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Fast liquid chromatographic–mass spectrometric determination of pharmaceutical compounds

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Abstract

We present fast LC–MS–MS analyses of multicomponent mixtures containing flavones, sulfonamides, benzodiazepines and tricyclic amines. Using a short microbore HPLC column with small particle size, five to eight compounds were partially resolved within 15 to 30 s. TurboIonSpray and atmospheric pressure chemical ionization interfaces were well suited to tolerate the higher eluent flow-rates of 1.2 to 2 ml/min. The methods were applied to biological sample matrices after clean-up using solid-phase or liquid–liquid extraction. Good precision and accuracy (average 8.9 and 97.7%, respectively) were achieved for the determination of tricyclic amines in human plasma. Benzodiazepines were determined in human urine with average precision of 9% and average accuracy of 95% for intra- and inter-assay. Detection limits in the low ng/ml range were obtained. An example for 240 injections per hour of demonstrated the feasibility of rapid LC–MS–MS analysis.

Keywords: Multicomponent mixtures; Flavones; Sulfonamides; Tricyclic amines; Benzodiazepines; Carbamazepine; Propranolol

1. Introduction

In today's pharmaceutical and biotechnology industries there is a need for fast sample analyses. Large numbers of compounds resulting from rapid high-throughput combinatorial synthesis and early drug discovery experiments [1] must be characterized to provide structural and purity information [2,3]. For these applications fast and reliable high-throughput analytical methods have to be developed and implemented. Furthermore, cassette dosing experiments [4] and other drug metabolism studies [5] require rapid determinations. Laboratories engaged in drug testing and toxicology are interested in high-throughput methodologies with concomitant high sensitivity and selectivity [6]. Large sample batches resulting from new drugs entering clinical trials also drive the need for high-speed analysis [7].

Liquid chromatography–mass spectrometry (LC–MS) and especially LC–MS–MS, now routinely applied in the pharmaceutical industry, are powerful tools to characterize complex samples. High sensitivity and selectivity make this technique well suited for high-throughput analysis; however, sample preparation, separation and data processing require time. Automatic sample preparation strategies, like 96-well plate technology [8,9], decrease the time for this phase of the work by parallel treatment of

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samples. Thus, robotic sample pretreatment can facilitate large numbers of samples. Direct solid-phase extraction (SPE)–LC–MS coupling has also been reported which integrates on-line sample preparation with analysis [10]. Shortening the analytical run times is an important step towards high sample throughput. To obtain maximum chromatographic resolution is no longer the major driving force, because run times of several minutes are increasingly not tolerable for truly high-throughput analyses. Increased emphasis is now directed towards adequate chromatographic resolution in a drastically reduced time. This may be accomplished by the use of short columns, high mobile phase flow-rate and small particle sizes in packed high-performance liquid chromatography (HPLC) columns [11].

Routine LC–MS analyses in the pharmaceutical industry are now achieved in less than 10 min [12–14] while 1.5–2 min separation times are increasingly common [15–17]. For a single component determination retention times of 20–30 s were reported [18]. Using LC–UV rapid gradient separations of combinatorial library mixtures in less than 1 min were obtained [3]. Fast LC–MS–MS runs of 60 samples per hour have been reported [6]. Turbulent flow chromatography achieved with high flow-rates and large particle size reportedly can remove the need for time-consuming sample preparation because direct injection of plasma or serum samples is possible. Reasonably high throughput with analysis times of 2.5 min has been reported [19]. Parallel chromatography using two analytical columns combined with one mass spectrometer is another approach for increased throughput [2]. By coupling four autosamplers to one column and MS–MS more than 1100 samples have been analyzed in less than 12 h [20].

Our goal in this work was to achieve separation times as short as 15 s for mixtures of five analytes to demonstrate the possibility of fast LC–MS analysis in selected real-world applications. We present in this paper separations of several synthetic mixtures of model compounds which are of pharmaceutical interest. These include separate mixtures of flavones, sulfa-drugs, benzodiazepines and tricyclic amines. The suitability of LC–MS and LC–MS–MS to determine these substances in biological samples was described previously [21–24]. The analytes studied in this report were monitored in the selected ion mode (SIM) or the selected reaction mode (SRM) using TurboIonSpray or atmospheric pressure chemical ionization (APCI) to better handle the high mobile phase flow-rates. Results of initial investigations of the applicability to urine and plasma samples are shown and an example demonstrating 240 injections per hour is given.

2. Experimental

2.1. Materials

Daidzein, genistein, 7-hydroxyflavone, 7-hydroxymethoxyisoflavone and flavone were purchased from ICN (Plainview, NY, USA). Oxazolam, chlordiazepoxide, carbamazepine, medazepam, estazolam, norfludiazepam, triazolam, delorazepam and bromazepam were obtained from Alltech-Applied Science (State College, PA, USA). Estazolam-d5, used as internal standard for the benzodiazepine quantitation and imipramine-d3 as internal standard for the tricyclic amines were purchased from Radian (Austin, TX, USA). Sulfadiazine, sulfamerazine, sulfamethoxazole, sulfadimethoxine, sulfasalazine as well as propranolol, doxepin, desipramine, imipramine, amitriptyline and trimipramine were obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and chloroform were from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA), hydrochloric acid from Mallinckrodt (Paris, KY, USA), and ammonium hydroxide from VWR Scientific (West Chester, PA, USA). Ammonium acetate and sodium carbonate were purchased from Sigma. Deionized water was generated in the laboratory with a Barnstead Nanopure II filtration system (Boston, MA, USA). For SPE Bakerbond SPE C_{18} cartridges (3 ml) (J.T. Baker) were used. The reconstituted extracts were filtered through 0.45-μm nylon syringe filters (Whatman, Clilfton, NJ, USA).

2.2. Sample preparation

All pipetting was done manually using 100-μl and 1000-μl pipettors (Rainin, Emeryville, CA, USA).

Stock solutions of standards at concentrations between 0.1 and 1 mg/ml were prepared in water–acetonitrile (50:50) and diluted with water to obtain separate standard working solutions of flavones, sulfonamides, tricyclic amines and benzodiazepines. Human control plasma was obtained from Lampire Biological Labs. (Pipersville, PA, USA). Human control urine was supplied by healthy volunteers in our research group. The highest concentration standards were prepared by adding stock solutions of the investigated compounds (tricyclic amines and benzodiazepines) to plasma or urine. The lower concentration standards were diluted from that solution with control plasma or urine. Blanks were prepared to investigate potential matrix influence and to do post-extraction spikes for the determination of extraction recovery.

