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Stir bar sorptive extraction-thermal desorption-capillary GC–MS for profiling and target component analysis of pharmaceutical drugs in urine

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Abstract

Stir bar sorptive extraction (SBSE) in combination with thermal desorption (TD) on-line coupled to capillary gas chromatography–mass spectrometry (CGC–MS) was applied to the analysis of pharmaceutical drug compounds and metabolites in urine. SBSE implies stirring of the aqueous sample (urine, blood, etc.) with a glass stir bar coated with a thick layer (24 μl) of polydimethylsiloxane (PDMS) for sorptive enrichment of the analytes of interest. In combination with quantitative TD, on-line coupled with CGC–MS, the technique showed to be very versatile and sensitive for the analysis of a wide range of drug substances. Moreover, the relative high enrichment efficiencies of SBSE allow to use mass spectrometric detection (MSD) in the full scan mode. In situ derivatization of polar compounds before SBSE is demonstrated for the analysis of paracetamol and this resulted in both improved chromatographic behavior and higher sensitivity. The quantitative performance of SBSE–TD–CGC–MS is illustrated with the analysis of some barbiturates in urine.
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Keywords: Stir bar sorptive extraction; Thermal desorption; Capillary gas chromatography–mass spectrometry; Urine; Drugs; Quantitative analysis

1. Introduction

Screening of pharmaceutical compounds in biological fluids often involves the multi-component analysis and profiling of target analytes that may possess relatively different characteristics, i.e. volatility and polarity. Moreover, they can occur within outsized concentrations ranges. In the analytical scheme, sample preparation is the most tedious and laborious step and new developments in this respect are more than welcome to cope with the increasing demand for accurate and precise data.

Stir bar sorptive extraction (SBSE) has recently been introduced as a versatile sample preparation

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technique for the enrichment of organic compounds from aqueous samples [1]. SBSE implies stirring of a bar that is coated with a layer of polydimethylsiloxane (PDMS) gum (Twister™, Gerstel, Müllheim a/d Ruhr, Germany) in a liquid sample for a preset time and results in sorptive extraction of the analytes into the PDMS coating. The Twister™ can be desorbed with a suitable solvent for CGC or LC analysis, but for volatile and semi-volatile compounds, thermal desorption on-line coupled with CGC–MS analysis provides a very sensitive tool for monitoring a wide range of analytes. The versatility of SBSE–TD–CGC–MS was already demonstrated for the detection and quantification of organic pollutants in water [2–5] and for the profiling of flavor compounds in and in the headspace of food samples [6–9].

Recently, SBSE was evaluated for the enrichment of organic compounds from biological fluids like urine, blood and sperm [10,11]. Principles and practical guidelines were discussed. Selected applications illustrated the high and sensitive performance of SBSE–TD–CGC–MS and proved its applicability for total profiling as well as for target compound analysis. The applicability of the extraction technique was further extended by in situ derivatization of analytes with ethyl chloroformate or acetic anhydride.

This contribution illustrates the use of SBSE–TD–CGC–MS for the screening and quantitative analysis of pharmaceutical drug compounds and metabolites in biological fluids. Possibilities and limitations are critically discussed and illustrated with the analysis of selected CGC-amenable drugs.

2. Experimental

2.1. Procedure for urine samples as such

Five ml urine was poured into a 20 ml headspace vial and 1 ml ammonium acetate buffer (1 M, pH 6.5) was added. 10 μl β-glucuronidase of *Escherichia coli K12* (Roche Molecular Biochemical, Mannheim, Germany) was added and the mixture was equilibrated at 37 °C for 90 min. For SBSE sampling, a stir bar (Twister™, Gerstel GmbH) containing 24 μl PDMS was stirred in the sample for 60 min at 1000 rpm. After sampling, the stir bar was taken out of the vial with tweezers and shortly dipped on a clean paper tissue to remove residual water droplets. The Twister™ was finally put in an empty glass thermal desorption tube of 187 mm l, 6 mm O.D. and 4 mm I.D. for thermal desorption.

2.2. Derivatization

2.2.1. Derivatization with acetic acid anhydride (AA)

Urine samples (5 ml) were poured into a 20 ml headspace vial and 1 ml ammonium acetate buffer (1 M, pH 6.5) and 10 μl β-glucuronidase of *E. coli K12* (Roche Molecular Biochemical) were added. After enzymatic hydrolysis (Chapter 2.1.), 0.5 g potassium carbonate (Sigma-Aldrich, Bornem, Belgium) and 0.5 ml acetic acid anhydride (Sigma-Aldrich) were added. SBSE sampling followed immediately after this mixing according to Section 2.1.

2.2.2. Derivatization with ethyl chloroformate (ECF) [12]

One ml urine sample was poured into a 20 ml headspace vial and buffered with 1 ml of ammonium acetate (1 M). Enzymatic hydrolysis was performed as described in Section 2.1. Afterwards, 0.5 ml of a 2:1 (v/v) mixture of ethanol (ChromaSolv, Merck Eurolab, Leuven, Belgium) and pyridine (Sigma-Aldrich) was added. The mixture was shortly vortex shaken and 0.1 ml of ethyl chloroformate (Sigma-Aldrich) was added. The mixture was then vortexed for 10 s and the open vial was put in an ultrasonic bath for 15 min. Attention should be paid after ECF addition because the reaction involves an intense gas production. After derivatization, the mixtures were sampled with SBSE as such (Section 2.1).

