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Solid-phase microextraction with capillary gas–liquid chromatography and nitrogen–phosphorus selective detection for the assay of antidepressant drugs in human plasma

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Abstract

Solid-phase microextraction (SPME) was tested as a sample preparation for the simultaneous assay of ten antidepressant drugs and metabolites (TADs) in human plasma. Aqueous NaOH (0.5 ml, 1 *M*) and chloramitriptyline (50 μl, 40 μg/ml) as internal standard (I.S.) were added to a 2-ml plasma sample. This mixture was extracted with a 100-μm polydimethylsiloxane SPME fiber (Supelco) for 10 min. After washing in water and methanol (50%) and subsequent drying at room temperature, desorption of the fiber was performed in the injection port of a gas chromatograph at 260°C for 1 min (HP 5890, DB-17 30 m×0.25 mm I.D., 0.25 μm capillary; 0.7 ml/min nitrogen; nitrogen–phosphorus selective detection). The recovery was found to be very low from plasma (0.3% to 0.8%) but considerably higher from water (about 15%). Therefore, the high protein binding of antidepressants appears to be the main limiting mechanism for a better extraction. However, the analytes were well separated and the calibrations were linear between 125 ng/ml and 2000 ng/ml. The limits of quantification were about 90 ng/ml for imipramine and desipramine, 125 ng/ml for amitriptyline, trimipramine, doxepine, nortriptyline and mianserine and about 200 ng/ml for maprotiline, clomipramine and desmethylclomipramine. The recovery was improved by increasing the extraction time. The influence of the concentrations of the sum of proteins and of α-acid glycoprotein on the peak-area ratios $A_{TAD}/A_{I.S.}$ and on absolute peak areas was studied. Peak-area ratios increased with decreasing protein concentration but were found to be independent on α-acid glycoprotein. A simple model for the explanation of the effect is presented. Measures for the improvement of sensitivity are discussed. As presented in this paper, which first describes SPME for the analysis of drugs in plasma, SPME with a short extraction time can be of only very limited value for therapeutic drug monitoring. Lower concentrations than the limit of quantification are usually found at therapeutic doses. The method can be useful for toxicological analysis after the accidental or suicidal intake of higher doses. However, an about 10-fold improvement of the sensitivity of the method seems to be possible.

Keywords: Imipramine; Desipramine; Amitriptyline; Trimipramine; Doxepine; Nortripyline; Mianserine; Maprotiline; Clomipramine; Desmethylclomipramine

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1. Introduction

Solid-phase microextraction (SPME) is a novel sample preparation method for gas–liquid chromatography (GLC). A small fused-silica fiber is coated with polydimethylsiloxane or polyacrylate, therefore, an inverse GLC-capillary is used for extraction [1]. Hitherto, the method was described mainly for the analysis of aqueous samples of environmental pollution [1–3]. However, applications for drugs and their metabolites have been tested in human urine [4–6], but not in serum or plasma. Because of the physicochemical properties of the polymer (melting point: −50°C, glass transition temperature: −126°C) extraction with this method obeys the physicochemical rules of classical liquid–liquid equilibrium, also:

$$\text{drug}_{aq} \overset{K^f}{\rightleftharpoons} \text{drug}_{fiber}$$

$$K^f = \frac{c_f}{c_{aq}} \quad (1)$$

where K^f is the equilibrium constant of liquid–liquid equilibrium, c_f is the equilibrium concentration of analyte in the stationary phase of the fiber (the coating) and c_{aq} is the equilibrium concentration of the analyte in the aqueous matrix (environmental sample, human urine or plasma).

According to Eq. (1a), which can be derived from Eq. (1), it is evident that the number of molecules in the fiber, n_f, is linearly dependent on the initial number of analyte molecules n_0 in the sample (the target variable of analysis). Therefore, the basis for a quantitative method is given.

$$n_f = \frac{K^f V_f n_0}{(K^f V_f + V_{aq})} \quad (1a)$$

where n_f is the number of molecules in the fiber in equilibrium, n_0 is the initial number of molecules of analyte in the sample, V_f is the volume of the fiber, and V_{aq} is the volume of the aqueous sample.

The values K^f and relative values $K^{f,rel}$ of the polydimethylsiloxane–water system for organic compounds are in good agreement with equilibrium constants of octanol–water. They are high for lipophilic compounds, e.g., K^f(benzene)=125, K^f(*p*-xylene)=831 [7]. The equilibrium is influenced by pH, salts and temperature. Because of the low volume of the coating SPME is an extraction with low capacity. However, this will hardly be a problem in trace analysis.

The extraction process was mathematically described using two models which were derived from two limiting cases: (1) perfectly agitated analyte solution and (2) unstirred analyte solution [7]. According to both models the sensitivity of the method was found to be proportional to K^f and the volume of the polymer coating of the fiber, V_f. As a result of both theoretical models and also of experimental data obtained under real conditions, therefore, where the results of both models have to be taken into account, the mass of analyte extracted is linearly proportional to the concentration of the analyte in solution not only for equilibrium but also for every non-equilibrium time-point of extraction. This is important for quantitative analyses because the application of the method is extremely simplified. The main difference of both models is that the extraction speed is proportional to either the diffusion coefficient in the fiber, D_f, or the diffusion coefficient in the solution, D_{aq}, in the well-agitated model (model 1) and in the non-agitated model (model 2), respectively. On the other hand, extraction speed is independent of D_{aq} (model 1) and D_f (model 2). However, in real systems the speed of extraction is expected to be simultaneously dependent on both, D_f and D_{aq}. Furthermore, the lower the thickness of the coating the faster the extraction was found for both models. In general, the speed of extraction is considerably higher for stirred solutions. The time to reach equilibrium is decreased by increasing the speed of stirring. This was e.g., about 120 s for benzene at the highest speed of a commercial stirrer. The theoretical value for perfectly stirred solutions of 20 s could not be achieved [7]. It even lasted several hours for higher molecular mass organic compounds such as the triazine herbicides prometryn, terbutryn and simetryn [8].

Besides direct dipping and stirring of the fiber in the liquid matrix several headspace methods for volatile compounds were applied [9].

Advantages claimed for SPME compared to other sample preparation methods are (1) no use of solvents, (2) easy handling, (3) little equipment necessary, (4) fast method, (5) easy automation

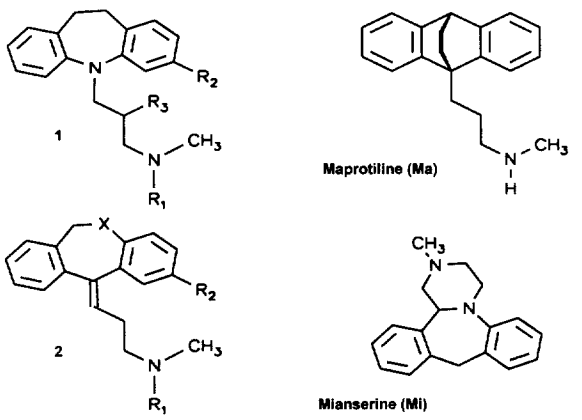
possible and (6) good linearity and high sensitivity for many analytes.

Antidepressant drugs are used in psychiatry for the therapy of depressive disorders, mainly endogenous major depression. For the group of the so-called tricyclic and tetracyclic antidepressant drugs (TADs, Fig. 1) distinct ranges of optimal plasma concentrations (therapeutic windows) were found. Lower and higher concentrations are associated with suboptimal or even no therapeutic effect. Furthermore, more severe adverse effects and even toxicity can appear at higher concentrations. Therefore, the measurement of TAD plasma levels is increasingly applied in clinical praxis (Therapeutic Drug Monitoring, TDM and clinical toxicology).

Gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) are established methods for the analysis of TADs in serum or plasma [10]. Liquid–liquid extraction and solid–liquid extraction are used for the sample preparation. However, the long time required for sample processing, for instance in liquid–liquid extraction, can be a problem. Recently, also methods without a special sample preparation (direct plasma injection) have been described. They are based on systems with restricted access materials (RAM), therefore, size-exclusion chromatography for the separation of proteins and drugs [11].

The fluorescence polarisation immunoassay (FPIA) shares the advantage of no sample preparation. However, the lack of sufficient specificity can cause, and even has caused, serious problems for the interpretation of pharmacokinetic/pharmacodynamic data in clinical research and in TDM. The cross-reactivity mainly occurs with phenothiazines and antihistamines, i.e., drugs often coadministered to TADs. Because of the very similar antibody the same problem also applies to enzyme linked immunoassay (EMIT). EMIT even needs sample preparation. Therefore, unlike for anticonvulsant drugs immunoassays only have a questionable value for TDM or toxicological screening of TADs.