2.2.1. Tricyclic amines

Human control plasma (500 μl) was spiked with the tricyclic amines (doxepin, desipramine, imipramine, amitriptyline and trimipramine) to final concentrations of 2, 5, 10, 20, 50, 100, 200 and 400 ng/ml. Quality control samples with concentrations of 8, 80 and 180 ng/ml (six replicates at each concentration level) were prepared. Imipramine-d3 was added to each sample for a final concentration of 20 ng/ml. Volumes (10 ng/ml) of tricyclic amines were spiked into blank control plasma extract after reconstitution to determine the recovery.

SPE of amines from the biological sample was carried out using a vacuum manifold VAC ELUT (Analytichem, Harbor City, CA, USA) and pump (KNF Neuberger, Princeton, NJ, USA) at a flow-rate of about 1 ml/min. For precision and accuracy studies 55 plasma samples containing the tricyclic amines were prepared. C_{18} cartridges were preconditioned sequentially with 1 mol/l HCl, methanol, water and ammonium acetate buffer, pH 8 (2 ml each). The spiked plasma (500 μl) was pretreated by adding 700 μl acetonitrile for protein precipitation and centrifugation using a VSMC-13 micro centrifuge (Shelton Scientific). Then the supernatant was applied to the cartridge. To remove endogenous chemical interferences, the cartridge was rinsed with 2 ml buffer followed by 500 μl acetonitrile before eluting the analytes with 1 ml methanol containing 1% formic acid. For the evaporation under nitrogen with the vials placed in a water bath at 40°C a Reacti-Vap blow down manifold and Reacti-Therm heater (Pierce, Rockford, IL, USA) were used. Reconstitution was carried out by dissolution in 500 μl water–acetonitrile (4:1) followed by filtration through 0.45-μm nylon syringe filters.

2.2.2. Benzodiazepines

Human control urine (500 μl) was spiked with the five benzodiazepines bromazepam, carbamazepine, estazolam, norfludiazepam and delorazepam to obtain standard solutions at nine concentration levels and quality control samples (low, medium and high QC) at concentrations shown in Table 3. To each sample estazolam-d5 was added yielding a final concentration of 60 ng/ml of internal standard. For the determination of recovery 202.2 ng/ml bromazepam, 19 ng/ml carbamazepine, 23.6 ng/ml estazolam, 92.4 ng/ml norfludiazepam and 97.6 ng/ml delorazepam were spiked into blank control urine extract.

Liquid–liquid extraction of benzodiazepines from urine was performed in 1.2-ml screwcap vials. Large sample sets were prepared in Matrix Technologies 1.1-ml 96-well disposable tube racks (Lowell, MA, USA) and transferred to Beckman deep-well collection plates (Fullerton, CA, USA). Two 96-well plates of benzodiazepines in urine containing duplicate standard sets and QC samples (six replicates at each of the three levels) were prepared for precision and accuracy determination. Standards and internal standard (final concentration of 60 ng/ml) were spiked into 500 μl urine. Then 50 μl ammonium carbonate (0.1 mol/l) was added. After mixing the sample solution, 500 μl of chloroform was added followed by mixing 5 min (manually) and centrifugation for 5 min at 8000 rpm. The chloroform layer was removed and evaporated under a gentle nitrogen stream at 40°C. The reconstituted extracts (500 μl water–acetonitrile, 4:1) were filtered through 0.45-μm nylon filters to prevent column pressure build-up due to particulate material. The pipetting and filtration in the 96-well-format was performed using the TOMTEC Quadra 96-320 workstation (Hamden, CT, USA).

To prepare the samples for the 240-injections-per-hour experiment, two spiked 5-ml urine volumes containing 400 ng/ml bromazepam, 80 ng/ml car-

bamazepine, 50 ng/ml estazolam, 184 ng/ml norfludiazepam and 200 ng/ml delorazepam were extracted in the same way using 20-ml vials, 500 μl ammonium carbonate and 5 ml chloroform.

2.3. Chromatography

A Waters Alliance 2690 separations module (Milford, MA, USA) with integrated autosampler and pump delivered the mobile phase containing ammonium acetate/acetic acid and acetonitrile (mixed by the gradient unit) at flow-rates of 1.2 to 2 ml/min. The resulting HPLC system pressures were between 20 and 27 MPa. An Endurance autosampler (Spark Holland, Emmen, The Netherlands) was used for the analysis of samples in 96-well plates. The analytical column was a SB-C_{18} Mac Mod Rapid Resolution 15×2.1 mm cartridge packed with 3 μm particles (Hewlett-Packard Analytical, Chadds Ford, PA, USA). Polyether ether ketone (PEEK) tubing of 0.008 in. I.D. was used to connect the column to the pump and the mass spectrometer (1 in.=2.54 cm). The tubing length was made as short as possible to minimize extra-column volume. The split of the eluent flow after the column was performed using a micro-splitter valve (Upchurch Scientific, Oak Harbor, WA, USA). The exact conditions for the special separations (mobile phase composition and flow-rates) are given in the text and figures for each application described. Prior to the LC–MS experiments the chromatographic separation methods were established with UV detection at 254 nm using a Spectroflow 783 detector (ABI Analytical, Ramsey, NJ, USA).

2.4. Mass spectrometry

The mass spectrometer used was a PE Sciex (Concord, Ontario, Canada) API 365 tandem triple quadrupole mass spectrometer, equipped with a TurboIonSpray and heated pneumatic nebulizer interface operated in the positive ion mode under SIM or SRM conditions. Nitrogen from a liquid nitrogen dewar boil-off at 80 p.s.i. was used as nebulizer, auxiliary and collision gas (1 p.s.i.=6894.76 Pa). Flavones and sulfonamides were investigated in the SIM mode with the TurboIonSpray probe temperature set at 450°C, the auxiliary gas flow-rate at 8 l/min and the nebulizer and curtain gas flow maintained at instrument setting 12 using a sprayer voltage of 5000 V. Benzodiazepines were monitored using SRM, setting the temperature at 450°C, the voltage to 4000 V and the auxiliary gas flow at 8 l/min while maintaining the nebulizer, curtain and collision gas flow at 12, 12 and 5, respectively. For APCI studies of the amines, the probe temperature was set at 450°C, and the needle current at 2 μA; using a maximum auxiliary gas flow of 8 l/min the nebulizer gas was maintained at setting 12 and the collision gas at 3. The mass resolution of Q1 was set at 0.7 m/z and Q3 at 0.8 m/z peak width at half-height for SRM experiments. The collision energy for each ion was optimized using infusion experiments prior to the LC–MS analysis. The analytes were monitored with a dwell time of 30 ms for each ion or selected precursor ion-product ion transition to obtain at least 10 data points per peak.

