phase extraction

3.3.1. Development of the solid-phase extraction method

The second procedure for the pretreatment of plasma samples is the enrichment of the analytes on an SPE column. As compared to the protein precipitation procedure, the advantage of this method is the possibility to transfer all analytes into an organic solvents (in our case methanol) within one step. In principle, it is possible to gain higher ratios of analyte enrichment than with the protein precipitation procedure.

Varian Mega Bond Elut C_{18}, Chromabond C_{18} ec and Water Oasis were tested for the use as column materials. The latter is a copolymer [poly(divinylbenzene-co-*N*-vinylpyrrolidone)] with hydrophilic and as well as lipophilic retention properties, which leads to the retention of a large variety of hydrophilic and lipophilic compounds [31]. In our experiments, we used 30 mg cartouches and the following conditions for the sample size, and the equilibration, washing and elution steps [31], i.e., equilibration: 1 ml methanol+1 ml water; sample: 500 μl plasma; washing: 1 ml water; elution: 1 ml methanol.

The following fractions were collected from the column (dead-volume: ca. 500 μl): F-0: effluent after load of 500 μl of plasma or reference solution; F-1: effluent after load of 500 μl water; F-2: effluent after load of additional 500 μl water and application of pressure to remove water completely, subsequently addition of 500 μl methanol to the 'dry' column; F-3: effluent after load of additional 500 μl methanol; F-4: effluent after load of additional 500 μl methanol.

Test compounds in the first experiment were DOPA, 3-Me-DOPA, salsolinol and salsolin. DOPA and 3-Me-DOPA were not enriched on the SPE phase, whereas 40% of salsolinol and 80% of salsolin were found in fractions 2 and 4 (Table 12).

In a second experiment, we tested the elution behavior of DOPA, tyrosine (Tyr), phenylalanine (Phe), salsolin and tryptophane (Trp) in the presence of HSA-H^+ (50 mmol/l). HSA-H^+ led to a marked increase of the content of two analytes, i.e., of salsolin and Trp, in the methanol-containing fractions 3 and 4, whereas DOPA, Tyr and Phe appeared in the aqueous fraction 2 (Table 13).

The additional use of 20 μl concentrated phosphoric acid (as recommended in Ref. [31]) also did not result in a shift of the test compounds DOPA,

Table 12
Recovery of four compounds of the amount added after enrichment on Waters Oasis and distribution into four fractions ($n=3$): F-1, F-2, F-3, F-4 (for details see text)

Compound	Recovery (%)			
	F-1	F-2	F-3	F-4
DOPA	90	15	<LOD	<LOD
3-Me-DOPA	28	65	<LOD	<LOD
Salsolinol	<LOD	40	<LOD	<LOD
Salsolin	<LOD	<LOD	<LOD	80

LOD=Limit of detection.

Table 13
Recovery and distribution of sample analytes and spike compounds between different fractions after elution from Waters Oasis in presence of 50 mmol/l HSA-H^+ (n=3), (*)=spike compounds

Compound	Recovery (%)			
	F-1	F-2	F-3	F-4
DOPA*	<LOD	85	<LOD	<LOD
Tyr	<LOD	100	<LOD	<LOD
Phe	<LOD	100	<LOD	<LOD
Salsolin*	<LOD	<LOD	95	<LOD
Trp	<LOD	<LOD	100	9

Tyr and Phe from fractions 1 and 2 into fractions 3 and 4.

Similar experiments were performed with Varian Mega Bond Elut C_{18} and Chromabond C_{18} ec. In these cases, cartouches with an increased length to diameter ratio (20 mm bed length×5 mm I.D., 300 mg material) showed better results than the commercial products. This lead to an increase of the contact between solid-phase material and sample.

In the case of an enrichment of a pure solution of standard compounds, DOPA and salsolin appeared completely in fractions 3 and 4. This means, that they were eluted completely from the extraction sorbent by applying 0.7–1.4 ml methanol. However, these values were obtained by using a standard solution without protein matrix. The plasma amino acids appeared in fractions 3 and 4 (Table 14).