The aim of the present work was to test SPME as a sample preparation method in the quantitative analysis by GLC of lipophilic drugs, such as TADs, in human serum or plasma. The influence of plasma proteins should be studied. The knowledge from environmental pollution analysis and human urine analysis provides evidence that SPME may be of advantage also for the analysis of drugs in plasma.



1

		R_1	R_2	R_3
Imipramine	**(Im)**	CH_3	H	H
Desipramine	**(De)**	H	H	H
Clomipramine	**(Cl)**	CH_3	Cl	H
Desmethylclomipramine	**(DMCl)**	H	Cl	H
Trimipramine	**(Tr)**	CH_3	H	CH_3

2

		X	R_1	R_2
Amitriptyline	**(At)**	CH_2	CH_3	H
Nortriptyline	**(Nt)**	CH_2	H	H
Internal Standard	**(I.S.)**	CH_2	CH_3	Cl
Doxepine	**(Do)**	O	CH_3	H

(ca. 85 % trans-doxepine (tDo) and 15 % cis-doxepine (cDo))

Fig. 1. Structure of tricyclic antidepressant drugs (TADs) and internal standard (I.S.).

2. Experimental

2.1. Chemicals

The internal standard (I.S.) chloramitriptyline·HCl was kindly donated by Lundbeck Laboratory (Kopenhagen, Denmark). Amitriptyline·HCl was from Rzeszowa Zhimizeska Fabrika, RZF (Rzeszow, Poland), nortriptyline·HCl was from Troponwerke (Cologne, Germany). Imipramine·HCl, desipramine·HCl and trimipramine·HCl were kindly donated by Arzneimittelwerk Dresden, AWD (Dresden, Germany). Clomipramine·HCl, desmethylclomipramine·HCl and maprotiline methanosulfate were kindly

donated by Ciba Geigy (Basle, Switzerland). Mianserine·HCl was from Diosynth (Oss, Netherlands) and doxepine·HCl was from ICN Biochemicals (Cleveland, OH, USA). NaOH p.a. was obtained from Merck. NaCl was obtained from Laborchemie Apolda (Apolda, Germany). Hexane p.a., methanol p.a. and iso-amyl alcohol p.a. were from Merck (Darmstadt, Germany). α_1-Acid glycoprotein (αAGP) (orosomucoid) was from Sigma (St. Louis, MO, USA). Drug-free plasma was taken from healthy volunteers. Human serum albumin was from Pharma Dessau (Dessau, Germany).

2.2. Solutions

The reference solution for ten analytes (10 μg/ml of each drug or metabolite, base) was prepared by mixing 10×1 ml of individual stock solutions of each drug or metabolite in water (100 μg/ml). Plasma was spiked with a volume of this 10 μg/ml reference solution plus a volume of water to keep a constant aqueous volume of 0.2 ml and adding 1.8 ml drug-free plasma from healthy volunteers, e.g., 100 μl reference solution + 100 μl water + 1.8 ml plasma yielding 500 ng/ml of each analyte. After vigorous shaking and standing for 10 min the sample was used for analysis. It should be kept in mind that this spiking means a dilution of the plasma sample with water by a factor of 10%. This may be important due to the influence of plasma proteins on calibration (see Section 2.8).

The internal standard solution for SPME (40 μg/ml) was prepared by diluting 1:2.5 a stock solution of 100 μg/ml chloramitriptyline-base (=111.6 μg/ml chloramitriptyline·HCl) in water. The reference and internal standard solutions were stored at 4°C. 1 *M* aqueous NaOH with 6% NaCl was prepared by dissolution of 20 g NaOH and 30 g NaCl in 500 ml water. A mixture of *n*-hexane–iso-amyl alcohol (98.5:1.5, v/v) was prepared by adding 1.5 ml iso-amyl alcohol to 98.5 ml *n*-hexane. Reference solutions of six and four analytes (10 μg/ml) and internal standard solutions (7.5 μg/ml chloramitriptyline and 7.5 μg/ml clomipramine) for the GLC reference method (standard method) were prepared analogously.

2.3. Sample preparation

In a 10-ml glass tube 50 μl internal standard solution (40 μg/ml chloramitriptyline) and 0.5 ml aqueous NaOH were added to 2 ml plasma and vortex-mixed for 10 s. About 1.5 ml was transferred to an autosampler vial (Hewlett-Packard; height 3 cm, diameter 1.1 cm) which fitted to a shaker (Thermomixer 5436, Eppendorf). The SPME-fiber (100-μm polydimethylsiloxane, Supelco, Bellefonte, USA) was placed in the vial by fixing the manual fiber assembly in a support. After 10 min shaking (700 rpm, 22°C) the fiber was washed 20 s with water and 20 s with 50% methanol. After 2 min drying at air and at room temperature the fiber was ready for injection to the chromatograph (desorption).

2.4. Apparatus and desorption

A Hewlett-Packard 5890 series II plus gas chromatograph equipped with a nitrogen–phosphorus detector (NPD) and a split-splitless injector port was used for the analysis. Separation was obtained with a 30 m×0.25 mm I.D., 0.25 μm DB-17 capillary (J&W Scientific) and N_2 (0.7 ml/min, automatic pressure control) as the carrier gas. Flow-rates of the detector gases were: air: 100 ml/min (275 kPa), H_2: 2.5 ml/min (100 kPa) and the auxiliary gas N_2: 27 ml/min (220 kPa). The NPD detector port was maintained at 300°C. The NPD was operated at a baseline of 25 to 30 pA. As an alternative detector system for SPME–GLC a mass spectrometer detector (MSD, HP 5972) was applied in the selective ion mode (SIM) and electron impact ionisation (EI, 70 kV): transfer-line temperature 300°C, ion source temperature 183°C, dwelling time per ion 100 ms. Six ions *m/z* were selected for the SIM: 58 (At, Tr, Im, cDo, tDo, I.S., Cl), 202 (At, Nt, Ma, I.S.), 249 (Tr, Mi), 193 (Tr, Im, Mi, De, DMCl), 277 (Ma) and 268 (Cl, DMCl). The injector was operated at 260°C in the splitless mode. After 1 min desorption the run was started by opening the split (30 ml/min) after 0.05 min. A temperature program was used for the oven (T_1=140°C, T_2=220°C, T_3=270°C, rate_1=20°C/min, rate_2=2°C/min). An integrator HP 3398 series II was used for the calculation of retention

times, peak widths and peak areas. After every analysis the fiber was cleaned by shaking it for 2 min in *n*-hexane–iso-amyl alcohol (98.5:1.5, v/v). Complete desorption of analytes, therefore, readiness for the next analysis was tested by a chromatogram.

2.5. Quantitation

Calibration curves were constructed by plotting the mean peak-area ratios (mean $A_{TAD}/A_{I.S.}$) of four measurements obtained from blank plasma spiked with the reference solution. The concentrations were 125 ng/ml, 250 ng/ml, 375 ng/ml, 500 ng/ml, 750 ng/ml and 1000 ng/ml. It should be noted that, strictly, due to the influence of protein concentration this calibration is valid only for plasma samples which have been 10% diluted by water.

2.6. Precision and accuracy

Precision was determined utilising the percentage coefficient of variation (C.V., %) of the within-day ($n=4$) and between-day ($n=4$) variations. Accuracy was assessed for amitriptyline, nortriptyline, imipramine and desipramine by means of a commercially available control serum (Lyphochek, BioRad).

2.7. Standard method

For the reference method a DB-35MS capillary (J&W Scientific) was applied. The injector was operated at 300°C in the split/splitless mode. The oven was operated isothermally at 250°C and the detector was held at 300°C. Because cDo and Im were not separated by this less polar phase and because it was the intention to use an alternative internal standard at least for part of the ten analytes, two variants of the reference method were used: variant 1 (six analytes: At, Im, Nt, Mi, De, Ma; clomipramine as I.S.), variant 2 (four analytes: Tr, Do, Cl, DMCl; chloramitriptyline as I.S.). The sample preparation by liquid–liquid extraction of the reference method has been described previously [12] with the difference that the last extract was reconstituted in 35 μl instead of 20 μl of solvent. Without further evaporation 2-μl aliquots were injected by an autosampler. The internal standard was 100 μl of 7.5 μg/ml clomipramine (instead of 1 μg/ml clomipramine [12]) for variant 1 and 100 μl 7.5 μg/ml chloramitriptyline for variant 2.