2.5. Hardware set-up for 240 samples per hour

Sample injections every 15 s are not possible with conventional autosamplers, because they require at least a 1-min cycle time. Therefore, we set up a flow-injection experiment to demonstrate an LC–MS run to include 240 samples per hour. A 10-ml syringe filled with urine extract containing the benzodiazepine mixture was placed into an infusion pump (Harvard Apparatus, South Natick, MA, USA). The syringe delivered sample solution continuously at 120 μl/min through the 5-μl sample loop installed on an automatic switching valve (PROSPEKT system, Spark Holland). This valve was programmed to switch every 13 s to the injection position followed by a rapid return to the load position. Every 2 min a signal was sent to the MS instrument to start another data collection sequence. Sample acquisition methods of 1.85 min length were created, allowing the MS to load the next file and be ready for data collection after 2 min. Therefore, 30 runs with eight chromatograms per run were acquired over the 60-min time period.

3. Results and discussion

3.1. Method development for synthetic standard mixtures

Short columns with small particles are well suited for fast chromatography. Their use can reduce method development and sample analysis time significantly not only because of the rapid separation, but also the reduced time for re-equilibration of the column after changing the mobile phase. A cartridge column of 15 mm length was used to obtain short analysis times for each sample. The inner diameter of 2.1 mm was chosen because of the rapid linear velocity achievable with increased flow-rates that are comparable to those often used for a 4.6 mm I.D. column. At high eluent flow-rates signal degradation of TurboIonSpray LC–MS can occur although it can tolerate flows up to 2 ml/min. A post-column split of the eluent flow was performed in some cases to obtain better ion current stability. A large additional column volume due to tubing and the post-column split device should be avoided to minimize deterioration of chromatographic peak resolution, broad peaks and peak tailing.

To demonstrate the feasibility of fast LC–MS and LC–MS–MS analyses, synthetic mixtures of different analytical standards were investigated. A Mac Mod SB-C_{18} rapid resolution column (15×2.1 mm, 3 μm particle size) was applied in all cases. SIM and SRM LC–MS modes were used for the requisite selectivity as well as sensitivity. For these experiments complete chromatographic separation of the analytes is not required. Mixtures of five compounds or more were selected and separated in 15 to 30 s (Fig. 1A–D). Structures of investigated compounds as well as the monitored selected ions and SRM transitions (where applicable) are shown in Tables 1–3. Prior to LC–MS experiments the separation conditions for all compounds were established using UV detection. First the composition of the mobile phase was varied with the flow-rate set at 1 ml/min in order to obtain good peak resolution in the shortest time possible. Then the eluent flow was increased stepwise to reduce the migration times while maintaining partial peak resolution. The higher column pressure did not allow flow-rates to exceed

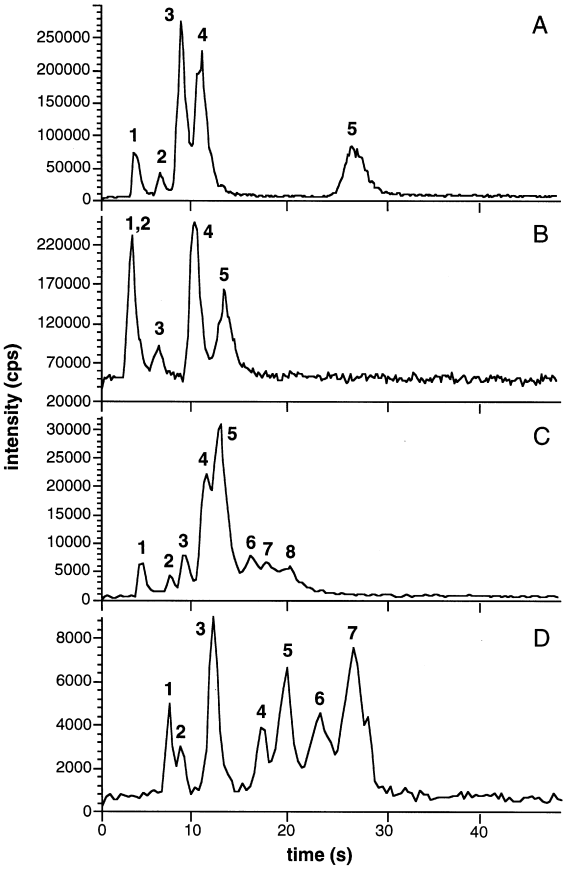


Fig. 1. Fast LC–MS separations of synthetic mixtures containing (A) five flavones, (B) five sulfonamides, (C) eight benzodiazepines and (D) five tricyclic amines, carbamazepine and propranolol. (A) 1=Daidzein, 2=genistein, 3=7OH-flavone, 4=7OH-methoxyisoflavone, 5=flavone; 6 ng each, 10 μl injection; acetonitrile–ammonium acetate (3 mmol/l, pH 5.5) (31:69), 2 ml/min (split 1.1 ml/min to MS); SIM; for monitored ions see Table 1. (B) 1=Sulfadiazine, 2=sulfamerazine, 3=sulfamethoxazole, 4=sulfadimethoxine, 5=sulfasalazine; 2 ng each, 10 μl injection; acetonitrile–ammonium acetate (3 mmol/l, pH 3.3) (20:80), 1.8 ml/min (split 1.1 ml/min to MS); SIM; for monitored ions see Table 1. (C) 1=Oxazolam, 2=chlordiazepoxide, 3=carbamazepine, 4=medazepam, 5=estazolam, 6=norfludiazepam, 7=triazolam, 8=delorazepam; 0.5–2.5 ng, 50 μl injection; acetonitrile–ammonium acetate (3 mmol/l, pH 3.3) (32:68), 1.2 ml/min (no split); SRM; for monitored transitions see Table 1. (D) 1=Propranolol, 2=carbamazepine, 3=doxepin, 4=desipramine, 5=imipramine, 6=amitriptyline, 7=trimipramine; 50 pg each, 20 μl injection; acetonitrile–ammonium acetate (3 mmol/l, pH 3.3) (32:68), 1.2 ml/min (no split); SRM; for monitored transitions see Tables 1 and 2. (A–D) C_{18} column (15×2.1 mm), TurboIonSpray, positive ion mode, total selected ion current profiles.