2.3. Instrumental conditions

2.3.1. Thermal desorption–capillary GC–MS (TD–CGC–MS)

A TDS-2 thermodesorption unit (Gerstel) was mounted on a 6890 Agilent GC (Agilent Technologies, Little Falls, DE, USA). A robotic sampling

system (TDS-A, Gerstel) was used in combination with the TDS-2 for automatic sample tube delivery. The thermally desorbed analytes were cryofocused in a programmed temperature vaporization injector (PTV, CIS-4, Gerstel) at −100 °C with liquid nitrogen prior to injection. An empty baffled liner was applied in the PTV. For splitless thermal desorption, the TDS-2 was ramped from 30 to 300 °C at a rate of 60 °C/min and the upper temperature was held for 10 min. Splitless injection (2 min) was performed by ramping the PTV injector from −100 to 300 °C at a rate of 600 °C/min. Capillary GC analyses were done on a 30 ml × 0.25 mm I.D., 0.25 μm d_f HP-5MS column (Agilent Technologies). The oven was programmed from 50 to 320 °C at a rate of 10 °C/min. Helium was used as carrier gas and the head pressure was calculated using the retention time locking (RTL) software, eluting ethyl palmitate at a constant retention time of 17.68 min [13]. The Agilent 5973 mass spectrometric detector was operated in the scan mode (*m/z* 40–500). Barbiturates were quantified in extracted ion chromatograms (EIC) at *m/z* 156 (amobarbital, pentobarbital), 168 (secobarbital) and 221 (hexobarbital). For selected ion monitoring of barbiturates, the MSD was programmed at *m/z* 156, 168 and 221 (dwell time = 100 ms).

3. Results and discussion

3.1. Multi-component profiling and target compound analysis

SBSE in combination with TD–CGC–MS was in first instance applied for multi-component analysis of biological fluids. The mass spectrometric detector (MSD) was used in the full scan mode to allow identification of the detected analytes. Fig. 1 shows the total ion chromatogram (TIC, *m/z* 40–500) of the SBSE–TD–CGC–MS analysis of a urine sample (5 ml) of an adult multi-drug user. Sampling and analysis was performed as described in Section 2.1. More than 200 compounds could be enriched and detected at the same time resulting in a complete profile of GC-amenable compounds in the urine sample. Some major peaks were identified and the numbers in the chromatogram refer to the identity of the compounds listed in Table 1. The urine mainly reflects the food consumption of the patient, since several terpenes (Fig. 1, peaks 1, 2, 4, 7-10) originating from the digestion of vegetables are highly abundant. Additionally, the presence of some lower fatty acids and their ethyl esters (Fig. 1, peaks 3, 5, 6) indicate the metabolism of fats. Benzyl salicylate (Fig. 1, peak 11), which is added in sunscreen preparations (Fig. 1, peak 14) [14] and as a fixer in perfumery, could also be detected in the urine. Steroids are reflecting the action and malfunction of endocrine glands [15] and some of them are clearly noticed in the TIC (Fig. 1, peaks 17, 19, 20, 24). The most abundant peaks, however, are pharmaceutical compounds and drugs of abuse. The occurrence of cannabichromene (Fig. 1, peak 12) proves that the person took cannabis [16] and methadone [16] and its major metabolite (Fig. 1, peak 17, 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine, metabolite I) [17] are also present.

The major pharmaceuticals in the urine sample are the clomipramine metabolites 5-chloro-3-phenyl-2,1-benzoisoxazole (Fig. 1, peak 13) and 8-hydroxy-5-chloro-3-phenyl-2,1-benzoisoxazole (Fig. 1, peak 15). The compounds can be screened out more clearly by the EIC at *m/z* 229 (Fig. 2). Clomipramine, distributed under the name Anafranil®, is a chlorinated tricyclic analogue of imipramine [18] with antidepressant and anti-obsessional properties [19] It is widely used in Western Europe because of its ability to inhibit serotonine into the central nerve terminals. Its major active metabolite desmethylclomipramine inhibits norepinephrine re-uptake. More than 90% of both compounds are recovered from an oral dose in the urine and faeces, essentially as metabolites. The metabolites are formed by desmethylation and aromatic hydroxylation, preferentially at the 8-position [20]. Clomipramine and its metabolites are phase II-metabolized and form water-soluble glucuronide conjugates [21]. Analysis of the urine samples was hence performed after enzymatic hydrolysis with β-glucuronidase complex of *E. coli K12*. The enzyme is not absorbed into the PDMS coating and does not interfere