2.8. Influence of plasma protein

For the investigation of the influence of plasma proteins on the assay plasma from healthy volunteers was diluted with isotonic solution of NaCl in water. For the calculation of the concentrations of protein in these samples an initial concentration of $x_{pr}=7.5\%$ for the healthy volunteers was supposed. Albumin was added to obtain two samples with $x_{pr}>7.5\%$. Samples for the investigation of αAGP have been prepared by adding 4 mg, 8 mg and 10 mg of αAGP to plasma samples of 2 ml. For the calculation, an initial concentration of αAGP of 1 mg/ml was supposed.

3. Results and discussion

3.1. Chromatographic conditions and recovery

Fig. 2 shows typical chromatograms obtained from blank plasma, plasma spiked with 375 ng/ml of each of the ten analytes and a sample of a patient after suicidal intoxication with amitriptyline (At: 766 ng/ml, Nt: 489 ng/ml). A relatively long desorption time of $t_{des}=1$ min was found to be necessary with the 100 μm fiber and at a temperature of desorption of $T_{des}=260$°C. Shorter t_{des} did not provide complete desorption. However, due to the refocussing of the analytes on the "cold" capillary ($T_1=140$°C) sharp peaks with good resolution were obtained. The antidepressants, metabolites and I.S. are well separated with the DB-17 capillary (50% diphenylpolysiloxane, 50% dimethylpolysiloxane). The retention times t_R and relative retention times t_R^{rel} are listed in Table 1. No interfering peaks appeared in chromatograms of blank plasma from healthy volunteers. Signals from the matrix were mainly in the range $t_R<12$ min. The peaks at $t_R=15.877$ min and $t_R=16.710$ min consistently also appeared in the chromatograms of calibration plasma. They obviously did not interfere with one of the analytes. A very

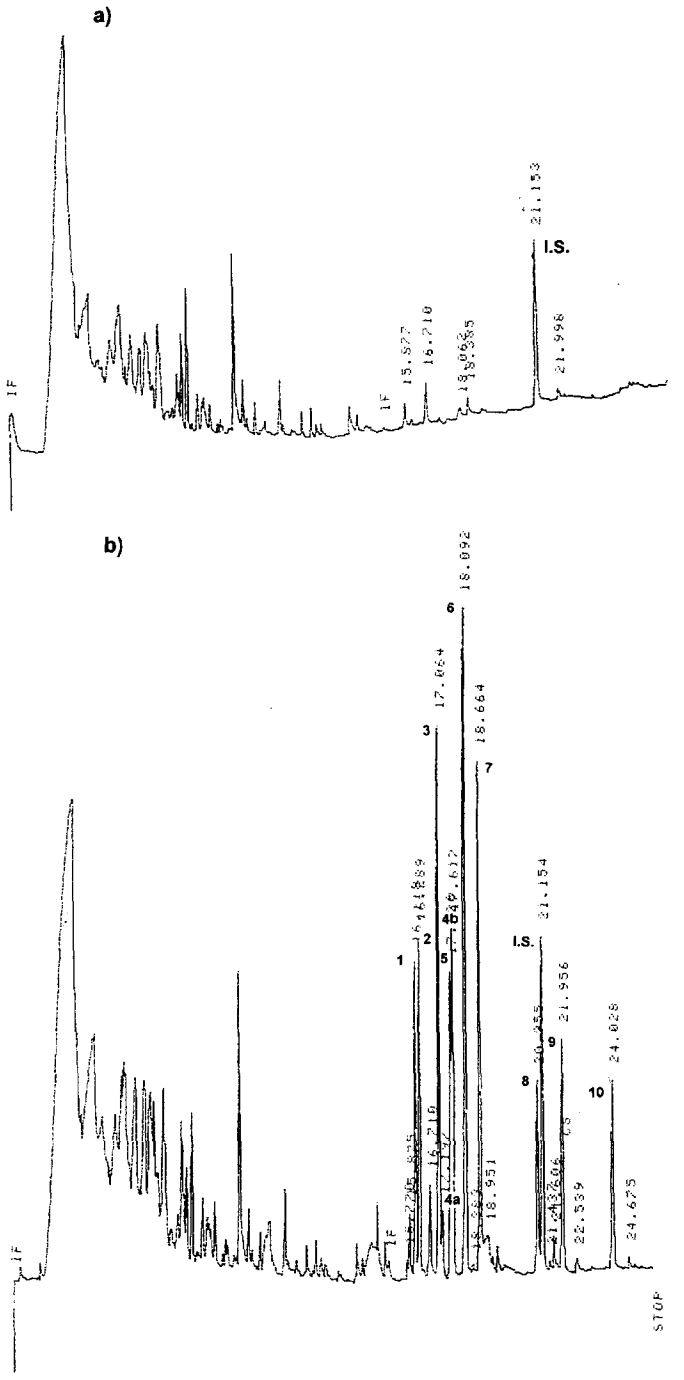


Fig. 2. Typical SPME–GLC–NPD chromatograms obtained from (a) blank plasma with I.S., (b) plasma spiked with ten antidepressant drugs and metabolites each 375 ng/ml and (c) a sample of a patient after suicidal intoxication with amitriptyline (At: 766 ng/ml, Nt: 489 ng/ml); Peaks: 1 = amitriptyline, 2 = trimipramine, 3 = imipramine, 4a = *cis*-doxepine, 5 = nortriptyline, 4b = *trans*-doxepine, 6 = mianserine, 7 = desipramine, 8 = maprotiline, I.S. = internal standard (chloramitriptyline), 9 = clomipramine, 10 = desmethylclomipramine.

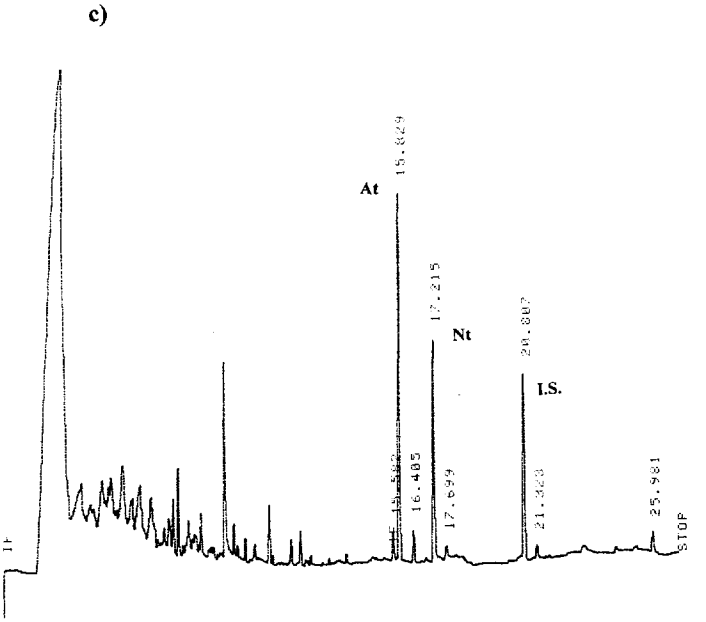


Fig. 2. (*continued*)

minor peak at $t_R = 18.062$ min occasionally appeared and interfered with mianserine. It did not affect the identification and quantification in the anticipated concentration range >125 ng/ml. In contrast, an insufficient separation of all analytes resulted with a less polar HP-5 capillary (5% diphenylpolysiloxane, 95% dimethylpolysiloxane). As an advantage, the time of analysis considerably decreased. Therefore,

Table 1
Retention time t_R, relative retention time t_R^{rel}, calibration parameters, limit of quantification and analytical recovery of ten antidepressant drugs and metabolites

TAD	t_R (min)	t_R^{rel}	Intercept *A*	Slope *B*	*r*	Limit of quantification (ng/ml)	Recovery (%)	
							Plasma	Water
At	16.118	0.762	0.0565	0.0021	0.989	125	0.46	13.7
Tr	16.289	0.770	0.0249	0.0023	0.992	125	0.34	10.9
Im	17.064[a]	0.807	−0.0196	0.0037	0.997	90	0.48	15.0
cDo	17.197	0.813	0.0312	0.0004	0.992	–	0.63	15.6
Nt	17.526[b]	0.828	−0.1036	0.0025	0.997	125	0.50	14.4
tDo	17.617	0.833	−0.0365	0.0026	0.999	125	0.64	15.7
Mi	18.092	0.855	0.1974	0.0054	0.998	125	0.82	16.1
De	18.664	0.882	−0.0287	0.0039	0.994	90	0.60	14.6
Ma	20.955	0.991	0.0129	0.0016	0.995	200	0.57	14.0
I.S.	21.154	1.000	–	–	–	–		
Cl	21.956	1.038	0.0119	0.0019	0.991	200	0.37	10.3
DMCl	24.028	1.136	−0.0127	0.0017	0.995	200	0.43	11.6

TAD = tricyclic and tetracyclic antidepressant drug, At = amitriptyline, Tr = trimipramine, Im = imipramine, cDo = *cis*-doxepine, Nt = nortriptyline, tDo = *trans*-doxepine, Mi = mianserine, De = desipramine, Ma = maprotiline, Cl = clomipramine, DMCl = desmethylclomipramine.