Table 1
Structures, monitored $[M+H]^+$ in SIM and transitions in SRM

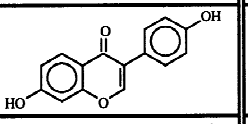
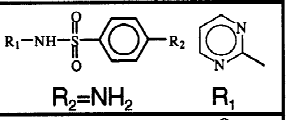
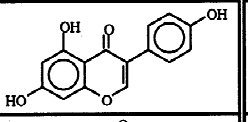
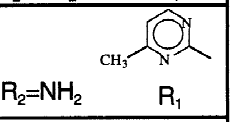
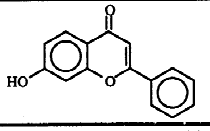
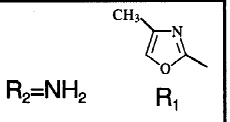
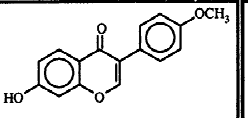
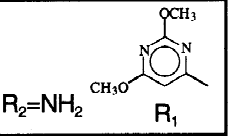
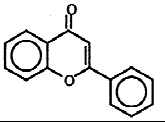
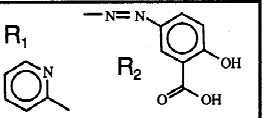
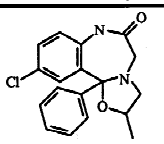
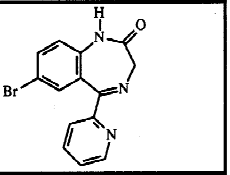
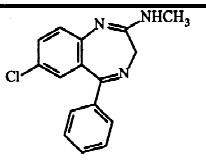
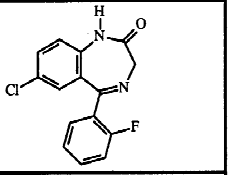
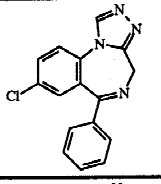
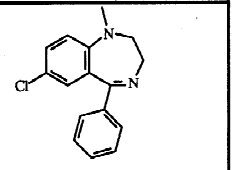
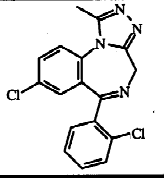
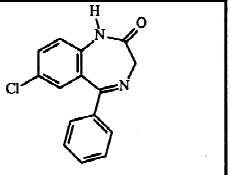
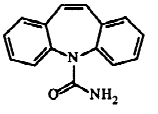
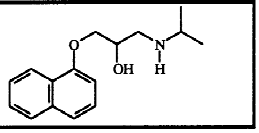
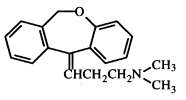
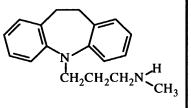
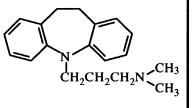
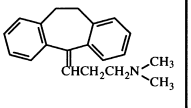
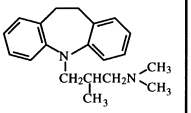
Compound	m/z	Structure	Compound	m/z	Structure
daidzein M_r=254	255.3		sulfadiazine M_r=250	251.2	
genistein M_r=270	271.1		sulfamerazine M_r=264	265.2	
7OH-flavone M_r=238	239.2		sulfa-methoxazol M_r=253	254.2	
7OH-methoxy-isoflavone M_r=268	269.2		sulfadi-methoxine M_r=310	311.2	
flavone M_r=222	223.3		sulfasalazine M_r=398	399.2	
oxazolam M_r=328	329.1→ 271.2		bromazepam M_r=315	316.2→ 182.3	
chlordiaz-epoxide M_r=299	300.0→ 282.3		norfludi-azepam M_r=288	289.2→ 140.1	
estazolam M_r=294	295.2→ 267.1		medazepam M_r=270	271.1→ 90.8	
triazolam M_r=342	343.2→ 308.1		delorazepam M_r=304	305.2→ 139.9	
carbam-azepine M_r=236	237.2→ 194.1		propranolol M_r=259	260.4→ 116.1	

Table 2
Structures, monitored SRM transitions and quantitation results for tricyclic amines in human plasma

	doxepin	desipramine	imipramine	amitriptyline	trimipramine
Structure					
M_r	279	266	280	277	294
Transition	280.4→107.1	267.4→72.1	281.3→86.4	278.4→91.1	295.3→100.2
concentration range (ng/ml)	2-400	2-400	2-400	2-400	2-400
R^2 (2 standard sets)	0.9984	0.9977	0.9964	0.9977	0.9943
LOQ (ng/ml)	2	2	2	2	2
RSD of LOQ (%)	19.5	17.2	12.1	15.7	23.7
Recovery (%)	87.5	96.6	100.3	88.9	104.5
QC1 Precision (%)	19.2	21.4	8.4	7.8	8.3
QC1 Accuracy (%)	111.5	96.7	91.7	94.0	90.2
QC2 Precision (%)	6.7	11.9	7.2	11.7	7.9
QC2 Accuracy (%)	102.2	103.0	98.6	89.4	97.9
QC3 Precision (%)	6.5	1.7	1.8	8.2	4.4
QC3 Accuracy (%)	100.6	98.7	100.3	94.2	90.4

about 2 ml/min, which varied as a function of the composition of the mobile phase. Acetonitrile was a better suited eluent than methanol because of its lower viscosity and, as a result, lower column pressure. Furthermore, it provided higher elution strength. A sufficient separation of the early eluting compounds from the solvent front was desirable because interferences can cause signal suppression under the experimental conditions used. Therefore, the increase of the flow-rate is a better approach for higher speed than an increased organic solvent content. However, a careful adjustment of the organic content in the mobile phase produced optimized results. Changes of only 1% can lead to changes in peak resolution as well as retention times that are critical in fast chromatography. Sample solutions have to be prepared in a weaker solvent than the mobile phase to obtain sharp chromatographic peaks. The injection of small volumes is preferable, although the application of higher volumes (up to 50 μl) is possible without disturbing the peak shape if the appropriate weak sample solvent is used.

