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Evaluation of an automated thermospray liquid chromatography–mass spectrometry system for quantitative use in bioanalytical chemistry

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ABSTRACT

An automated thermospray liquid chromatography–mass spectrometry system is described, including an autosampler and a gradient liquid chromatography system controlled from the mass spectrometer data system. The performance and reliability of the equipment during unattended operation were evaluated by repeated injections of standard solutions of some antiasthmatic drugs, using deuterium-labelled analogues as internal standards. High sensitivity and reproducibility were achieved during a 19-hour run, incorporating gradient elution and a total of 54 injections. The relative standard deviation of the peak area measurement of the internal standards was in the range of 6.5–8.2%. The corticosteroid budesonide can be routinely measured in plasma down to 0.1 nmol/l. Direct injection of a small plasma volume into the thermospray liquid chromatography–mass spectrometry system could be used to monitor drug plasma levels during a toxicity study in dogs.

INTRODUCTION

During the last few years liquid chromatography (LC) combined with thermospray (TSP) mass spectrometry (MS) has been extensively used for the identification of polar and thermally labile compounds of biomedical interest [1–5]. The technique has been less widely used for quantitative analysis, possibly because careful optimization of thermospray conditions is necessary to obtain high sensitivity and reproducibility [1,6–10]. Early applications of LC–TSP-MS demonstrated it to be potentially useful as a quantitative technique [11], and during recent years a number of quantitative or semi quantitative bioanalytical methods of various degrees of sophistication have been published [12–34]. Reports on more thoroughly validated methods, however, are scarce, and LC–TSP-MS does not seem to have found wide acceptance as a routine technique for the determination of drugs in biological fluids.

There are several attractive features of LC–TSP-MS which make it an interesting bioanalytical technique. The sensitivity, although widely varying for different compounds, is in many cases excellent, permitting measurement in the low nmol/l range. LC affords mild conditions for the separation of polar compounds, thereby reducing the risk of artefact formation. The high selectivity of the mass spectrometer generally allows simple sample clean-up procedures to be used. In addition, short

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chromatographic columns often give adequate separation, leading to short analysis time. LC–TSP-MS is a reliable and robust technique which has been shown to be useful for routine drug analysis using automated procedures [18,23,31,34].

This paper describes an automated LC–TSP-MS system, including an autosampler and a gradient LC system controlled from the mass spectrometer data system. The performance and reliability of the equipment during unattended operation were evaluated by repeated injections of standard solutions of some antiasthmatic drugs. Examples of the quantification of drugs in plasma are also given. Deuterium-labelled analogues were used as internal standards in all cases.

EXPERIMENTAL

Chemicals

The prodrug bambuterol and its metabolic hydrolysis products D2439 and terbutaline [35–37], from Draco (Lund, Sweden), were used to test the performance of the LC–MS system. The chemical structures of the compounds are shown in Fig. 3. [$^2H_6$] Terbutaline, [$^2H_6$]D2439, and [$^2H_6$]bambuterol, labelled with deuterium in the *tert.*-butyl group, were used as internal standards. The corticosteroid budesonide [38,39], also from Draco, and its internal standard [$^2H_8$]budesonide were analysed by LC–TSP-MS as the 21-acetyl esters (Fig. 3) after derivatization with a mixture of acetic anhydride and triethylamine [34,40]. Ammonium acetate and acetic acid (Gold Marke quality) were purchased from Aldrich Chemie (Steinheim, Germany) and methanol (HPLC grade) from Rathburn (Walkerburn, UK). Water was purified in a Milli-Q system (Millipore, Molsheim, France).