[a] R_s(Im, cDo) = 0.99, [b] R_s(Nt, tDo) = 0.70 (coefficient of separation).

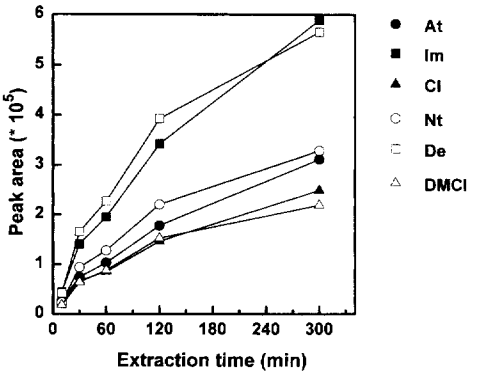


Fig. 3. Dependence of peak areas of six selected TADs on the time of extraction by SPME.

this may be an alternative for samples with well-known anamnesis where the identification of the TAD is not questioned.

The dependence of the peak areas of six exemplary TADs on the time of extraction is shown in Fig. 3. A curvilinear increase of peak areas is evident, but a plateau is not reached even after 5 h of extraction. The about 10-fold higher peak areas after 5 h compared to 10 min extraction time reflects the possible degree for an improvement of the method. However, because low extraction times of SPME are regarded as an advantage of the method in comparison to liquid–liquid and solid–liquid extraction, the method with 10 min of extraction time was further investigated. The peak-area ratios ($A_{TAD}/A_{I.S.}$) did not change up to 120 min extraction time as visible in Table 2. Differences were within the precision of the method as calculated by the percentage coefficient of variation (Table 3). Only after 5 h of extraction a slight decrease of peak-area ratios was found. It was most pronounced for mianserine which has the highest slope in the calibration curve.

Table 2
Peak-area ratios $A_{TAD}/A_{I.S.}$ in dependence on extraction time, relative values to $t=10$ min extraction time

TAD	Extraction time (min)				
	10	30	60	120	300
At	1.00	1.18	1.06	1.04	1.06
Tr	1.00	1.18	1.07	0.99	0.95
Im	1.00	1.24	1.15	1.15	1.15
cDo	1.00	0.96	0.88	0.84	0.78
Nt	1.00	1.07	1.08	1.07	0.92
tDo	1.00	1.17	1.10	1.04	0.95
Mi	1.00	1.02	1.01	0.90	0.76
De	1.00	1.01	1.09	1.08	0.90
Ma	1.00	0.95	0.99	0.97	0.81
Cl	1.00	1.13	1.07	1.05	1.04
DMCl	1.00	0.99	1.00	0.99	0.83
Mean	1.00	1.08	1.04	1.01	0.92
S.D.	0.00	0.10	0.07	0.09	0.12

Abbreviations as in Table 1, S.D. standard deviation.

The recoveries from plasma and water were calculated by comparison with the direct injection of the compounds to the chromatograph (Table 1). The recoveries from plasma are very low (<1%!) at 10 min extraction time. In contrast, considerably higher recoveries have been found for aqueous TAD solutions (10 to 16%). The recoveries from plasma are just about three to five percent of the recoveries from water. Protein precipitation either by perchloric acid or by uranyl acetate did only very slightly increase the recoveries from plasma. In the case where the remaining aqueous phase from plasma after protein precipitation was spiked with TADs the recoveries were comparable to the values of water. Therefore, the low recoveries from plasma can be explained by the high protein binding of TADs which is known to be about 85 to 98% at physiologic pH (At: 90–95%, Tr: 94–96%, Im: 88–93%, Nt: 94%, Do: 80–85%, Mi: 90%, De: 86%, Ma: 89%, Cl: 98%, DMCl: not found) [13,14]. Although a dependence of protein binding on pH is well-known for TADs [15], it can be assumed that binding is also high at strong basic pH as used for the extraction. Because an activation barrier exists for this association–dissociation reaction equilibrium this bound portion of drug does not seem to be accessible by SPME in a fast extraction. A liquid–liquid equilibrium only occurs with the free TADs. Eq. (1) applies only to the free concentration of drug c_{fr}. The bound drug is extractable from plasma water after a coupled but kinetically slow chemical reaction equilibrium:

$$\text{drug}_{aq} + \text{protein} \underset{\text{slow}}{\overset{K^{pr}}{\rightleftharpoons}} \text{drug}_{\text{protein}}$$

$$K^{pr} = \frac{c_b}{c_{fr}(S_V^0 - c_b)} \quad (2)$$

where K^{pr} is the equilibrium constant of association–

Table 3
Within-day precision of the assay at various concentrations (within-day C.V. of four measurements, %)

TAD	Concentration (ng/ml)						Mean±S.D.
	125	250	375	500	750	1000	
At	11.5	11.8	2.8	3.4	10.1	18.5	9.7±5.9
Tr	10.9	7.1	10.9	3.3	4.8	15.2	8.7±4.4
Im	7.3	1.9	7.1	7.6	3.6	16.5	7.3±5.1
cDo	44.6	8.1	2.8	9.9	5.3	15.8	14.4±15.4
Nt	13.5	3.8	3.5	12.0	5.6	31.1	11.6±10.5
tDo	6.4	4.1	3.7	11.9	4.7	17.7	8.1±5.6
Mi	7.1	4.2	4.3	5.8	5.0	25.8	8.7±8.4
De	16.5	6.8	14.6	13.2	12.0	14.1	12.9±3.3
Ma	26.7	5.2	2.0	9.9	3.9	17.4	10.8±9.5
Cl	6.1	11.6	7.2	5.0	5.9	17.1	8.8±4.7
DMCl	39.6	3.3	6.7	8.8	3.9	12.8	12.5±13.7
Mean±S.D.	17.3±13.7	6.2±3.3	6.0±3.9	8.2±3.5	5.9±2.7	18.4±5.4	

Abbreviations as in Table 1, S.D. standard deviation.

dissociation reaction equilibrium according to the global association function [16], c_b is the concentration of drug bound to plasma proteins, c_{fr} is the concentration of unbound drug, free concentration, and S_V^0 is the maximal number of vacant sites for drug binding per unit volume.

Besides this influence of protein binding on the recovery, diffusion has to be discussed, too. Compared with water, diffusion coefficients D_{aq} are smaller in the more viscous protein solution. Therefore, also the flux of drug into the fiber and the recovery at a distinct extraction time are decreased. However, according to the well-agitated model viscosity of the analyte solution has no influence on the extraction time and may be of minor influence also in a real stirred solution.

3.2. Calibration, precision and accuracy, selectivity

Calibrations were found to be linear for all analytes in the investigated range between 125 and 1000 ng/ml. Linearity was exemplarily confirmed also in a separate experiment for amitriptyline, nortriptyline and mianserine up to 2000 ng/ml. The parameters of the calibration curves were the same either with simultaneous measurement of ten TADs or measurement of three TADs alone. Therefore, the calibration of an individual TAD was not affected by the coextraction of other TADs. Calibration curves were typically described by the formula $y=Bx+A$, where y is the peak-area ratio $A_{TAD}/A_{I.S.}$, x is the concentration of TAD and A and B are the intercepts and slopes as shown in Table 1, respectively. The lower limits of quantification at a signal-to-noise ratio of 9 are arranged in the order Im, De<At, Tr, Nt, tDo, Mi<Ma, Cl, DMCl (Table 1). Due to the highest slope in the calibration curve mianserine could have the lowest limit of quantification. However, this is compensated by the higher degree of chemical noise (a small interfering peak).