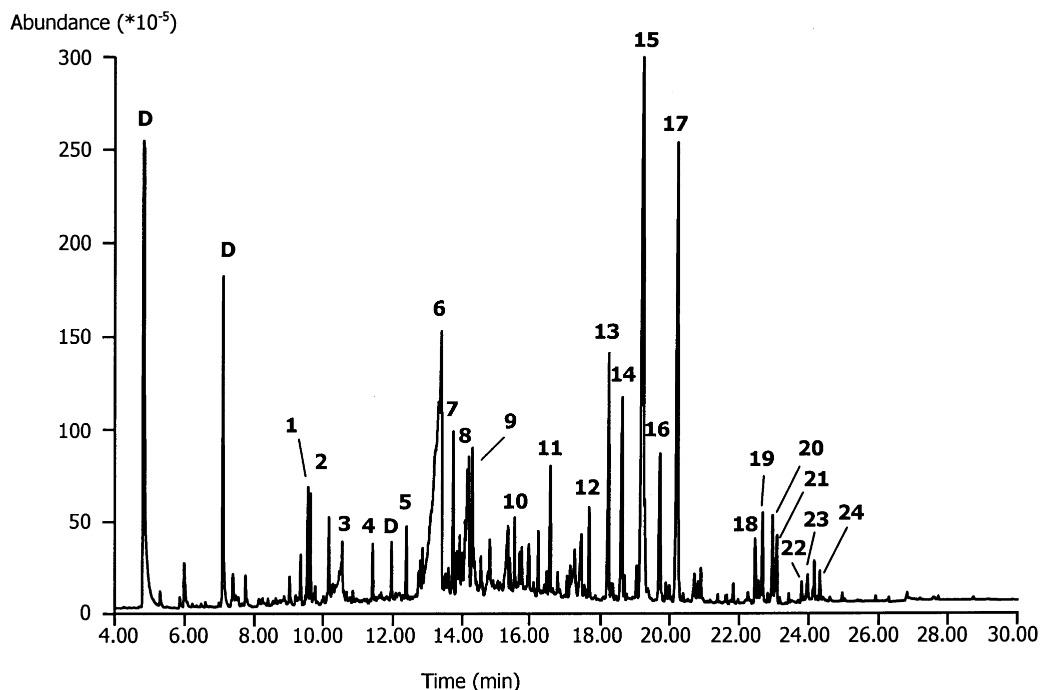

Fig. 1. SBSE–TD–CGC–MS analysis of 5 ml urine of a patient under treatment of drugs. The numbers refer to the compound names in Table 1. Sampling and chromatographic conditions see text.

during subsequent sampling and TD–CGC–MS analysis. Both 5-chloro-3-phenyl-2,1-benzoisoxazole (Fig. 2, peak 13) and 8-hydroxy-5-chloro-3-phenyl-2,1-benzoisoxazole (Fig. 2, peak 15) show favorable characteristics towards sorption into the PDMS coating since they posses relative high PDMS–water distribution coefficients ($K_{\mathrm{PDMS-water}}$). This value can be approximated by the K_{ow}-value and can be calculated by software programs like KOWWIN [22]. The metabolites 13 and 15 have log K_{ow}-values of 3.5 and 4, respectively, and this results in nearly quantitative extraction of the analytes from the urine into the stir bar under the given experimental conditions, i.e. 5 ml urine and 24 μl PDMS coating on the stir bar.

In the same urine sample, benzodiazepines could also be traced. Fig. 3 shows the EIC at m/z 317 (bromazepam, peak **1**), 283 (diazepam, peak **2**), 269 (nordiazepam, peak **3**), 271 (temazepam, peak **4**) and 284 (O-methyloxazepam, peak **5**). It is known that the clomipramine response may be increased by a synergic effect when this drug is used in combination with antidepressants like benzodiazepines and barbiturates. Benzodiazepines are hydrophobic (log $K_{\mathrm{ow}} = 2.3–5.1$) and are, therefore, suitable to be enriched by SBSE with extraction recoveries between 50 and 100%. As will be illustrated later, barbiturates can also relatively easily be screened and quantified with SBSE–TD–CGC–MS.

Another example concerns the analysis of venlafaxine. This bicyclic phenethylamine is a representative of a new class of antidepressants that inhibits selectively the uptake of serotonine and noradrenaline, but in contrast to tricyclics like clomipramine, shows no affinity for neurotransmitter receptors (muscarinic, histaminergic, α1-adrenergic receptors) [23]. It is partially metabolized to its active O-desmethyl derivative (ODV). Approximately 87% of a single dose of venlafaxine is recovered in urine, of which 5% is present in its non-conjugated form. Goeringer et al. identified venlafaxine in urine samples of post mortem cases

Table 1
Compounds detected in the total ion chromatogram (Fig. 1) and EIC at *m/z* 229 (Fig. 2) and *m/z* 317, 283, 269, 271, 284 (Fig. 3) of the SBSE–TD–CGC–MS analysis of urine of a multi-drug user

Number	Compound
1	4-Vinyl-2-methoxyphenol
2	Eugenol
3	Decanoic acid
4	Cis-eugenol
5	Ethyl 4-ethoxybenzoate
6	Dodecanoic acid
7	Cadinene
8	Junipene
9	Bulnesol
10	Citronilide
11	Benzylsalicylate
12	Cannabichromene
13	Clomipramine metabolite I
14	Methadone metabolite I
15	Clomipramine metabolite II
16	Methadone
17	Androstenol
18	Diazepam
19	Androsterone
20	Epiandrosterone
21	Nordiazepam
22	Temazepam
23	*O*-Methyloxazepam
24	Allopregnanediol
25	Bromazepam (EIC)

The numbers refer to Figs. 1–3.

and concentrations varied between 0.05–21 mg/l [24]. Analyses were performed by liquid chromatography–mass spectrometry (LC–MS) following extraction with *n*-butyl chloride. An alternative method was described by Bickeboeller-Friedrich et al. and implied acetylation prior to CGC–MS analysis [25]. Venlafaxine shows good affinity for the PDMS extraction phase ($\log K_{ow} = 2.88$) and can be extracted from the urine sample for about 78%. Fig. 4 shows the EIC at *m/z* 58 for venlafaxine (peak 2) in the urine sample of an adult under treatment for drugs of abuse.