Instrumentation

A schematic view of the LC–MS instrumentation is given in Fig. 1. Pieces of equipment are identified by letters (A–M) which are referred to in the text. The LC equipment included two LKB 2150 pumps for eluent pumping (B) (Pharmacia LKB, Uppsala, Sweden) controlled by a VAX-based Autochrom M340 program (version 1.13) via a CIM 114 interface (A) (Autochrom, Milford, MA, USA). The solvents were pumped through two SSI Model LP-21 pulse dampers (C) (Scientific Systems, State College, PA, USA) and joined in an LKB mixer (D). The system included three injectors. A Rheodyne Model 7125 injector (E) with a 5-ml loop was used to inject a tuning solution into the mass spectrometer, a Valco Model C6W (F) with a Model A60 air actuator and a 100-μl loop was used for manual sample injection, and a CMA 200 autosampler (G) (Carnegie Medicin, Stockholm, Sweden) was used for automatic sample injection. The LC column was connected to a Rheodyne Model 7000 valve (H), acting as a column-bypass valve, to allow direct injection of samples into the mass spectrometer. A third LKB 2150 pump (I) was included to allow pumping of a make-up solvent, which was added post-column through a 10-μl Lee Visco mixer (J) (The Lee Company, Westbrook, CT, USA). A Waters Model 440 UV detector (K), connected in-line with the mass spectrometer, was used to check the performance of the chromatographic system. Besides the standard safety vent valve (L) (Rheodyne Model 7001) controlled by the mass spectrometer vacuum system, the automated LC–MS system was fitted with a Valco Model C6W valve (M) with a Model A60 air actuator, which allowed switching of the chromatographic front to waste. The MS

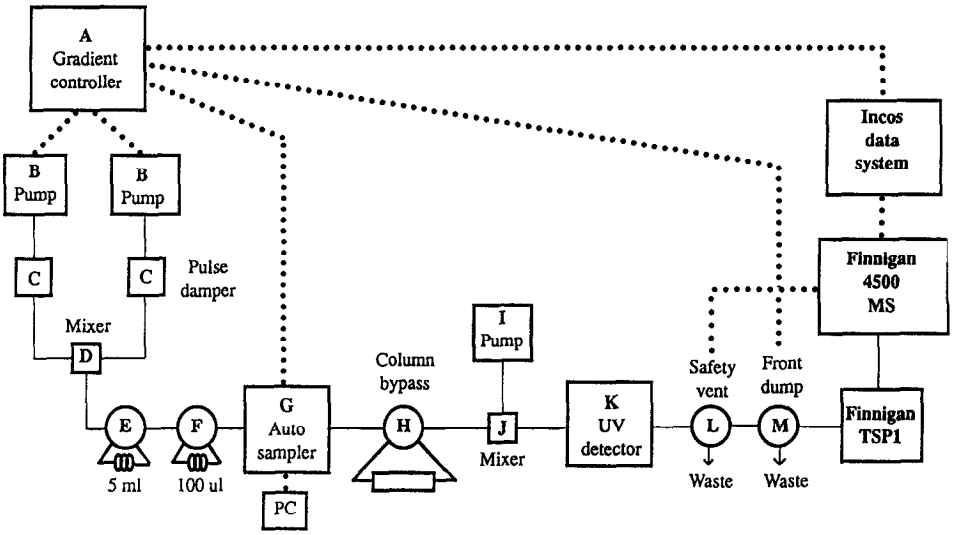

Fig. 1. Schematic view of the automated LC–TSP-MS system. Pieces of equipment labelled with letters A–M are described in the text. Dotted lines: control lines; solid lines: fluid lines.

equipment consisted of a Finnigan 4500 quadrupole instrument (Finnigan MAT, San José, CA, USA) equipped with a Finnigan thermospray interface (TSP1) and an Incos data system (SuperIncos revision 6.5 software). User-defined Incos procedures were used to start the acquisition, each sample being acquired into a separate file, and to send a start pulse to the gradient controller (A). Besides control of the LC pumps, the gradient controller was used to control the autosampler (G) and the front dump valve (M). The work described in this paper was performed with vaporizers, the tips of which had to be manually crimped to achieve satisfactory spray performance.