Precision as determined by the percentage coefficient of variation of the within-day variations at six concentrations between 125 ng/ml to 1000 ng/ml ($n=4$ each) is shown in Table 3. In the range from 250 ng/ml to 750 ng/ml the precision was consistently about 1.9% to 14.6%. The decreased precision at 125 ng/ml indicates the limitations of the method due to sensitivity. The lower precision at 1000 ng/ml may be an artefact due to the ageing of the fiber during the experiments (1000 ng/ml samples were measured as the last with one fiber). Therefore, with a fairly new fiber the precision at 1000 ng/ml is expected to be the same as between 250 ng/ml to 750 ng/ml. A more general view can be derived from the means of precision at a given concentration for all ten TADs. This appears to be reasonable because of the very similar physicochemical properties of each representative of this class of drugs. The conclusions drawn above are confirmed

Table 4
Between-day precision of the assay at 375 ng/ml of an internal control plasma and accuracy assessed by an external control serum (Lyphochek, BioRad)

TAD	Between-day precision C.V., $n=4$ (%)	Accuracy			
		Content[a] (ng/ml)	Acceptable range[a] (ng/ml)	Found (ng/ml)	Accuracy (%)
At	3.2	236	189–283	190	80.5
Tr	5.2				
Im	5.9	270	216–324	216	80.0
cDo	12.0				
Nt	14.7	242	193–290	257	106.2
tDo	3.8				
Mi	5.8				
De	14.0	260	208–311	240	92.3
Ma	15.5				
Cl	7.3				
DMCl	13.7				

Abbreviations as in Table 1.
[a] As given by the manufacturer, mean value of independent laboratories measured by HPLC, C.V. coefficient of variation.

but easier to overlook by the reader. However, some differences are evident for the means of precisions through all concentrations for each single TAD. Secondary amine TADs (Nt, De, Ma, DMCl) consistently have mean precisions >10% (C.V.) but tertiary amine TADs (At, Tr, Im, Do, Mi, Cl) have better values <10% (C.V.). This is an experience also from classical liquid–liquid extraction with GLC-analysis of TADs and can be explained by the potency to form H-bonds of the secondary amine compounds.

The data of the between-day variation are shown in Table 4. The precision is in the range 3.2 to 15.5%. The between-day precision of tertiary amine TADs is consistently better than of secondary amine TADs. The accuracy as tested by an external control serum was within the acceptable ranges. These ranges have been chosen by the producer of the control serum and are based on the mean±20% of the values of independent laboratories which have used HPLC methods (Table 4). However, amitriptyline and imipramine were just at the lower border of the acceptable range.

The method was tested for selectivity with regard to several classes of psychotropic drugs: other TADs (dibenzepine, opipramol, mirtazapine), a selective serotonine reuptake inhibitor (fluoxetine), phenothiazine neuroleptics (promethazine, chlorpromazine, levomepromazine, thioridazine, flufenazine), a thioxanthene neuroleptic (chlorprothixene), a butyrophenone neuroleptic (haloperidol), the important atypical neuroleptic clozapine, benzodiazepines (diazepam, oxazepam etc.) and zolpidem (Table 5). No interfering drug was detected. Therefore, the method is selective as it is necessary for the safe identification and quantification of multiple TADs. A false-positive result caused by these often coadminis-

Table 5
Retention times t_R and relative retention times t_R^{rel} of potentially interfering drugs

TAD	t_R (min)	t_R^{rel}
I.S.	21.15	1.00
Fluoxetine	8.10	0.38
Dibenzepine	27.35	1.29
Opipramol	no signal	–
Mirtazapine	20.02	0.95
Citalopram	21.66	1.02
Desmethylcitalopram	23.60	1.12
Promethazine	19.64	0.93
Levomepromazine	26.78	1.27
Chlorpromazine	25.69	1.21
Chlorprothixene	22.75	1.08
Thioridazine	no signal	–
Flufenazine	no signal	–
Clozapine	no signal	–
Haloperidol	no signal	–
Diazepam	27.13	1.28
Alprazolam	no signal	–
Oxazepam	22.80	1.08
Zolpidem	no signal	–

Table 6
Comparison with standard method, measurement of eight identical samples between 200 ng/ml and 900 ng/ml of TAD by both methods and analysis by linear regression (SPME results plotted in the abscissa)

TAD	A	B	r
At	−0.9	0.90	0.992
Tr	−36.3	1.02	0.986
Im	0.0	0.96	0.995
cDo	−2.4	0.90	0.965
Nt	38.0	0.94	0.986
tDo	−16.8	0.94	0.966
Mi	27.6	0.96	0.994
De	48.1	0.90	0.982
Ma	47.0	0.91	0.990
Cl	4.5	0.91	0.989
DMCl	−6.9	1.12	0.983

Abbreviations as in Table 1; A = intercept, B = slope, r = correlation coefficient.

tered to TADs drugs can be excluded. This is an important advantage over FPIA or EMIT assays of TADs.

The method was compared with a standard method which is based on traditional liquid–liquid extraction and GLC–NPD analysis [12]. A different capillary and a different internal standard were used. The standard method was shown to have an accuracy of 94 to 105% and a precision of <12% (C.V.). Eight identical samples between 200 ng/ml and 900 ng/ml of each TAD were measured with both methods and the results have been analysed by linear regression. With the SPME method slightly elevated results were obtained. However, as can be seen in Table 6 a very good agreement was found as indicated by slopes $B = 0.9$ to 1.0 (1.1), correlation coefficients $r > 0.96$ and intercepts A close to the original. Thus, the accuracy of the novel SPME–GLC–NPD method has been confirmed. A very good agreement was obtained also for a sample of a patient after suicidal intoxication with amitriptyline (Fig. 2c). The values were for SPME: At 766 ng/ml and Nt: 489 ng/ml vs. values for standard method: At: 731 ng/ml and Nt: 441 ng/ml. Deviations are below 10%.

3.3. Mass spectroscopy as alternative detection

The method was tested whether mass spectroscopy can be applied as an alternative detector. This should be of advantage for the analysis of toxicological samples because of the higher selectivity of this detector system. The chromatographic conditions were identical unless helium instead of nitrogen was used as the carrier gas. The mass spectrometer was used in the single ion monitoring mode (SIM) with electron impact ionisation (EI). For the tertiary amine TADs, m/z 58 was the unanimously used ion for detection. For the secondary amine TADs and mianserine five selected ions as indicated in Section 2.4 were used. A typical chromatogram is shown in Fig. 4. All TADs were chromatographically separated also with helium as the carrier gas. The

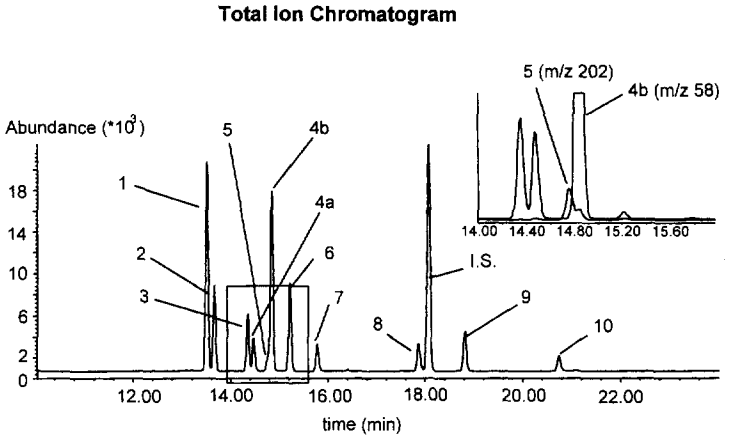


Fig. 4. Typical SPME–GLC–MSD chromatogram obtained from plasma spiked with ten antidepressant drugs and metabolites each 375 ng/ml. Peaks: 1 = amitriptyline, 2 = trimipramine, 3 = imipramine, 4a = *cis*-doxepine, 5 = nortriptyline, 4b = *trans*-doxepine, 6 = mianserine, 7 = desipramine, 8 = maprotiline, I.S. = internal standard (chloramitriptyline), 9 = clomipramine, 10 = desmethylclomipramine.

separation of Nt and tDo seems to be decreased. This is caused by the great difference of peak-heights. However, Nt and tDo could be easily separated by the different ion traces (Nt: *m/z* 202, tDo: *m/z* 58). In contrast to GLC–NPD the sensitivity with GLC–MSD is considerably more differentiated between the single analytes. In general, tertiary amine TADs are detected considerably more sensitive than secondary amine TADs because of the high intensity of the dimethylamine methylene cation base-peak in the mass spectra [$(CH_3)_2NCH_2^+$, *m/z* 58].