Under the elution conditions described above, similar results could be obtained with Chromabond C_{18} ec (Table 14) and Varian Mega Bond Elut C_{18} (Table 15).

The amount of methanol needed for the elution of the various compounds from the column was comparatively high. The reason for this phenomenon may be the large amount of column material used or the fact that a significant proportion of the protein and lipid matrix was also adsorbed to the column material. For this reason we tested also a plasma sample after membrane ultrafiltration (cut-off: molecular mass=5000) and spiking with standard compounds. In the presence of low-molecular-mass proteins (<5000) the components were eluted by the first 700 μl methanol, hence they appeared in fraction 3 (Table 16). The results indicated that the protein matrix of the sample had an influence of the distribution of certain standard compounds, since they appeared in fractions 3 or 4 in the presence of all proteins (Tables 14 and 15).

The distribution of the analytes was not changed significantly when the volume of the plasma sample or the reference solution of 500 μl was increased to a volume of 2000 μl. After removal of methanol to dryness and dissolving the analytes in 100 μl buffer solution, an enrichment ratio of 20 was achieved.

Table 14
Recovery and distribution of analytes and spike compounds between different fractions after elution from Chromabond C_{18} ec (self packed column) in presence of 50 mmol/l HSA-H^+ (n=3), (*)=spike compounds

Compound	Recovery (%)			
	F-1	F-2	F-3	F-4
DOPA*	<LOD	<LOD	55	60
Tyr	<LOD	<LOD	50	50
Phe	<LOD	<LOD	50	50
Salsolin*	<LOD	<LOD	52	58
Trp	<LOD	<LOD	55	45

Table 15
Recovery and distribution of analytes and spike compounds between different fractions after elution from Varian Mega Bond Elut C_{18} (self packed column) in presence of 50 mmol/l HSA-H^+ (n=3), (*)=spike compounds

Compound	Recovery (%)			
	F-1	F-2	F-3	F-4
DOPA*	<LOD	<LOD	118	<LOD
Tyr	<LOD	<LOD	45	55
Phe	<LOD	<LOD	54	46
Salsolin*	<LOD	<LOD	51	53
Trp	<LOD	<LOD	54	46

Table 16
Recovery and distribution of analytes between different fractions after elution from Varian Mega Bond Elut C_{18} (self packed column) in presence of 50 mmol/l HSA-H^+ [a]

Compound	Recovery (%)			
	F-1	F-2	F-3	F-4
DOPA*	<LOD	<LOD	95	10
Tyr	<LOD	<LOD	100	<LOD
Phe	<LOD	<LOD	100	<LOD
Salsolin*	<LOD	<LOD	95	<LOD
Trp	<LOD	<LOD	91	9

[a] Samples were ultrafiltered. Recovery in relation to amount applied to column (n=3), (*)=spike compounds.

Fig. 3 represents the complete procedure for the pretreatment of plasma samples based on SPE.

The results of our experiments show that basic compounds which can form an ion pair with HSA, interact strongly with the C_{18}-chains of the stationary phase of the column in the presence of HSA which is a hydrophobic ion pair reagent. This is caused by adsorption of HSA to the surface of the reversed-phase material and by its function as an ion exchanger. Hence, the interaction of the analytes with the surface of the reversed-phase is stronger than in the absence of HSA. As a consequence, the analytes were eluted only by methanol. The affinity of the alkyl sulfonic acids to the counterion is determined by the strength of the sulfonic acid on the one hand and by the degree of its interaction with the hydrophobic surface of the solid phase on the other [32].