3.1.1. Flavone derivatives

The first compound mixture studied consisted of flavones and isoflavones, phytoestrogens which occur in many fruits and vegetables, especially in soy products. They inhibit tumor growth, stimulate apoptosis and have been proposed to be responsible for the low breast cancer rate of Asian women [25]. Flavone was shown to possess antiproliferative effects on human lung adenocarcinoma [26]. For that reason, these substances are under investigation as possible anti-cancer drugs, and their determination is of importance for the pharmaceutical industry. Flavone and hydroxylated derivatives (see Table 1) were analyzed by SIM LC–MS using a TurboIonSpray interface operating in the positive ion mode. Employing a mobile phase containing 3

Table 3
Quantitation results for benzodiazepines in human urine

	Bromazepam	Carbamazepine	Estazolam	Norfludiazepam	Delorazepam
Concentration range (ng/ml)	50.55–15170.0	4.75–1422.0	5.9–1770.0	23.1–6930.0	24.4–7320.0
R^2 (4 sets on 2 days)	0.9930	0.9957	0.9976	0.9973	0.9940
LOQ (ng/ml)	50.55	4.75	5.9	23.1	24.4
RSD of LOQ (%)	14.3	5.1	24.1	9.8	5.5
Recovery (%)	58.3	87.7	85.3	69.8	77.8
Concentration QC1 (ng/ml)	606.6	57	70.8	277.2	292.8
Concentration QC2 (ng/ml)	4044	380	472	1848	1952
Concentration QC3 (ng/ml)	8088	760	944	3696	3904
Intra-day					
QC1 Precision (%)	4.5	18.3	10.8	8.2	8.9
QC1 Accuracy (%)	78.0	76.6	104.5	102.6	117.3
QC2 Precision (%)	7.6	16.1	4.0	6.3	8.7
QC2 Accuracy (%)	104.7	69.7	100.9	98.1	103.1
QC3 Precision (%)	8.2	8.5	4.4	7.2	4.4
QC3 Accuracy (%)	108.2	75.5	104.0	86.7	101.3
Inter-day					
QC1 Precision (%)	13.0	19.5	12.6	7.6	7.4
QC1 Accuracy (%)	90.1	68.9	107.3	105.9	113.2
QC2 Precision (%)	8.4	13.8	3.7	7.5	8.7
QC2 Accuracy (%)	108.1	67.0	102.8	98.6	101.5
QC3 Precision (%)	10.0	10.9	4.6	7.5	10.2
QC3 Accuracy (%)	102.0	70.7	104.8	91.3	94.2

mmol/l ammonium acetate, pH 5.5–acetonitrile (69:31) at a flow-rate of 2 ml/min (post-column split 1.1 ml/min to MS), the first four peaks eluted within 15 s (Fig. 1A). Flavone is more hydrophobic than the other compounds (e.g., genistein with three OH groups, while flavone lacks an OH group), so its retention time is significantly longer. Gradient elution is usually the method of choice for such mixtures, but is not desirable for fast cycle times required for high-throughput analyses. Isocratic elution is preferred to preclude reequilibration after the gradient run which would require too much time to accomplish analysis in less than 30 s. A disadvantage of the flavone compounds investigated under these experimental conditions is their high limit of detection (50 to 100 ng/ml). They are neutral compounds, so their proton affinities are much lower than for basic compounds. Therefore, these compounds do not display particularly good sensitivity, even with ''conventional'' chromatographic conditions [27,28]. To demonstrate the potential usefulness of the method, calibration was performed in the range of 50 to 12 500 ng/ml, resulting in R^2 values of at least 0.99 and reproducibilities between 0.5 and 10%. Although the detection limits are probably too high for biological samples, a potential application could be the determination of flavone derivatives in soy-based food formulations.

3.1.2. Sulfonamides

Another important compound class is bacteriostatic sulfonamide drugs which are applied in the treatment of human infections such as bronchitis or urinary tract infections [29]. These antibiotics are also used to prevent infections in livestock, and the analysis of undesired residues in milk and meat has been reported [30]. In our experiment the five sulfonamide drugs shown in Table 1 were resolved into four peaks within 15 s using ammonium acetate (3 mmol/l, pH 3.3)–acetonitrile (80:20) at 1.8 ml/min (split 1.1 ml/min to MS). Fig. 1B shows the total selected ion chromatogram for the TurboIonSpray SIM LC–MS analysis of these analytes. Each compound was detected by monitoring the corresponding protonated molecule. This example demonstrates the possibility for fast LC separation of

compounds with large differences in polarity. Adjusting the organic solvent content in the mobile phase so that the nonpolar sulfasalazine elutes in 15 s caused the two very polar compounds, sulfadiazine and sulfamerazine, to co-elute. Here also gradient elution would be required for optimum separation results, but this would lead to longer run times. A complete separation of the analytes is not necessary due to the selectivity of MS detection, which provides selected ion current profiles. The applicability of the established conditions for real-world analysis of sulfonamide drugs may be demonstrated in future experiments.

3.1.3. Benzodiazepines

The separation of eight benzodiazepines (structures and transitions shown in Table 1) with eight distinguishable peaks in less than 25 s achieved by TurboIonSpray positive ion SRM LC–MS is presented in Fig. 1C. Benzodiazepines are of importance as sedative, hypnotic and antiepileptic drugs [29,31] and were among the first small molecule combinatorial libraries synthesized [32]. These substances were chosen for our studies because they are good model compounds to develop analytical methods for combinatorial library characterization and drug metabolism studies, where the applicability of these fast LC–MS experiments may be utilized. The investigated benzodiazepines possess a similar structure with slight differences in polarity leading to similar retention behavior. Therefore, they are well suited for fast isocratic separations, here achieved at 1.2 ml/min ammonium acetate (3 mmol/l, pH 3.3)–acetonitrile (68:32) without post-column split. More analytes can be fitted into the time frame of 25 s, because overlaying peaks of non isobaric compounds can be resolved in SRM when extracted ion current profiles are used. Several benzodiazepine mixtures are separable within 15 s (e.g., first five components in Fig. 1C, or a mixture of bromazepam, carbamazepine, estazolam, norfludiazepam and delorazepam). The sensitivity for the different compounds varies due to different proton affinity and fragmentation behavior. Carbamazepine and estazolam, for example, have good response factors leading to lower limits of detection for these analytes.

3.1.4. Amines

A mixture of five tricyclic amines together with propranolol and carbamazepine (for structures and transitions see Tables 1 and 2) was separated within 30 s using TurboIonSpray SRM LC–MS in the positive ion mode (Fig. 1D). The mobile phase, consisting of 3 mmol/l ammonium acetate, pH 3.3–acetonitrile (68:32), was directed to the mass spectrometer at a flow-rate of 1.2 ml/min without split. Tricyclic amines are applied as antidepressants in the treatment of one of the most common psychiatric disorders and play an unfortunate role as suicidal drugs [29]. Therefore, their determination in biological samples is of importance for monitoring drug levels after administration and in toxicological analysis. Tricyclic amines possess only minor differences in structure and therefore show a similar retention behavior with similar retention times. When only the tricyclic amines were studied using a higher flow-rate (1.5 to 1.9 ml/min) and/or organic solvent content (35%), retention times as short as 15 to 20 s were achieved (data not shown here). Amines have an affinity to surfaces leading to peak tailing and carryover which can be problematic in quantitation. In our experiments no significant chromatographic tailing was observed, and carryover was nearly eliminated through the use of appropriate autosampler rinsing steps. The tricyclic amines as basic compounds are well suited for atmospheric pressure ionization (API) LC–MS applications because of their good response and therefore, low detection limits under these conditions.