3.4. Influence of plasma proteins

Besides water and electrolytes human plasma of healthy individuals consists of 7 to 8% of plasma proteins. Albumin is the main component with about 55% of proteins. An important component with regard to protein binding of TADs is αAGP which composes just about 1% of plasma proteins. However, for comparison, albumin binds with low effectivity but has high capacity whereas αAGP binds with high effectivity but has low capacity. There are only very minor differences between plasma and serum. Serum proteins are almost the same as plasma proteins, i.e., plasma proteins minus fibrinogen and some clotting factors (2.5 to 5% of proteins). Therefore, it is concluded that the method can be applied in serum and plasma samples.

As discussed above protein binding considerably influences the recovery of SPME in plasma or serum. Deviations from normal in the content of proteins in plasma can, therefore, change the absolute peak areas in chromatograms and cause serious bias of the analytical method. Pathological changes of plasma albumin occur in hepatic and nephrotic diseases. The content of albumin can be decreased to about 50% of normal. On the other hand, e.g., in inflammation diseases the content of αAGP can be increased by a factor of up to about six. In contrast to albumin the concentration of αAGP can also vary in healthy individuals by a factor of about three [17]. However, this serious problem is circumvented if an internal standard with very similar physicochemical properties is used. This applies to the internal standard as used in the present method because chloramitriptyline is a chemical analog of the group of TADs. Another important advantage of chloramitriptyline is that this compound is not used as an TAD and can, therefore, be excluded as an interfering substance in human plasma. This application of an internal standard in drug analysis is an important difference compared to the analysis of environmental pollution because "taylor-made" internal standards are easier available and the number of possible interfering chemicals is lower.

Nevertheless, because of minor differences of protein binding of TADs and I.S. little changes of the calibration curve can be expected with changes of the composition of the matrix, even for an internal standard method. Briefly, a theoretical examination of this problem may be performed as follows: in equilibrium Eqs. (3) and (4)

$$K^{f} = \frac{c_f}{c_{fr}} = \frac{n_f \cdot V_{pl}}{n_{fr} \cdot V_f} \tag{3}$$

$$K^{pr} = \frac{c_b}{c_{fr}(S_V^0 - c_b)} = \frac{n_b \cdot V_{pl}}{n_{fr} \cdot (n_V^0 - n_b)} \tag{4}$$

with n_f is the number of drug molecules in the fiber (in mol), n_{fr} is the number of drug molecules in plasma water, n_b is the number of drug molecules bound to the protein, V_{pl} is the volume of plasma, V_f is the volume of polymer coating; n_V^0, the maximal number of vacant sites for drug binding, can be derived from Eqs. (1) and (2) because of:

$$c_f = n_f / V_f \tag{5}$$

$$c_{fr} = n_{fr} / V_{pl} \tag{6}$$

$$c_b = n_b / V_{pl} \tag{7}$$

$$S_V^0 = n_V^0 / V_{pl} \tag{8}$$

Eq. (4) is in agreement with the association–dissociation reaction equilibrium for drugs and proteins according to the global association function [16]. The discrete values of the apparent association constants for several groups of proteins of the classical models (e.g., the usual Scatchard's equation [18]) are replaced in the sense of an apparent overall equilibrium and, therefore, can be more easily described mathematically. However, the main assumption of a rigid structure of proteins with a definite

number of preformed binding sites is made in both models.

From Eq. (4) the amount of drug bound to the protein is:

$$n_b = \frac{n_V^0}{\frac{V_{pl}}{K^{pr} n_{fr}} + 1} \tag{9}$$

Introducing the sum of drug molecules in each of the three phases, n_0, as given by Eq. (10)

$$n_0 = n_f + n_b + n_{fr} \tag{10}$$

$$n_0 - n_{fr} - n_f = \frac{n_V^0}{\frac{V_{pl}}{K^{pr} n_{fr}} + 1} \tag{11}$$

Eq. (11) resulted. Note that n_0 is also the number of drug molecules in plasma before extraction and, therefore, the target variable of the analysis. With an expression for the number of free drug molecules (Eq. (12)), which is available from Eq. (3), and substitution of n_{fr} in Eq. (11), Eq. (13) can be derived.

$$n_{fr} = \frac{n_f \cdot V_{pl}}{K^f \cdot V_f} \tag{12}$$

$$\left(n_0 - \frac{n_f \cdot V_{pl}}{K^f \cdot V_f} - n_f\right) \cdot \left(\frac{K^f \cdot V_f}{K^{pr}} + n_f\right) = n_V^0 \cdot n_f \tag{13}$$

By multiplication and setting

$$A = \frac{K^f V_f}{K^{pr}} \tag{14}$$

and

$$B = \frac{V_{pl}}{K^f V_f} \tag{15}$$

a quadratic equation for the drug in the polymer coating of the fiber, n_f, is obtained:

$$-An_0 - n_0 n_f + \frac{n_f V_{pl}}{K^{pr}} + n_f^2 B + n_f A + n_f^2 + n_V^0 n_f = 0 \tag{16}$$

Summing up n_f^2 and n_f, dividing by $(B+1)$ and using the expression Eq. (17) yields Eq. (18).

$$b = \frac{\left(n_0 - (V_{pl}/K^{pr}) - A - n_V^0\right)}{2(B+1)} \tag{17}$$

$$n_f^2 - 2bn_f = \frac{An_0}{(B+1)} \tag{18}$$

By solving the quadratic equation a dependence of the number of drug molecules in the fiber, n_f, on n_0 and on the content of plasma proteins $x_{pr} = f(n_V^0)$ is given. (As can be easily shown the second result after extracting the root gives a physically meaningless negative n_f):

$$n_f = \frac{n_0}{2(B+1)} - \frac{(V_{pl}/K^{pr}) + A + n_V^0}{2(B+1)} + \sqrt{\frac{A \times n_0}{(B+1)} + \frac{\left(n_0 - (V_{pl}/K^{pr}) - A - n_V^0\right)^2}{4(B+1)^2}} \tag{19}$$

It should be noted that the first term of Eq. (19) equals half of the expression of Eq. (1a).

A linear dependence of the number of binding sites on the portion of protein in plasma is supposed:

$$n_V^0 = u \cdot x_{pr} = u \cdot m_{pr}/m_{pl} \tag{20}$$

where u is the proportional factor for binding sites per concentration of protein; x_{pr} is the concentration of protein in plasma as mass of protein per mass of plasma; m_{pr} is the mass of protein and m_{pl} is the mass of plasma.

With Eq. (19) for the peak-area ratio $A_{TAD}/A_{I.S.}$ the following complex formula can be derived:

$$A_{TAD}/A_{I.S.} = f\left(\frac{n_f^a}{n_f^{is}}\right) = \frac{F_{GC}^a\left(\frac{n_0^a - u\cdot x_{pr} - A^a - (V_{pl}/K^{pr,a}) + \sqrt{4(B^a+1)A^a n_0^a - (V_{pl}/K^{pr,a})^2 - n_0^a + A^a + u\cdot x_{pr})^2}}{2\cdot(B^a+1)}\right)}{F_{GC}^{is}\left(\frac{n_0^{is} - u\cdot x_{pr} - A^{is} - (V_{pl}/K^{pr,is}) - \sqrt{4(B^{is}+1)A^{is} n_0^{is} - ((V_{pl}/K^{pr,is}) - n_0^{is} + A^{is} - u\cdot x_{pr})^2}}{(2B^{is}+1)}\right)} \tag{21}$$

F_{GC}^a and F_{GC}^{is} are the experimental factors of the GLC equipment connecting mass of drug and peak area. The values A^a, A^{is} etc., indicate the individual constants from Eq. (19) for the analyte and internal standard, respectively.

It should be emphasised that several assumptions are made if this equation is applied: e.g., (1) preformed sites for drug binding at the protein, (2) linear dependence of the number of sites on the concentration of protein in plasma, (3) the direct interaction of proteins and bound drug with the fiber was neglected. This appears to be reasonable because of the low concentration of protein (<8%), (4) neglect of the competition of TAD and I.S. for the binding sites at the protein, (5) no change of equipment factors F^{a}_{GC} and F^{is}_{GC} with increasing n^{a}_{f} and n^{is}_{f}, (6) this equation was derived for equilibrium, but equilibrium may not be achieved. As discussed above, it has been shown that the ratio of peak areas remains unchanged during extraction. However, for Eq. (21) it has to be assumed further that the diffusion coefficients of TADs and I.S. have the same dependence on viscosity, i.e., on the portion of protein in plasma, x_{pr}.