The main compound in the urine sample (Fig. 4) was phenyltoloxamine (*N*,*N*-dimethyl-2-[2-(phenylmethyl)phenoxy]ethanamine), showing a highly overloaded peak 1. The medicine is typically administered orally as its dihydrogen citrate and acts as an oral vasoconstrictor and bronchodilator. The compound provides symptomatic relief of allergic symptoms caused by histamine release [26]. Antihistamines are in most cases analyzed by LC [27], but CGC is a valid alternative. Because of their high $\log K_{ow}$-values, generally between 2.0 (pheniramine) and 3.9 (phenyltoloxamine), they provide relative good properties for sampling with SBSE from aqueous samples. Theoretical recoveries from a 5 ml water sample, using a stir bar which contains 24 μl PMDS, are between 32 and 97%, respectively. These types of compounds can thus also easily be monitored at trace levels.

On the contrary, codeine, which is in some cases administered in conjunction with phenyltoloxamine (Sinutab® with codeine, Pfizer Inc., New York, USA) and paracetamol, shows less favorable extraction efficiencies. The morphine-like analyte ($\log K_{ow} = 1.41$) is only extracted for 11%. Still, it can easily be detected in the EIC at *m/z* 299 of a enzymatic hydrolyzed (β-glucuronidase K12) urine sample taken after oral intake of a single dose of codeine (Fig. 5, peak 1). Its major metabolite morphine [28], which can only be recovered for 2.5%, could not be detected. Fig. 5 also shows the trace at *m/z* 271 for racemethorphan (Fig. 5, peak 2). This structure analogue to codeine has a $\log K_{ow}$ of 3.97 and is extracted with an efficiency of 96%. These examples show that recoveries highly depend on the polarity of the solutes. Recoveries of polar compounds can strongly be improved by in situ derivatizing the target solutes prior to SBSE sampling.

This is demonstrated by the analysis of acetaminophen (paracetamol) in urine. This *N*-acetylamino phenol shows very low affinity for PDMS as expressed by its low octanol–water distribution coefficient ($\log K_{ow} = 0.46$). This corresponds, under the given experimental conditions (5 ml urine, stir bar containing 24 μl PDMS) to a maximum recovery of 0.7%. Fig. 6A shows the EIC at *m/z* 109 of a urine sample containing approximately 200 μg/l of acetaminophen. Paracetamol is intensively phase II metabolized to sulfate, cysteine, mercapturate but mainly glucuronic acid conjugates [29]. Enzymatic deglucuronidation with β-glucuronidase was, therefore, performed before SBSE sampling. Because of the polar nature of

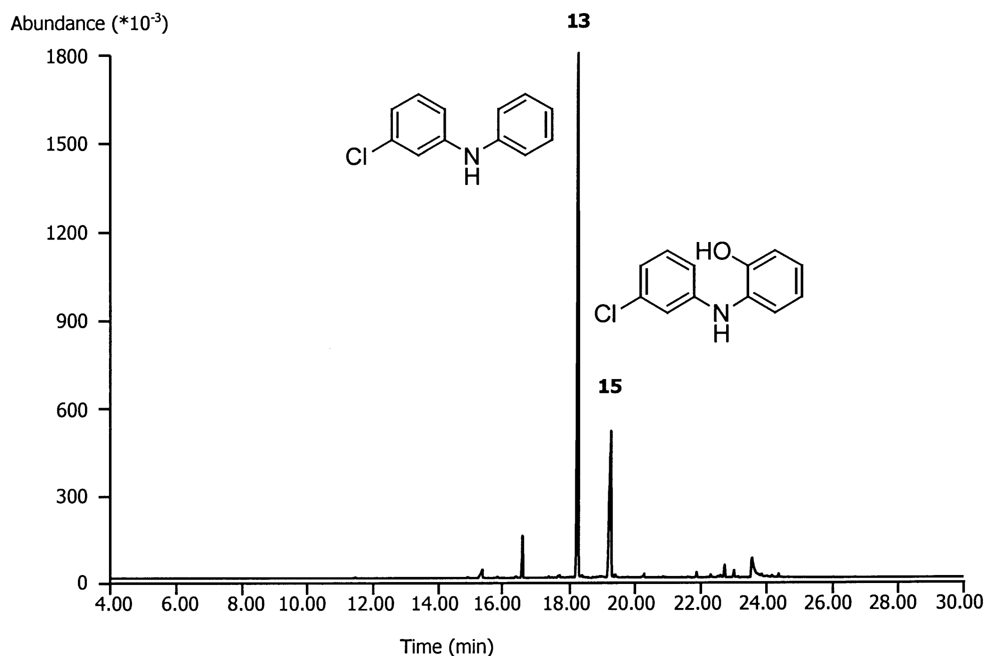

Fig. 2. EIC at m/z 229 of the SBSE–TD–CGC–MS analysis of the clomipramine metabolites 5-chloro-3-phenyl-2,1-benzoisoxazole [13] and 8-hydroxy-5-chloro-3-phenyl-2,1-benzoisoxazole [15] in urine under treatment of drugs. The numbers refer to Fig. 1 and Table 1. Sampling and chromatographic conditions see text.