To delay contamination of the repeller surface and to ensure reproducible positioning in the source block, the thermospray ion source repeller was modified as shown in Fig. 2. The stainless-steel electrode, insulated with a polyimide sleeve, was

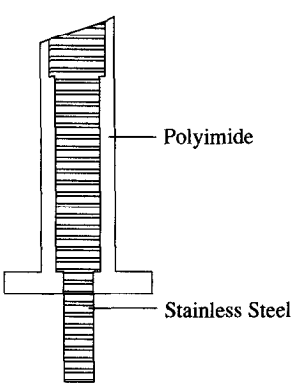

Fig. 2. Thermospray repeller electrode.

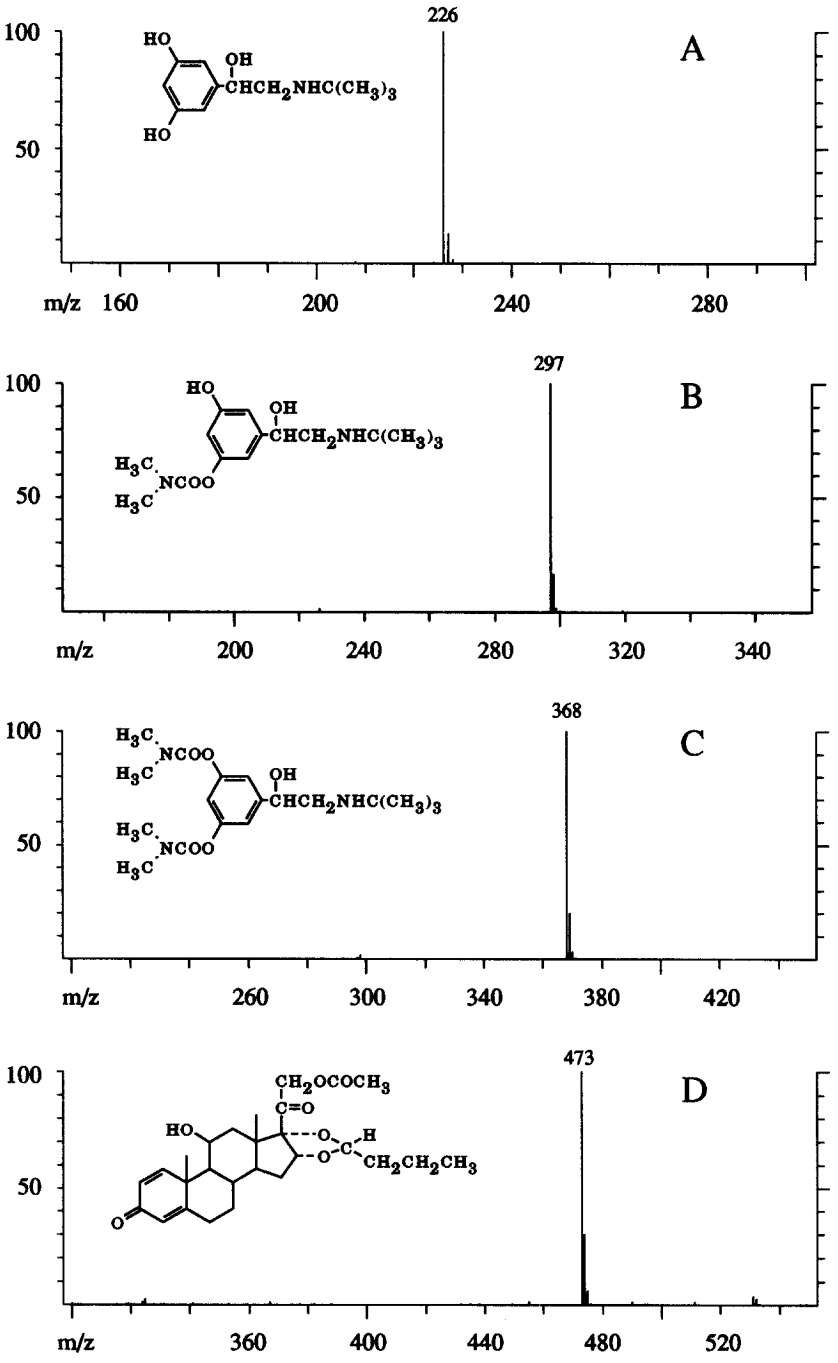

Fig. 3. Thermospray mass spectra and chemical structures of (A) terbutaline, (B) D2439, (C) bambuterol and (D) budesonide 21-acetate.

placed in the source block with the tip of the repeller facing the incoming vapor stream at an angle of about 30°. The exhaust line of the thermospray source was evacuated with a Rietschle CLF26 mechanical pump. Solvent vapor was trapped in a Savant RT490 refrigerated trap (Savant Instruments, Farmingdale, NY, USA), equipped with a 4-l flask kept at −90°C. To provide prolonged LC–MS operation, the inlet tube of the flask was shortened, allowing *ca.* 2000 ml of solvent to be trapped. During thermospray operation the analyzer pressure was $3.5 \cdot 10^{-5}$ Torr, the manifold forepressure 0.20 Torr, and the exhaust line pressure 1.1 Torr. Thermospray mass spectra of terbutaline, D2439, bambuterol, and budesonide 21-acetate are shown in Fig. 3. For quantitative analysis the $[M+H]^+$ ions of the compounds studied plus their deuterated internal standards were monitored, one pair at a time. The instrument was scanned in the selected-ion monitoring mode over a 0.5-a.m.u. window for each mass. The scan time was 400 ms for budesonide 21-acetate and 200 ms for the other compounds.

Liquid chromatography

Terbutaline, D2439 and bambuterol were chromatographed on a 50 × 4 mm LiChrospher RP-select B (5 μm) cartridge (E. Merck, Darmstadt, Germany) fitted with a 4 × 4 mm LiChrosorb RP-select B precolumn. The compounds were eluted during a 13-min gradient running from 3% up to 38% methanol in 0.1 *M* ammonium acetate buffer, pH 5, at a flow-rate of 1.40 ml/min. Budesonide 21-acetate was chromatographed on a 33 × 4.6 mm Supelcosil LC-8-DB (3 μm) cartridge fitted with a 10 × 3 mm