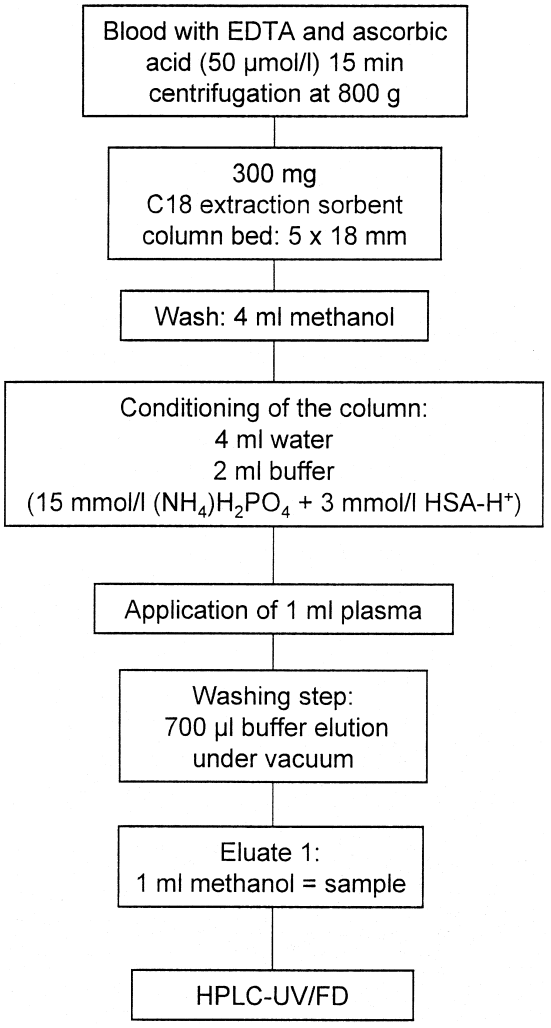

Fig. 3. Processing of plasma samples based on SPE.

The transfer of the analytes into the methanol fraction could be achieved only by HSA-H^+, but not by the Na^+ salt, despite of the fact that HSA in general is a strong ion-pairing reagent. Apparently, the equilibrium is shifted considerably more to the side of the ion-pair formation, when HSA is used as free acid than when used as Na^+ salt. HSA-H^+, showing a pK value of 1, is a strong acid and therefore is able to form ion pairs with the analytes easily. When HSA-Na^+ is used, apparently there is a competition between the protonated analytes and the Na^+ counterion.

3.3.2. Extraction efficiency of the solid-phase extraction method

The efficiency of the SPE method was tested under the conditions mentioned above on Chromabond C_{18} ec columns. The test mixture

Table 17
Recovery and SD of the SPE method for six reference compounds (n=5)

Compound	Spike (ng/ml)	Recovery (%)	SD
DOPA	22.7	60.4	4.8
	45.5	56.5	3.3
	90.9	73.8	7.3
Norsalsolinol	22.7	86.5	10.0
	45.5	74.3	7.8
	90.9	95.6	8.9
3-Me-DOPA	22.7	72.8	6.5
	45.5	54.8	7.2
	90.9	73.5	11.6
Salsolinol	22.7	73.2	10.0
	45.5	61.4	5.1
	90.9	86.7	12.5
Salsolin	22.7	54.4	3.2
	45.5	48.2	5.2
	90.9	66.4	9.5
Salsolidin	22.7	60.2	8.1
	45.5	50.9	5.1
	90.9	69.9	12.3