the target drug, the analyte peak shows low abundance and additionally a very bad peak shape on the apolar column is noted. For the in situ conversion of phenolic compounds in biological fluids followed by SBSE–TD–CGC–MS, two derivatization procedures, namely acetylation and reaction with ethyl chloroformate (ECF) were described [10]. Both reactions can be performed in aqueous medium and hence improve both extraction efficiencies and chromatographic behavior of the analytes. In situ acetylation implies the addition of 0.5 g potassium carbonate and 0.5 ml acetic anhydride to 5 ml of sample. Derivatization and sampling can be done simultaneously by stirring the reaction mixture with a Twister™ immediately after the addition of the reagents. The *O*-acetylated acetaminophen results in a better peak shape, but the extraction recovery increases only minimally ($\log K_{ow} = 0.58$, recovery = 1.8%). Derivatization of urine (1 ml) with ECF (100 µl) is typically performed in the presence 0.5 ml of a mixture of ethanol and pyridine (2:1, v/v). The mixture is put in an ultrasonic bath for 15 min prior to SBSE sampling. The reaction yields the *O*-alkoxycarbonyl derivative of acetaminophen (Fig. 6B) and shows an increased affinity for the PDMS coating. The $\log K_{ow}$ of 1.1 corresponds to a theoretical recovery of 5%. However, the addition of relative large amounts of ethanol and pyridine to the aqueous sample distinctively decreases the distribution of the solutes into the PDMS layer of the stir bar. For that reason, 10 ml of water was added to the mixture after derivatization and before SBSE sampling. Fig. 6B shows the EIC at m/z 109 of the same urine sample (Fig. 6A) that was treated with ECF. The mass spectrum of the derivatized acetaminophen is shown in Fig. 6C. The M+72 (151+72 = 253) ion is clearly present in the mass spectrum and proves the *O*-alkoxycarbonyl structure. The m/z 109 ion originates from the cleavage of the amide bound. The derivative shows a better peak shape and sensitivity is improved by a factor 50 compared with the pure urine sample. Note that only 1 ml of sample is used for derivatization instead of 5 ml.

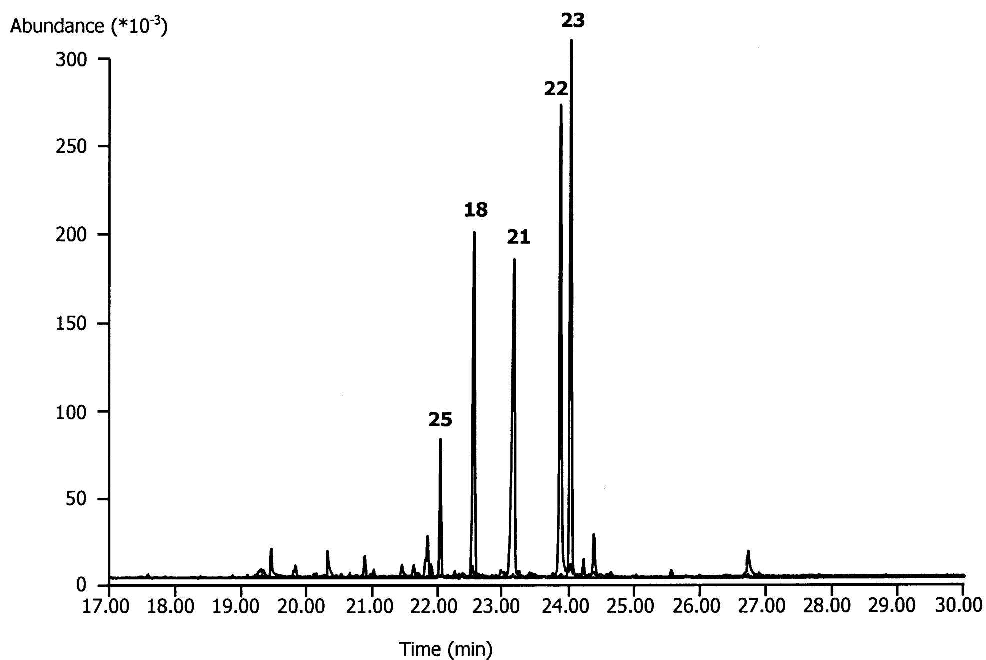

Fig. 3. Benzodiazepines in urine under treatment of drugs: EIC at m/z 317 (bromazepam, **25**), 283 (diazepam, **18**), 269 (nordiazepam, **21**), 271 (temazepam, **22**) and 284 (*O*-methyloxazepam, **23**). The numbers refer to Fig. 1 and Table 1. Sampling and chromatographic conditions see text.