Chromguard R precolumn. The mobile phase was 64% methanol in 0.1 *M* ammonium acetate buffer, pH 5, pumped at a flow-rate of 1.40 ml/min. Since it was difficult to crimp the vaporizer tip in a perfectly reproducible manner, the flow-rate of the make-up solvent (0.1 *M* ammonium acetate buffer, pH 5) was adjusted (0–0.20 ml/min) for each vaporizer to achieve optimum flow conditions. The mobile phases were filtered through a 0.22-μm Durapore filter (Millipore) before use and continuously degassed with helium during LC–MS operation.

Calibration and optimization

Mass calibration was performed with a polyethylene glycol (PEG) mixture (PEG200, PEG300, and PEG600, 2:1:1) dissolved in 30% methanol in 0.1 *M* ammonium acetate buffer, pH 5, at a concentration of about 0.1%. At a source block temperature of 220°C and a repeller voltage of 45 V this mixture produced $[M+NH_4]^+$ ions of PEG oligomers of relatively equal intensity over the mass range 150–1000. To achieve maximum sensitivity for target compound analysis the mass spectrometer was tuned by injecting 2–5 ml (from valve E) of a drug solution (1–5 μmol/l in mobile phase) with valve H in the column-bypass mode. In this way a stable flow of analyte into the ion source was achieved for a couple of minutes. Besides the "normal" adjustment of the lenses and the quadrupole parameters it was essential to optimize the vaporizer temperature, the time constant for the vaporizer temperature controller, and the repeller potential. Optimization of the last two parameters was facilitated by modifications of the wiring so that the potentiometers could be adjusted from outside the TSP1 electronics control module. The optimum repeller potential was found in the range 20–60 V, depending on the contamination of the repeller. Terbutaline, D2439 and bambuterol were analyzed at a vaporizer temperature of

115°C and a jet temperature of 200°C, giving an indicated aerosol temperature of about 270°C. Budesonide 21-acetate was analyzed with the vaporizer at 105°C and the jet block at 180°C, giving an aerosol temperature of about 220°C.