Two examples of theoretical calculations with Eq. (21) are demonstrated graphically in Fig. 5. The volume of polymer was calculated to $V_f = 6.6 \cdot 10^{-7}$ l, a volume of plasma $V_{pl} = 0.002$ l was used. The initial amounts of analyte and I.S. are $n^{a}_{0} = 2.71$ nmol (e.g., 375 ng/ml At in 2 ml) and $n^{is}_{0} = 6.43$ nmol (e.g., 2000 ng chloramitriptyline as in the present method), respectively. With a molecular mass of 60 000 g/mol (albumin) as a mean value for proteins and hypothetically ten binding sites per molecule of protein (compare e.g., [19]) $S^{0}_{V} = 0.0125$ M and $u = 3.33 \cdot 10^{-4}$ mol can be calculated. A hypothetical $K^{f} = 12121.2$ was used, therefore, a recovery from plasma water in equilibrium of 80% was assumed (about 10 to 16% were achieved from water after 10 min of extraction). For the calculations, e.g., for a ratio 95:5 bound to free drug and with $S^{0}_{V} = 0.0125$ M a theoretical $K^{pr} = 1520$ M^{-1} can be estimated by means of Eq. (2). Due to the high capacity for drug binding $S^{0}_{V} >> c_{b}$ was also assumed. The ratio of bound to free I.S. was set to 95:5 in Fig. 5a and 50:50 in Fig. 5b. The shares of curves in Fig. 5 show the dependence of peak-area ratios according to Eq. (21) for drugs which are more, equal or less bound than the I.S. to plasma proteins. As expected, the peak-area ratio does not change for drugs with the same plasma binding as the internal standard. Drugs which are less bound to proteins have higher peak-area ratios. They are decreasing with pathologically decreasing plasma proteins. Drugs which are more bound to proteins have a lower peak-area ratio and increasing peak-area ratios with decreasing plasma proteins. It should be recognised, that in dependence on individual parameters of each drug and the I.S., the proportional change of peak-area ratios can be low in the interesting for practical problems range $x_{pr} = 4$ to 8%. The effect can be limited mainly to very low values x_{pr}. For the present example, the proportional change in the interesting range between 4 to 8% of plasma proteins is low for highly bound drugs (Fig. 5a) but more pronounced for less bound drugs (Fig. 5b). Note that equal factors F^{a}_{GC} have been used in the theoretical examples. Therefore, in

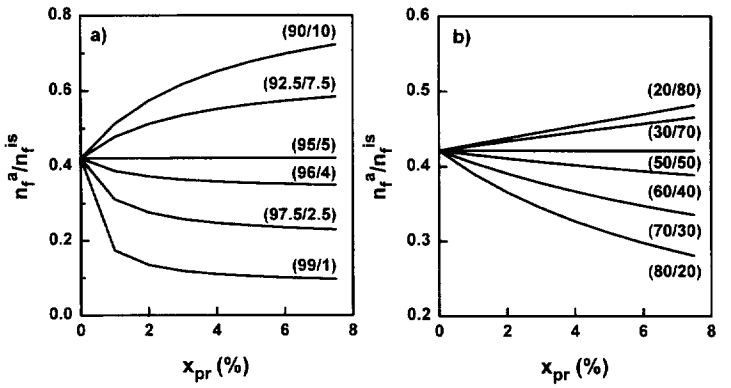


Fig. 5. Graphical demonstration of the theoretical dependence of peak-area ratios on protein concentration according to Eq. (21), (a) hypothetical highly bound drugs and I.S. as indicated in parenthesis with a constant ratio 95:5 bound to free drug for the I.S. and (b) hypothetical low bound drugs and I.S. as indicated in parenthesis with a constant ratio 50:50 bound to free drug for the I.S.

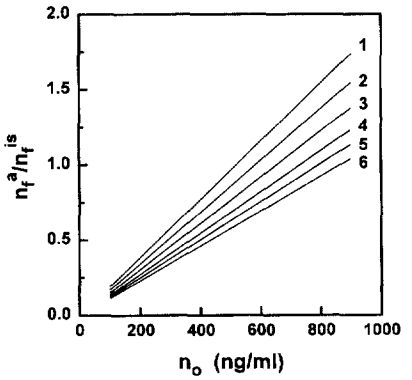


Fig. 6. Theoretical dependence according to Eq. (21) of the peak-area ratio on the amount of analyte n_0, $n_0=100$ to 900 ng/ml equals a range of 0.7 to 6.5 nmol of At, concentration of protein as indicated: 1: $x_{pr}=7.5\%$, 2: $x_{pr}=3.75\%$, 3: $x_{pr}=2.0\%$, 4: $x_{pr}=1.0\%$, 5: $x_{pr}=0.5\%$, 6: $x_{pr}=0.1\%$, ratio bound to free drug as in Fig. 5a: I.S.: 95:5, TAD: 90:10.

contrast to experimental curves of different TADs the theoretical curves end in one point at $x_{pr}=0$.

Eq. (21) describes a nonlinear dependence of peak-area ratios on n_0 at a given x_{pr}. However, a linear dependence has been found for all ten analytes in the experimental calibration. A calculation of theoretical peak-area ratios also provides linear relationships between n_f^a/n_f^{is} and n_0 for various x_{pr} (Fig. 6, parameters as in Fig. 5a). Therefore, it can be concluded that, in spite of the problems due to protein binding, the basis for a quantitative method is given for the present analytes and I.S.. Nonlinear calibrations may be found for other analytes or for larger ranges of concentration of analyte.

Experimental data of the dependence of the peak-area ratios of TADs (375 ng/ml each) on the concentration of protein in plasma is shown in Fig. 7. All TADs, except cDo, exhibited linearly increased peak-area ratios at decreased x_{pr}. Linear correlations revealed fairly high correlation coefficients $0.77<r<0.91$ (Table 7). No difference occurred between secondary and tertiary amine TADs. In accordance with Eq. (21) it can be concluded that, at the conditions of extraction (strong basic), the protein binding of the internal standard is lower than of the TADs. Only minimal changes of calibration occur within normal variations of plasma proteins between 7% to 8% and the method presented in this paper will be valid for the majority of samples. However, it is evident that the method is not robust with regard to extreme changes of the protein concentration in plasma. Pathological deviations from normal of the composition of plasma considerably interfere with

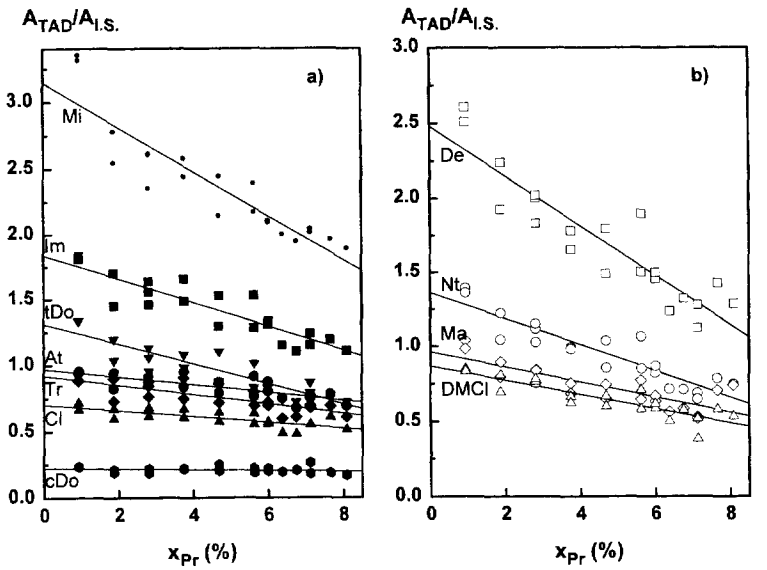


Fig. 7. Experimental dependence of peak-area ratios of ten TADs (375 ng/ml each) on the concentration of protein in plasma, (a) tertiary amine TADs, (b) secondary amine TADs.