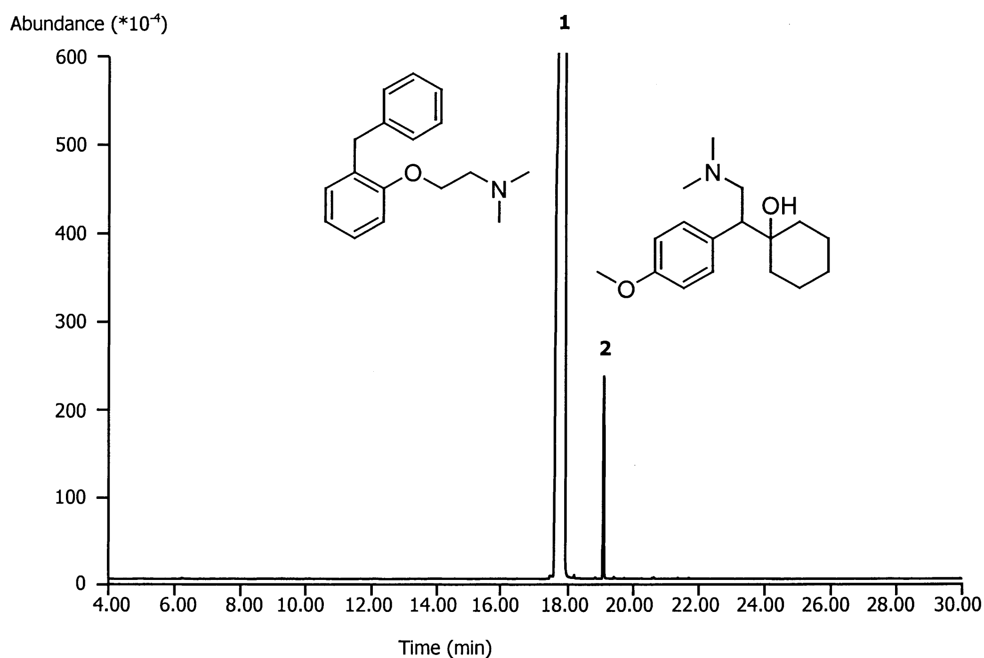

Fig. 4. EIC at m/z 58 for phenyltoloxamine (*N*,*N*-dimethyl-2-[2-(phenylmethyl)phenoxy]ethanamine) (**1**) and venlafaxine (**2**) in the urine of an adult under treatment for drugs of abuse. SBSE sampling and chromatographic conditions see text.

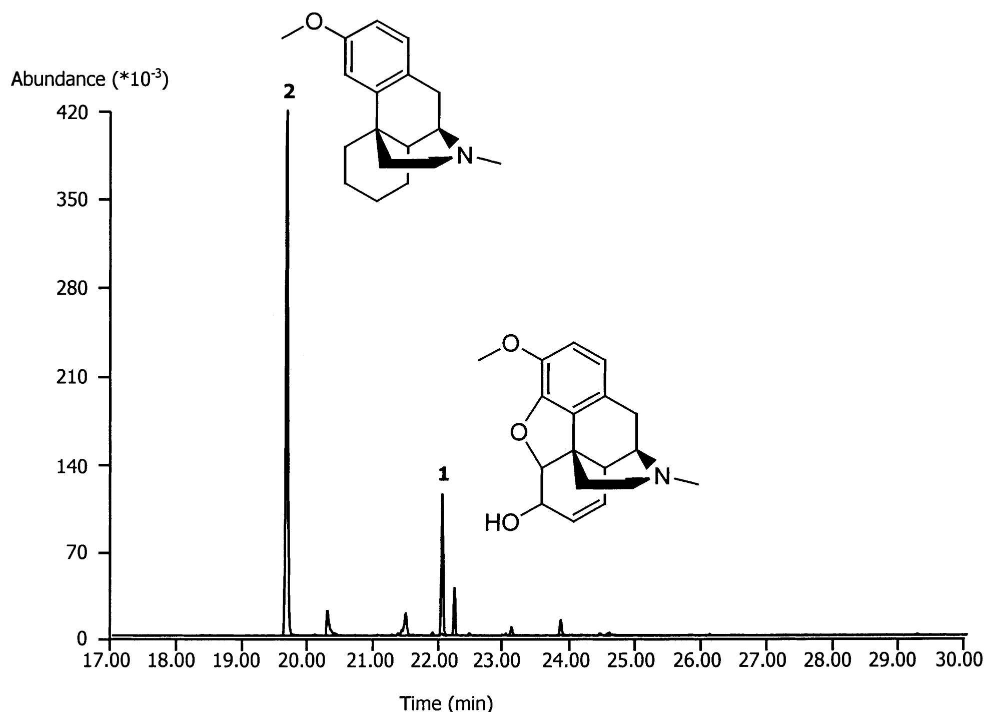

Fig. 5. EIC at *m/z* 299 (**1**, codeine) and *m/z* 271 (**2**, racemethorphan) of a urine sample taken after oral intake of a single dose of codeine. Sampling and chromatographic conditions see text.