RESULTS AND DISCUSSION

Band-broadening effects

The equipment connected between the injector and the mass spectrometer may cause significant extracolumn effects, seriously affecting the chromatographic efficiency, unless the system is well-plumbed and the dead volumes kept to a minimum. The number of theoretical plates, measured from the UV response of terbutaline with the UV detector connected directly to the analytical column, was 2000. This figure decreased to 1900 when the UV detector was connected in the system as shown in Fig. 1. A further decrease to 1800 theoretical plates was noted when the chromatographic efficiency was determined for terbutaline by LC–TSP-MS. A similar investigation with budesonide 21-acetate gave an estimate of 2000 theoretical plates when the mass spectrometer was used as the detector compared with 2200 plates using the UV detector. The mass chromatograms showed somewhat increased tailing, possibly as a result of adsorption of the compounds onto the walls of the ion source [18]. The results obtained for both terbutaline and budesonide, using columns with 2000 theoretical plates, showed that only about 10% of the chromatographic efficiency was lost in the thermospray interface.

Optimization

Optimization of operating conditions is important to obtain high sensitivity in LC–TSP-MS and should be performed with the target compound for quantitative analysis [7]. As observed by others [18] the vaporizer temperature and the repeller potential could not be set for maximum signal intensity, because this resulted in a flickering response. A compromise between intensity and stability had to be found, giving the best signal-to-noise ratio for the chromatographic peak. Trimming of the time constant for the vaporizer temperature controller also had a profound effect on signal stability. Once optimal conditions had been established the system proved to be very stable and did not require retuning over a period of several weeks. Efficient pulse dampers were used, and essentially no pump pulsation could be measured after the LC column. We feel that imperfections in the heating of the vaporizer [9] are the major source of signal instability in the system described, rather than pump pulsation. During high-pressure gradient mixing the built-in pulse compensation of the LKB 2150 pumps cannot be used.

Reliability test of the LC–MS system

The stability and reliability of the automated LC–TSP-MS system was tested by repeated injections of aqueous standard solutions containing a mixture of terbutaline, D2439 and bambuterol at equal concentrations, together with their deuterated internal standards. The standard curve comprised six standard concentrations, covering the range 1–32 pmol expressed as amount injected. The amount injected of each of the internal standards was 10 pmol. Fig. 4 shows a chromatogram of a 4-pmol sample together with a profile of the methanol content of the gradient used. The six

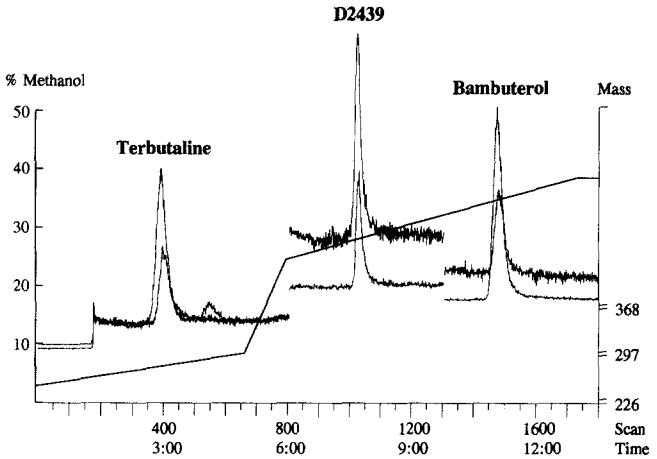

Fig. 4. Thermospray mass chromatogram of a 4-pmol standard solution containing terbutaline, D2439, and bambuterol. The amount of each of the internal standards was 10 pmol. The solid line shows the methanol content of the mobile phase during the gradient elution. Time in min.