3.2. Analysis of biological samples

Fast SIM LC–MS and SRM LC–MS analyses of a variety of standard mixtures have been shown above, although the realistic suitability of fast analyses can only be demonstrated through the analysis of real sample matrices, such as urine and plasma. There is of course a concern to determine whether these fast separation conditions are able to deal with endogenous or other interferences including metabolites occurring in biological samples [7,17,33]. So it was important to verify the absence of negative matrix influences on each analyte. Two examples were chosen to show the applicability: tricyclic amines in plasma and benzodiazepines in urine, with the objec-

tive to stay within 15 s sample analysis time. This strategy requires careful sample pretreatment, which is a critical step especially for compounds with short retention times. Filtration of the extracts was necessary to prevent column pressure increase during the analysis of larger sample sets. Using a simple plasma protein precipitation interferences might be still present which could adversely influence the early eluting analytes. Furthermore, matrix suppression effects are more likely when the samples are not sufficiently clean. The high selectivity provided by SRM may be preferred over SIM to avoid interferences. The same concerns are, of course, also relevant for ''conventional'' chromatographic conditions, and even more important to consider when applying flow injection analysis (FIA) MS techniques. However, modern equipment allows for robotic, parallel sample preparation with minimum time consumption. In this case, the sample preparation time has only a minor contribution to the overall analysis time.

3.2.1. Tricyclic amines

The ability of APCI to tolerate flow-rates up to 2 ml/min without significant deterioration of sensitivity and less influence of matrix interferences makes it very useful for such fast LC–MS analyses [34,35]. A method employing 15 s separation using APCI SRM LC–MS in the positive ion mode was developed for the determination of tricyclic amines in human plasma. Analyses were carried out on a C_{18} column (15×2.1 mm) with a mobile phase composition of 3 mmol/l ammonium acetate, pH 3.3–acetonitrile (67:33) at 1.9 ml/min without using a post-column split. Although interferences were not observed in our experiments using a simple protein precipitation of plasma, a more careful sample pretreatment was carried out for increased sensitivity and selectivity. After investigation of different extraction methods including liquid–liquid extraction [36,37] and SPE [38,39] a modified C_{18} SPE extraction procedure was applied. Recoveries between 87 and 104% were obtained. Fig. 2 presents the extracted ion profiles for a plasma spike of the amines at 10 ng/ml which is close to the lower end of the calibration curve (Fig. 2A) and a blank (Fig. 2B). The analyte peaks are separated and resolved from interferences observed in the blank. Table 2 summarizes the quantitation results for the tricyclic amines in human plasma. Good calibration results with $R^2>0.99$ in the range 2–400 ng/ml were obtained using imipramine-d3 as internal standard for all analytes. Reproducible results with low bias between the two standard curves were achieved (e.g., the calibration curve for imipramine from the beginning of a sample tray of 55 samples is described by $y=0.0307x-0.1696$, the calibration function from the end is $y=0.032x-0.163$). Some carryover was observed, but it was less than 10% of the limit of quantitation (LOQ). The sensitivity was sufficient for real samples (therapeutic plasma concentrations have been reported at about 100 ng/ml [29]), where 2 ng/ml could be easily detected. Reproducibilities of area ratios of the lowest standard (six replicates) were determined to be between 12 and 23%. Good precision and accuracy (average 8.9 and 97.7%, respectively) were obtained for the quality control samples with higher standard deviations for doxepin and desipramine at the low QC (19 and 21%, respectively).

3.2.2. Benzodiazepines

The TurboIonSpray interface operated in the positive ion mode under SRM conditions was used for the determination of benzodiazepines in urine. The applied liquid–liquid extraction procedure was fast and produced very clean extracts with recoveries of the analytes between 58 and 88%. Separations were carried out on a C_{18} column (15×2.1 mm) with a mobile phase composition of 3 mmol/l ammonium acetate, pH 3.3–acetonitrile (67:33) and a flow-rate of 1.9 ml/min (post-column split 1.1 ml/min to MS). Two 96-sample sets were analyzed on two different days using estazolam-d5 as internal standard. Fig. 3 shows the SRM chromatograms (selected ion current profiles) of a spiked (A) and a blank urine sample (B) with no chemical interferences observed from the biological matrix. Table 3 presents the summarized quantitative parameters. The calibration curves showed good linearity and reproducibility with no significant bias from the beginning to the end of the analysis (e.g., for estazolam the calibration function on day 1 for the first set of standards is $y=0.0065x-0.1783$, the calibration function on day 2 for the last set is $y=0.0062x-0.0503$). Reproducible results were also obtained for quality control samples [average 8.4% relative stan-