3.2. Quantitative analysis

The quantitative performance of SBSE–TD–CGC–MS was evaluated for the analysis of some barbiturates in urine. Barbiturates are used as diagnostic and therapeutic drugs and are commonly applied as anticonvulsant and anaesthetic agents [30]. Excretion amounts and rates of barbiturates can differ significantly in function of the patient's health condition and depend on the duration of their therapeutic action. A mixture of short- (pentobarbital, hexobarbital, secobarbital) and medium long-acting (amobarbital) barbiturates was selected and standard solutions were prepared in methanol. The analytes all possess log K_{ow}-values of around 2 and SBSE sampling using a Twister™ that contains 24 µl PDMS results in theoretical recoveries between 32 and 52% (Table 2). For calibration, quantitative extraction into the PDMS is not required. It is, however, important that calibration occurs under the same conditions as the analysis of real samples, i.e. sampling time, temperature, sample volume, stir bar dimensions, pH, sample ion strength, etc. Blank urine samples were buffered with ammonium acetate (pH 6.5) and spiked with the barbiturate standard solutions (10 µl) to individual concentrations of 1, 5, 10, 50, 100 and 500 µg/l and analyzed by SBSE–TD–CGC–MS. The mass spectrometric detector was used in the full scan mode. Quantitation was performed in the EIC at *m/z* 156 (amobarbital, pentobarbital), 168 (secobarbital) and 221 (hexobarbital). Fig. 7 shows a

Table 2
log K_{ow}-values and theoretical recoveries (%) of barbiturates

Number	Compound	log K_{ow}	Theoretical recovery (%)
1	Amobarbital	2.00	32
2	Pentobarbital	2.00	32
3	Secobarbital	2.36	52
4	Hexobarbital	2.02	33

Experimental conditions: 5 ml sample, 24 µl PDMS Twister™.

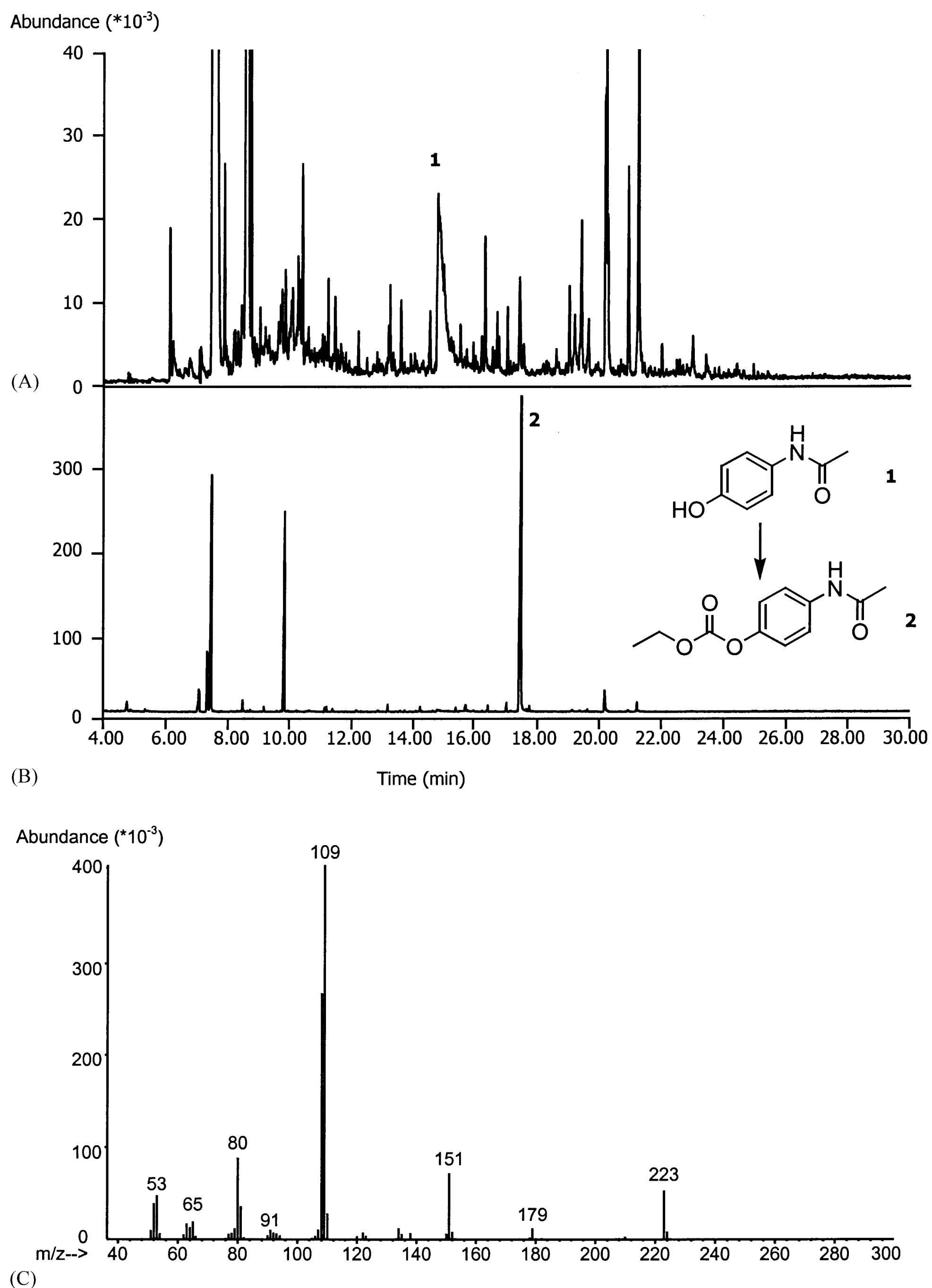

Fig. 6. EIC at m/z 109 of a pure (A) urine sample and the same urine sample that was treated with ECF (B). The mass spectrum and structure of the O-alkoxycarbonylacetamidophen are included. Derivatization, sampling and chromatographic conditions see text.