standard samples were injected from low to high concentration, the series being repeated nine times, giving a total of 54 injections and a total run time of 19 h. Fig. 5 shows a plot of the measured peak areas of the internal standards *versus* injection number. The slopes of the regression lines were positive for all three compounds, indicating an increase in mass spectrometer response during the experimental period. Contamination of the ion source, leading to decreased thermospray response, occurred only slowly and, even if high sensitivity was required, the system could be operated daily for a couple of weeks before it was necessary to clean the repeller electrode. The coefficient of variation (C.V.) of the internal standard peak area, calculated on all 54 injections, was 7.9% for $[^2H_6]$terbutaline, 8.2% for $[^2H_6]$D2439, and 6.5 for $[^2H_6]$bambuterol. Calculation of the response factors showed that the internal standards compensated for the drift in mass spectrometer response. The variation of the response factor was 6.6% (C.V.) for terbutaline, 8.5% for D2439, and 9.1% for bambuterol. The calibration curves showed excellent linearity for all three compounds. The correlation coefficient was 0.9986 for terbutaline, 0.9971 for D2439, and 0.9993 for bambuterol. Table I shows the precision of the method at each of the six standard concentrations. The C.V. ranged from 10–14% at the low end of the standard curve to 2–4% at the high end.

In the study described above the vaporizer temperature was optimized for a mobile phase containing 6% methanol, corresponding to optimal conditions for terbutaline. Ramping of the vaporizer temperature during gradient elution, to compensate for the changing methanol content, could not be accomplished during unattended operation, because the TSP1 controle module does not allow automatic reset and restart of the temperature program. The results, however, show that acceptable sensitivity and reproducibility could be achieved for D2439 and bambuterol as well, in spite of suboptimal conditions for these compounds.