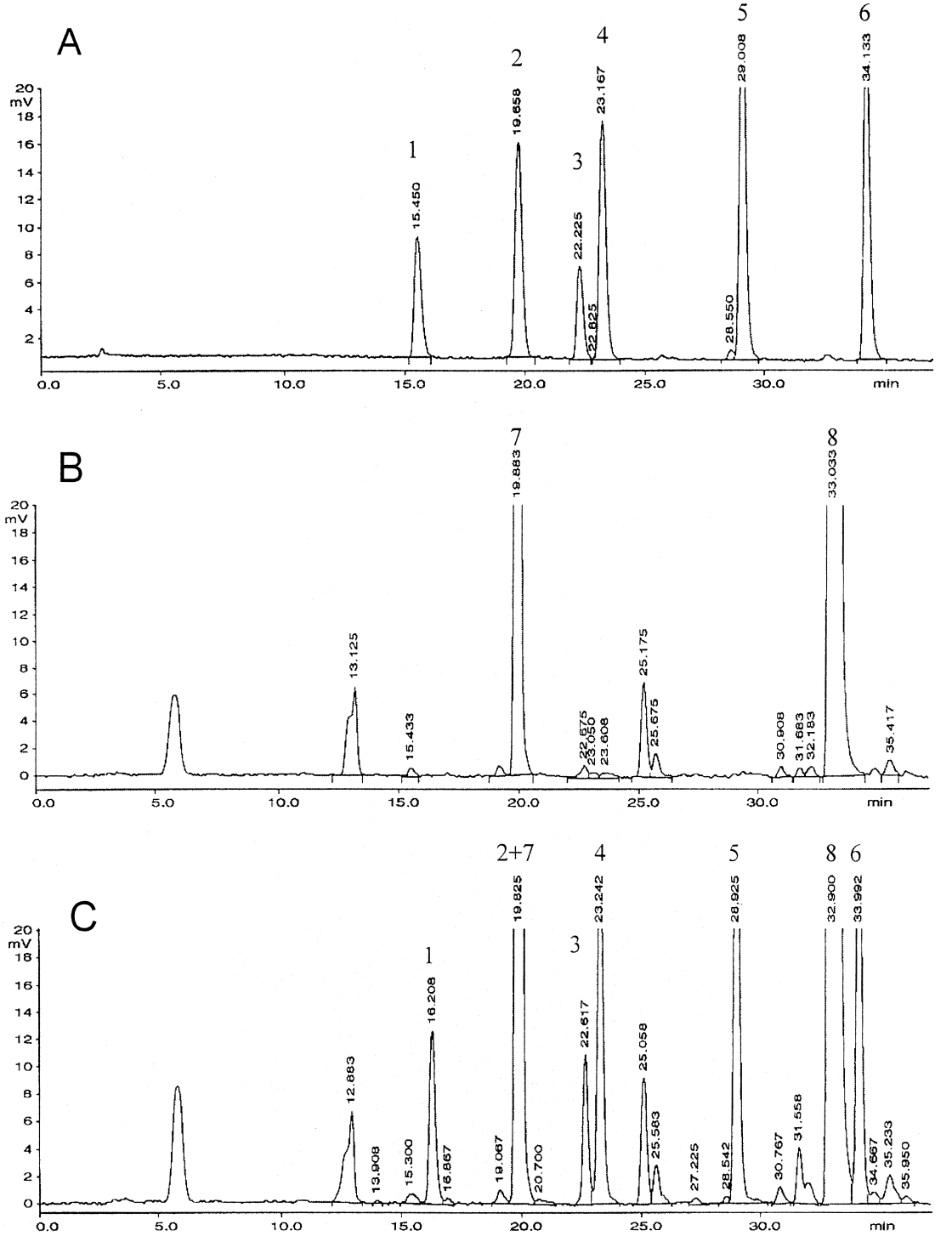

Fig. 4. Chromatogram of (A) bovine serum albumin spiked with the test mixture (see text), (B) plasma of a control person, and (C) the plasma sample (B) spiked like sample (A).

described in Sections 2.5 and 3.2.2 was spiked to the bovine serum albumin at concentrations ($n=5$) of 22.7 ng/ml, 45.5 ng/ml and 90.9 ng/ml. The recovery rates ($\pm$SD) are summarized in Table 17.

3.4. Removal of protein from plasma samples by precipitation

The precipitation method described here is useful to analyze human plasma samples. With our standard test mixture all other examined standard components could be detected selectively in spiked plasma, with the exception of norsalsolinol. In the case of norsalsolinol we observed a coelution with Tyr in the HPLC method applied. By changing the elution conditions it might be also possible to separate these analytes. In Fig. 4, chromatograms of spiked bovine serum albumin, plasma of a control person, and the same plasma spiked like the bovine serum albumin are shown as an example.

The high recovery rates, combined with the good selectivity (with the exception of norsalsolinol), shows that precipitation is indeed a suitable method for the analysis of THIQs and similar compounds in human plasma.

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