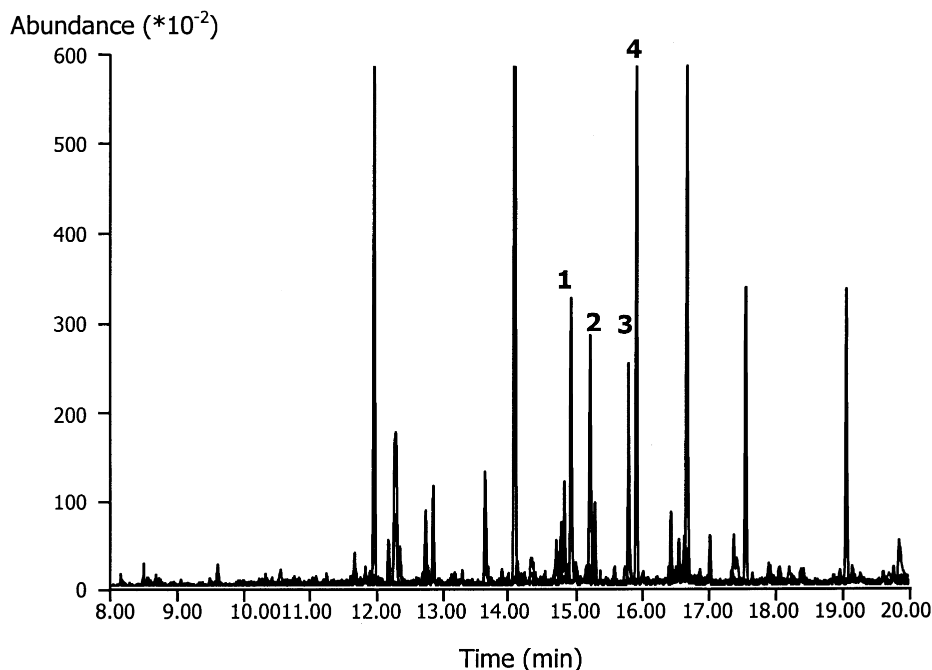

Fig. 7. EIC at m/z 156 (**1**, amobarbital; **2**, pentobarbital), 168 (**3**, secobarbital) and 221 (**4**, hexobarbital) of a blank urine sample spiked with barbiturates to a concentration level of 50 μg/l. The numbers refer to Table 2. Sampling and chromatographic conditions see text.

typical profile of the barbiturate mixture spiked to a level of 50 μg/l. The limit of detection (LOD) for the selected solutes was 1 μg/l (signal-to-noise S/N = 3) using the MSD in the full scan mode. The limit of quantitation (LOQ) was set to 5 μg/l. Linear regression of the peak areas was performed in a concentration range between 5 and 500 μg/l (Fig. 8) and the correlation coefficients of the individual regression lines were all larger than 0.99. When the MSD was used in the SIM mode at

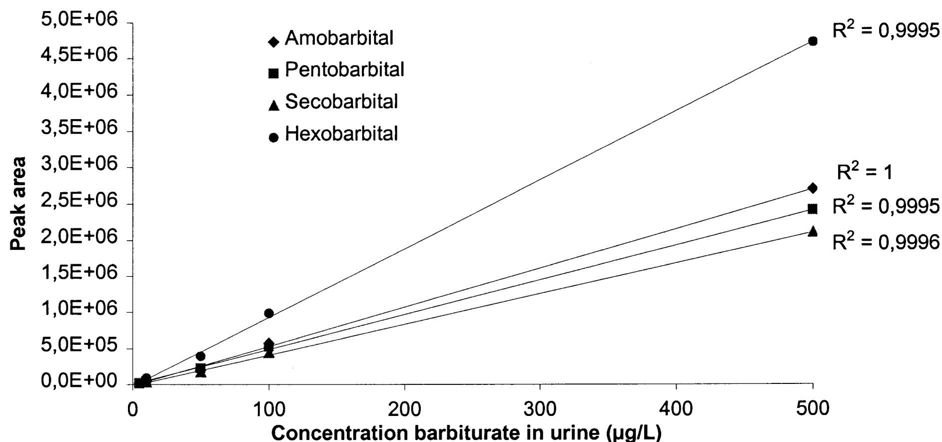

Fig. 8. Calibration curves of the SBSE–TD–CGC–MS analysis of some barbiturates spiked in blank urine to concentration levels between 5 and 500 μg/l.

m/z 156, 168, 221 (dwell time = 100 ms), a LOD of 10 ng/l could be obtained.

4. Conclusion

A wide range of pharmaceutical drug compounds can be screened in urine with SBSE in combination with thermal desorption–capillary gas chromatography–mass spectrometry (TD–CGC–MS) analysis. The high enrichment of SBSE and quantitative TD of the analytes allow to use the MSD in the full scan mode for complete profiling of both original drugs and metabolites. Enrichment and chromatographic behavior of polar solutes can be improved by in situ derivatization with ethyl chloroformate extending the versatility of the technique. Barbiturates could be quantified with good linearity in a concentration range between 5 and 500 μg/l. Limits of detections are in the order of 1 μg/l in the ion extraction mode and of 10 ng/l in the selected ion monitoring mode.

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