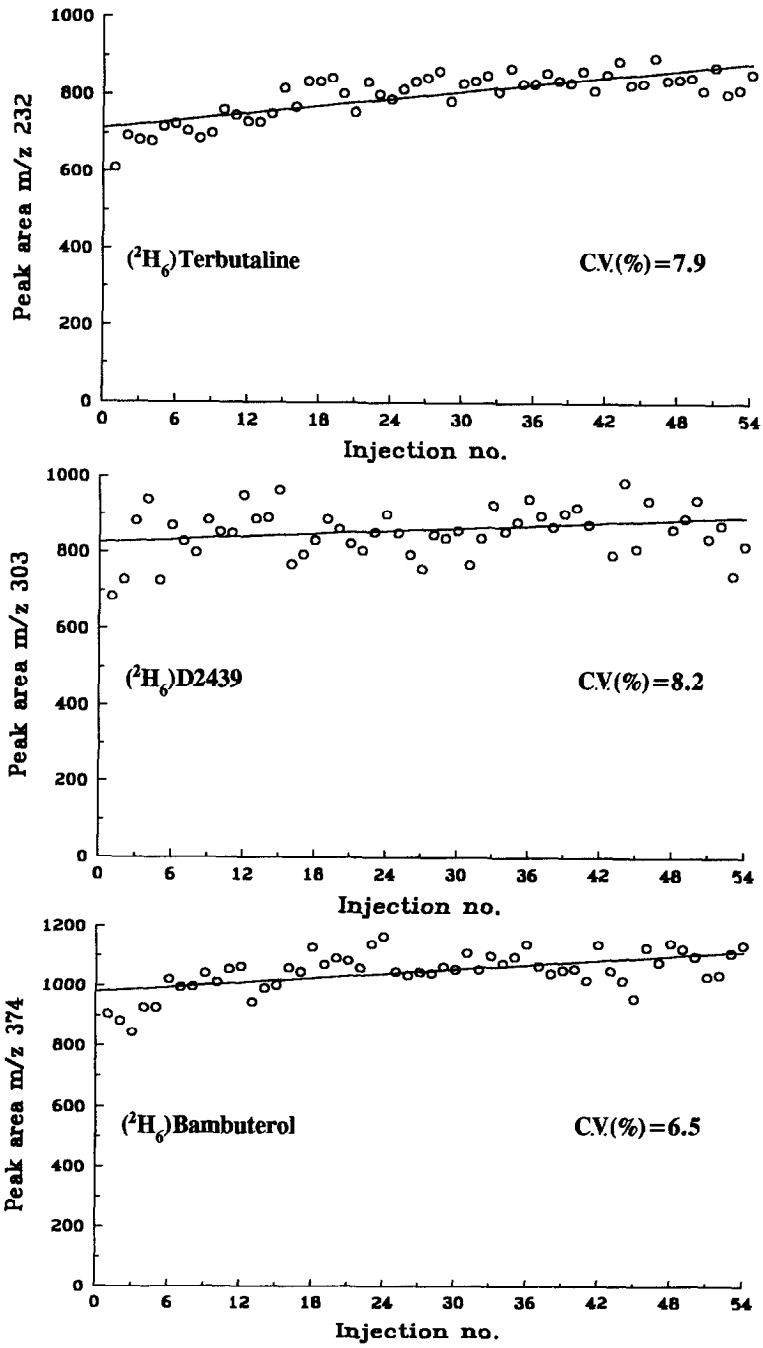

Fig. 5. Measured peak area of the internal standards for terbutaline, D2439, and bambuterol plotted *versus* injection number during the 19-h reliability test of the automated LC–TSP-MS system.

TABLE I

ANALYTICAL PRECISION FOR TERBUTALINE, D2439, AND BAMBUTEROL DURING LONG-TERM (19 h) LC–TSP-MS OPERATION

Compound	Amount injected (pmol)	Area ratio		C.V. (%)	*n*
		Mean	S.D.		
Terbutaline	1	0.1178	0.0114	9.67	9
	2	0.2390	0.0135	5.65	9
	4	0.4843	0.0402	8.30	9
	8	0.9429	0.0576	6.11	9
	16	1.8556	0.0740	3.99	9
	32	3.6781	0.1306	3.55	9
D2439	1	0.1172	0.0117	10.03	9
	2	0.2457	0.0173	7.04	9
	4	0.4977	0.0539	10.83	9
	8	1.0054	0.0753	7.49	9
	16	2.0481	0.1881	9.18	9
	32	4.0747	0.1762	4.32	9
Bambuterol	1	0.0966	0.0138	14.32	9
	2	0.2047	0.0181	8.83	9
	4	0.4360	0.0293	6.72	9
	8	0.8977	0.0281	3.13	9
	16	1.8251	0.0681	3.73	9
	32	3.6201	0.0824	2.28	9

Application to biological samples

LC–TSP-MS can afford elegant solutions to many bioanalytical problems encountered within the pharmaceutical industry. Selected-ion monitoring affords a detection system with high selectivity, making the sample extraction procedure less critical. This will generally facilitate rapid method development, which is important during the early tests of new drug candidates. Later, if the drug candidate is found safe and taken into extended clinical studies, the LC–MS method may be replaced by a simpler and less expensive method. The high selectivity of the mass spectrometer together with the compatibility of the thermospray interface with reversed-phase chromatography, opens the possibility of direct injection of biological samples onto the LC column.