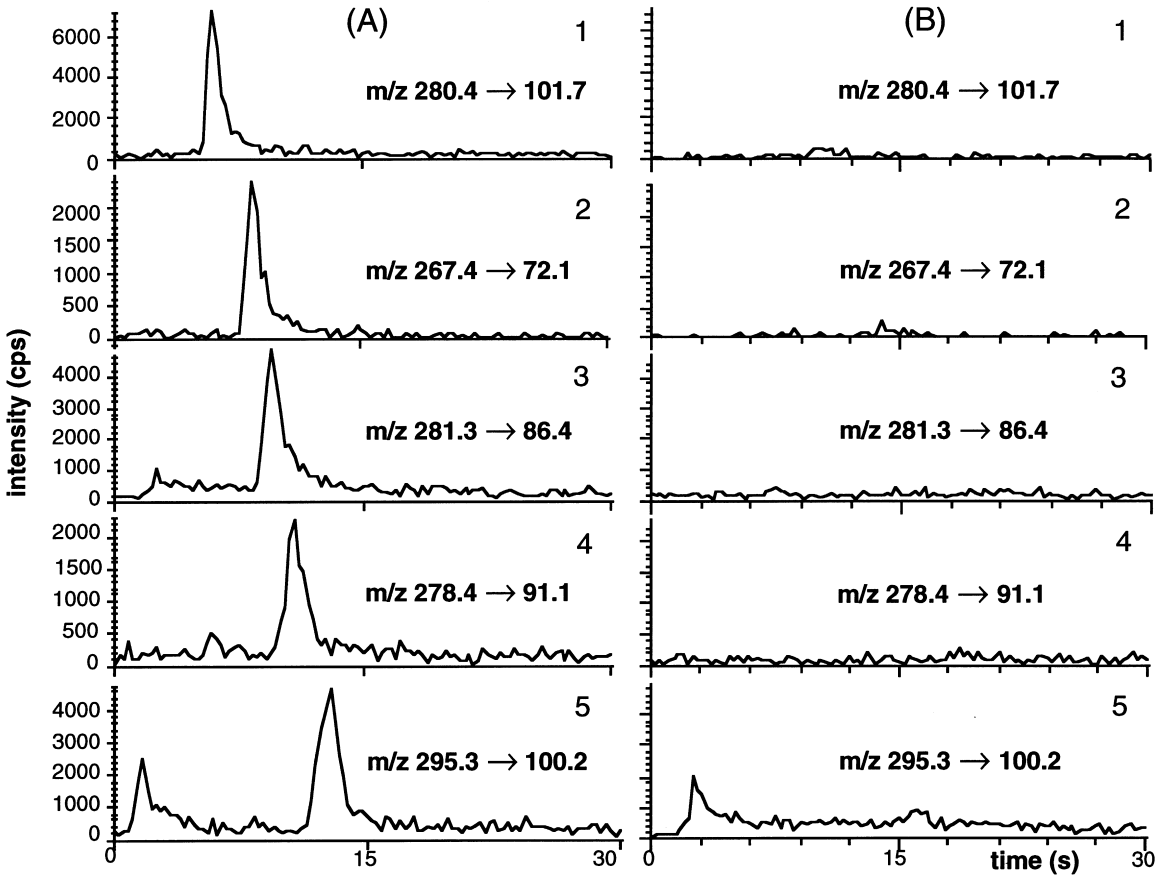


Fig. 2. SRM LC–MS determination of tricyclic amines in human plasma. (A) Extract from plasma spiked at 10 ng/ml doxepin (1), desipramine (2), imipramine (3), amitriptyline (4) and trimipramine (5), precursor ion-product ion transitions are included in each panel. (B) Same data for the plasma blank; 20 μl injection; SPE; C_{18} column (15×2.1 mm), acetonitrile–ammonium acetate (3 mmol/l, pH 3.3) (33:67), 1.9 ml/min (no split); APCI, positive ion mode.

dard deviation (RSD)]. High accuracy was achieved for all analytes (average 96–107%), but carbamazepine showed only about 74% accuracy for all QC samples. This is attributed to a systematic error in sample preparation. The reproducibility of six replicates of the lowest calibration level (5 to 50 ng/ml) was determined to be between 5 and 24% RSD. The results are acceptable for this preliminary study of fast LC–MS which is not considered a full rigorous method validation. The LOQ values achieved for the benzodiazepines are sufficient for combinatorial library characterization and drug metabolism studies, where the main application field can be seen. Improvements in sample preparation (e.g., further enrichment of the analytes) could enhance the detection limits if higher sensitivity is required.

3.3. 240 injections-per-hour experiment

The above described examples for the analysis of amines and benzodiazepines in biological matrices demonstrate the applicability of fast SRM LC–MS analyses. From the separation time of 15 s the possibility to analyze 240 samples per hour can be predicted. The only way to show real feasibility and robustness of this approach is to perform an experiment where sample extracts are injected every 15 s over a 1-h time period. Because most commercial autosamplers require nearly 1 min or more cycle

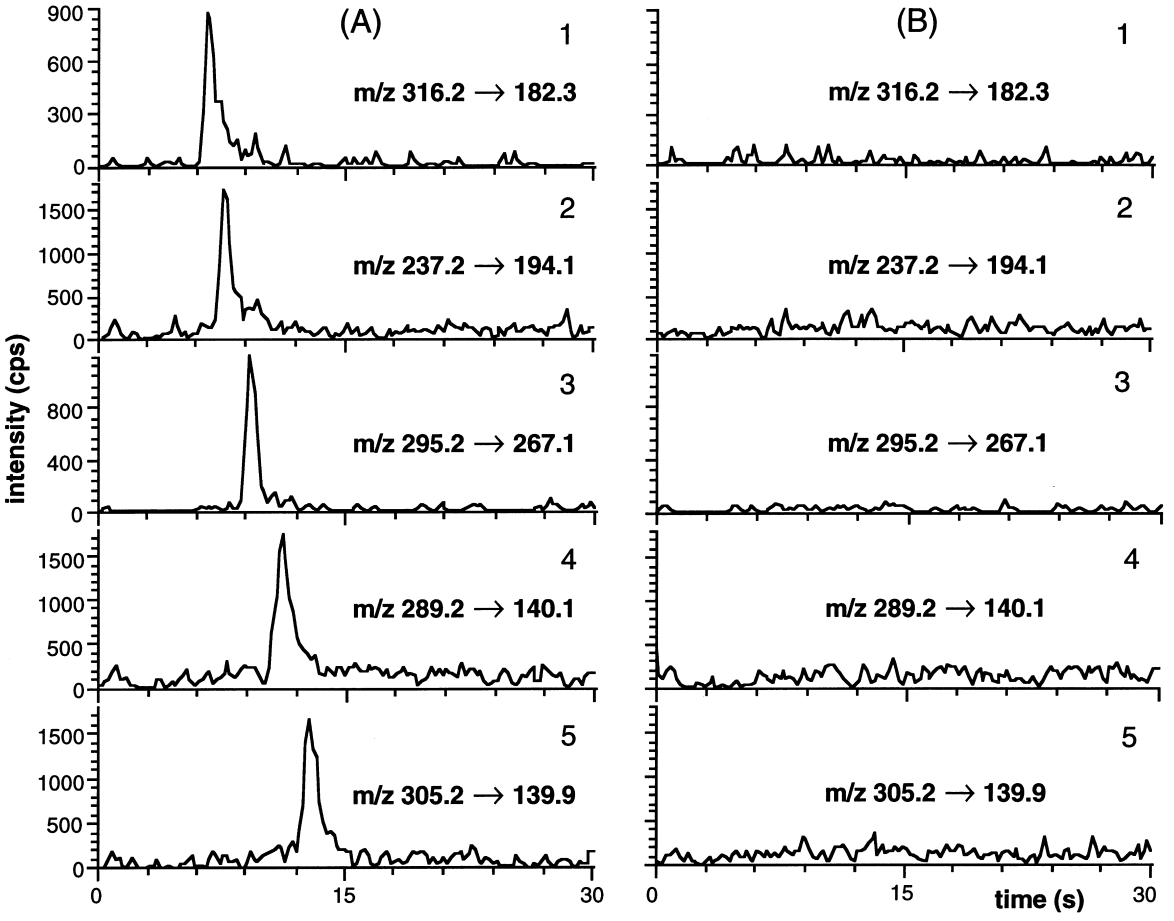


Fig. 3. SRM LC–MS determination of benzodiazepines in human urine. (A) Extract from urine spiked at 100 ng/ml bromazepam (1), 7.8 ng/ml carbamazepine (2), 11.8 ng/ml estazolam (3), 56.2 ng/ml norfludiazepam (4) and 58.8 ng/ml delorazepam (5), precursor ion-product ion transitions are included in each panel. (B) Same data for the plasma blank; 50 μl injection; liquid–liquid extraction; C_{18} column (15×2.1 mm), acetonitrile–ammonium acetate (3 mmol/l, pH 3.3) (33:67), 1.9 ml/min (split 1.1 ml/min to MS); TurboIonSpray, positive ion mode.