Table 7
Results of linear regression of the dependence of peak-area ratios $A_{TAD}/A_{I.S.}$ on protein concentration x_{pr} in plasma, %Diff(7.5→3.75) percent difference of peak-area ratios at normal (x_{pr}=7.5%) compared to extremely pathological (x_{pr}=3.75%) protein concentration

TAD	A	B	r	%Diff (7.5→3.75)
At	0.97	−0.030	0.781	14.8
Tr	0.92	−0.036	0.791	20.4
Im	1.84	−0.091	0.895	29.5
cDo	0.22	−0.003	0.294	5.8
Nt	1.36	−0.088	0.914	47.0
tDo	1.31	−0.076	0.898	38.0
Mi	3.15	−0.168	0.911	33.2
De	2.48	−0.167	0.911	51.0
Ma	0.96	−0.051	0.817	32.4
Cl	0.70	−0.023	0.776	15.8
DMCl	0.87	−0.048	0.878	34.8

Abbreviations as in Table 1; A=intercept, B=slope, r=correlation coefficient.

the method for some TADs (compare percent difference of peak-area ratios for x_{pr}=7.5% vs. x_{pr}=3.75% in Table 7). Values measured are e.g., about 50% above normal for Nt and De, whereas the deviations for At and Cl are low (about 15%). Although this problem will only occur in very few patients, an accurate assay of the concentration of the drug is only possible after additional information on the plasma protein concentration of the sample. Results for patients with a hepatic or renal disease must be regarded with caution. On the other hand, this dependence on plasma proteins can bear advantages. First, the action of TADs is more correlated with the free concentration in plasma water than with the sum plasma level (free plus bound) because the concentration in the central nervous system is better correlated with free concentrations. Therefore, the experimental deviation towards higher serum levels at decreased plasma proteins may even better describe the pharmacological action of the drug. However, a theoretical treatment of the relationship of SPME measured peak-area ratios and free drug concentration is necessary for an exact discussion of this problem, but was outside the scope of the present paper. Second, absolute peak areas are increased at lower concentrations of plasma proteins. Thus, adding of water to the sample, i.e., a simple "sample preparation step" by the "extraction of proteins with water", should be a way to increase the sensitivity of the method. Furthermore, also the robustness of the method due to changes of plasma proteins of the sample would decrease.

The experimental dependence of absolute peak areas on the content of plasma proteins is shown in Fig. 8a. A 5-fold dilution of the sample by water, for example, yields an about 4-fold increase of the sensitivity of the assay. Simultaneously, for De with the strongest dependence of peak-area ratios on plasma proteins, pathological changes of plasma proteins would result to only a difference of 5.6%. Therefore, deviations of the calibration are within the precision of the method. The theoretical dependence of peak areas (expressed by n_f) according to Eq. (19)

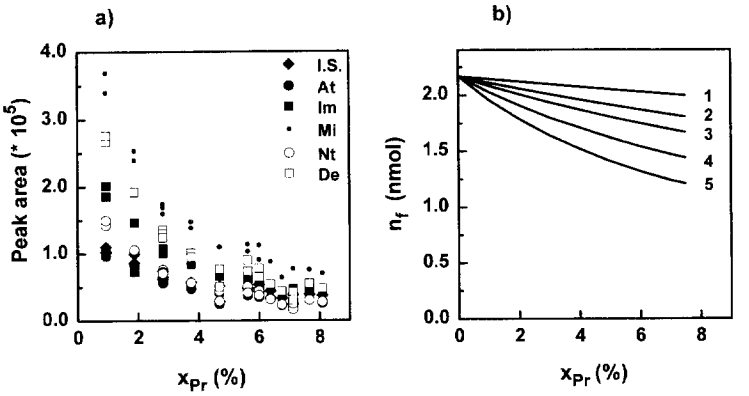


Fig. 8. (a) Experimental dependence of absolute peak areas of TADs (375 ng/ml each) and I.S. on the concentration of protein in plasma, (b) graphical demonstration of the theoretical dependence of n_f on protein concentration according to Eq. (19), hypothetical low bound drugs with ratio bound to free drug as indicated: 1: 20:80, 2: 50:50, 3: 60:40, 4: 70:30, 5: 80:20.

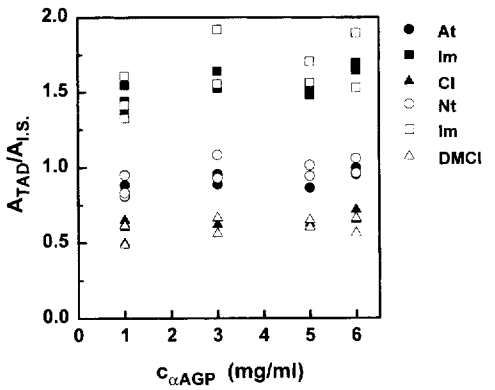


Fig. 9. Scatterplot of peak-area ratios of six selected TADs (375 ng/ml each) with dependence on the concentration of αAGP.

is shown in Fig. 8b. The chosen variables were as in Fig. 5b. However, it should be taken into consideration that the agreement between theoretical and experimental data will be lower for absolute peak areas than for peak-area ratios. It can be assumed that to some degree absolute peak areas are also influenced by the decrease of viscosity, i.e., the diffusion in aqueous solution is increased with decreasing protein concentration. According to the non-stirred model this results in enhanced peak areas. Furthermore, it should be noted that in the share of theoretical curves equal factors of the GLC-equipment F^{a}_{GC} have been supposed for each TAD.

Table 8
Results of linear regression of the dependence of peak-area ratios $A_{TAD}/A_{I.S.}$ on the concentration of αAGP in plasma, p-value as measure of significance, %Diff(1→6) percent difference of peak-area ratios at normal (1 mg/ml) compared to extremely pathological (6 mg/ml) αAGP-concentration

TAD	A	B	r	p	%Diff(1→6)
At	0.867	0.014	0.517	0.154	7.9
Tr	0.806	0.013	0.733	0.025	7.9
Im	1.440	0.031	0.644	0.061	10.5
cDo	0.174	0.005	0.651	0.058	14.0
Nt	0.861	0.027	0.631	0.069	15.2
tDo	1.025	0.020	0.549	0.126	9.6
Mi	2.313	0.045	0.395	0.291	9.5
De	1.450	0.046	0.494	0.177	15.4
Ma	0.634	0.019	0.599	0.088	14.5
Cl	0.614	0.008	0.446	0.228	6.4
DMCl	0.528	0.018	0.559	0.118	16.5

Abbreviations as in Table 1; A=intercept, B=slope, r=correlation coefficient.

Therefore, in contrast to the experimental data the curves end in one point at $x_{pr}=0$.

Because of the well-known influence of αAGP on the protein binding of TADs [20], this dependence was also studied in an experiment. As can be seen in Fig. 9 only a very low difference of the peak-area ratios was found between normal concentrations (about 1 mg/ml) and pathologically increased concentrations of αAGP (up to 6 mg/ml). The results of linear regression are shown in Table 8. A significant linear dependence was found only for one TAD (Tr). However, the increase due to the change of αAGP-concentration was only 7.9%. It is concluded that the influence of αAGP-binding of TADs can be neglected in the present SPME method. This result may be explained by the strong basic medium where the extraction is performed.

4. Conclusions

In principle, SPME is an alternative to traditional liquid–liquid and solid–liquid extraction for the sample preparation for GLC analysis of basic, lipophilic drugs in plasma. This is the main result of the first application of this novel sample preparation method for drug analysis in plasma. However, because of limitations due to low analytical recovery and, therefore, low sensitivity an application just in toxicological problems seems to be possible. The plasma levels found after normal doses in a therapeutic drug monitoring programme are too low for this, at present, insensitive method. The mechanism responsible for the low recovery is the high protein binding of TADs. Furthermore, extreme changes from normal in the matrix, i.e., very low protein concentrations due to pathological alterations of protein metabolism can interfere with the method. Values of calibration samples up to 50% above normal have been found for some analytes. However, the sensitivity of SPME–GLC–NPD can be considerably improved by dilution of serum samples with water. This way, also the problem resulting from changes of the matrix is circumvented. Other measures for an improvement of sensitivity are optimising of the rate of stirring and improving the dimensions of the fiber (e.g., 3 cm long instead of 1 cm but 3-fold decreased thickness). Altogether, it is

predicted that the sensitivity can be improved by a factor of about 10 to 12. Then, a high sensitive and selective method with very fast sample preparation for the analysis of tricyclic antidepressant drugs in human plasma or serum will be provided. The sensitivity could also be improved by increasing the extraction time (about 3- to 10-fold). However, then the main advantage of SPME, the short time of sample preparation, is abandoned.

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