We have used automated LC–TSP-MS for about one year for the determination of budesonide in plasma [34]. The plasma sample is mixed with the internal standard solution ([$^2H_8$]budesonide) and transferred to a conditioned Bond Elut C_{18} column. The column is rinsed in three steps with aqueous ethanol, water, and heptane, and finally eluted with a mixture of ethyl acetate in heptane. The eluate is evaporated to dryness and the residue treated with a mixture of acetic anhydride and triethylamine in acetonitrile to give the 21-acetyl derivative of budesonide. After evaporation of the derivatization reagent the extract is dissolved in mobile phase and injected into the LC–MS system by the CMA 200 autosampler. Batches of about 50 samples are routinely loaded into the autosampler and analyzed overnight. The drug can be measured down to 0.1 nmol/l with a relative standard deviation of less than

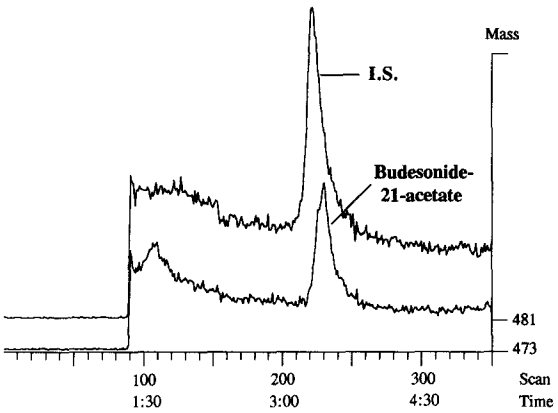

Fig. 6. Chromatogram of budesonide 21-acetate obtained by LC–TSP-MS after extraction and derivatization of a plasma sample containing 1.5 pmol of budesonide. [$^2H_8$]Budesonide (3 pmol) was added as internal standard (I.S.). Time in min.

20%. Fig. 6 shows a chromatogram of a plasma sample containing 1.5 pmol of budesonide. The example illustrates that high sensitivity can be achieved in spite of the use of a mobile phase with high (64%) methanol content. The details of the method will be reported elsewhere.

We have also used LC–TSP-MS to monitor the plasma levels of bambuterol during a toxicity study in dogs, in which high doses of bambuterol were administered, resulting in high plasma concentrations. A small plasma sample was diluted with buffer, containing the internal standard, and an aliquot of the solution, corresponding to 0.5 μl of dog plasma, was directly injected into the LC–MS system. Fig. 7 shows a chromatogram of such a sample containing 32 pmol of bambuterol, equivalent to a plasma concentration of 64 μmol/l. In spite of the absence of any sample clean-up the chromatographic baseline was free from interfering peaks. We do not recommend direct injection of crude plasma samples on a routine basis, because the efficiency of

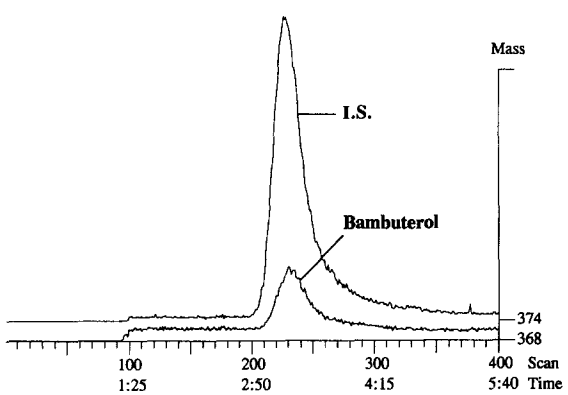

Fig. 7. Chromatogram of bambuterol (32 pmol) obtained by LC–TSP-MS after direct injection of 0.5 μl of dog plasma. [$^2H_6$]Bambuterol (160 pmol) was added as internal standard (I.S.). Time in min.

the chromatographic column will deteriorate relatively quickly. To preserve acceptable peak shape in the bambuterol study mentioned above the guard column had to be changed after less than 100 injections. Raw urine samples, on the other hand, can be injected over extended periods without deleterious effects. Direct injection of biological samples without any purification, or after only minimal sample pretreatment, reduces the risk of artefact formation and makes LC–TSP-MS an almost ideal reference method for the validation of other bioanalytical methods.

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