time (a multi-valve injector with a maximum of eight injections per minute has been introduced recently by Gilson), we set up an apparatus that could implement continuous flow injection of one sample solution to the 15×2.1 C_{18} column (see Experimental). Benzodiazepines (mixture of bromazepam, carbamazepine, estazolam, norfludiazepam and delorazepam) extracted from human urine were chosen as model compounds. Separation was achieved in less than 13 s using 3 mmol/l ammonium acetate, pH 3.3–acetonitrile (65:35) at 1.35 ml/min (no split). Fig. 4 shows the SRM LC–MS chromatograms obtained from the first, the 120th and the 240th injection. The retention times showed very good reproducibility, which is essential for these fast separations. Chromatographic peak shape and resolution was maintained over the entire run. The peak area reproducibilities were measured to be 12.1% RSD for bromazepam, 7.1% for carbamazepine, 9.4% for estazolam, 10.4% for norfludiazepam and 10.1% for delorazepam. The results suggest that the analysis of 240 samples per hour could be practical in the future,

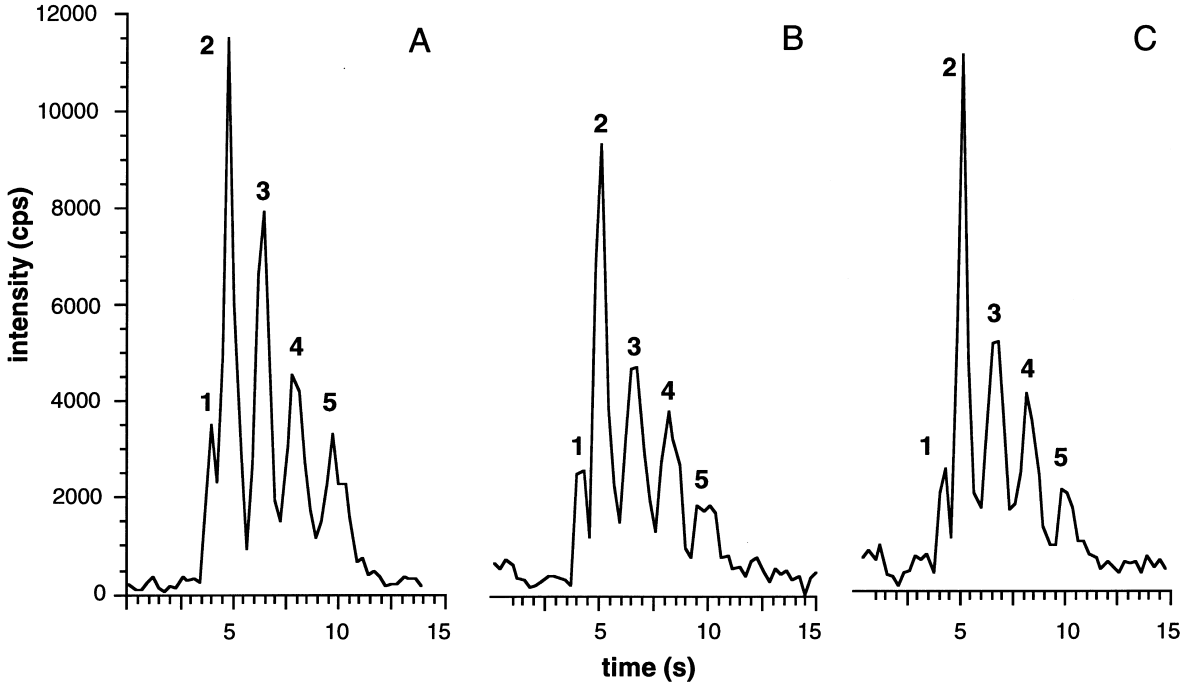


Fig. 4. Two hundred and forty injections per hour – benzodiazepines in human urine; (A) injection 1, (B) injection 120, (C) injection 240. 1=Bromazepam, 2=carbamazepine, 3=estazolam, 4=norfludiazepam, 5=delorazepam; 50–400 ng/ml, 10 μl injection; liquid–liquid extraction; C_{18} column (15×2.1 mm), acetonitrile–ammonium acetate (3 mmol/l, pH 3.3) (35:65), 1.35 ml/min (no split); TurboIonSpray, positive ion mode, SRM, total selected ion current profiles, for monitored transitions see Table 1.

leading to the possibility of SRM LC–MS analysis of as much as 5760 samples per 24 h. Further experiments would be required to demonstrate that continuous analysis could be performed at this rate over such a long period of time.

4. Conclusions

In today's trend of pushing the limits of chromatography we have shown the feasibility for considerably faster SIM LC–MS and SRM LC–MS analyses with separation times as short as 15 s for multicomponent mixtures. This may be a useful strategy for re-engineering the routine liquid chromatographic process from run times of several minutes to less than 1 min, and to provide high-throughput analytical methods for drug discovery and related areas. When these compromised chromatographic conditions are employed, it will become increasingly important to demonstrate that matrix interferences and other potential problems are not occurring. The next step is to do a full, rigorous method validation for a relevant analytical problem applying these fast separations. For that goal, an autosampler which can inject every 15 s or faster is desirable. A possible alternative could be the coupling of six autosamplers to one tandem LC–MS system, although not without high operative expenditure [20]. Efforts are needed to improve sample preparation and data processing throughput, which has to be highly automated to accommodate the high data volume produced by the high throughput analyses. Investigations regarding the applicability of parallel on-line SPE–fast-LC–MS–MS could decrease the entire analysis time even further. Simultaneous parallel chromatographic separations coupled

with one mass spectrometer may also be an interesting alternative in the future.

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